ANNALI
DELL'ISTITUTO SUPERIORE DI SANITÀ

A SCIENCE JOURNAL *for* PUBLIC HEALTH

ISTITUTO SVPERIORE DI SANITÀ

www.iss.it

Annali
dell'Istituto Superiore di Sanità
A SCIENCE JOURNAL FOR PUBLIC HEALTH

Graphic design of the cover: Massimo Delle Femmine, Istituto Superiore di Sanità
*The photograph on the cover is a transmission electron microscopy image of human colon carcinoma cells (LoVo) treated with the
natural product, voacamine and the chemotherapy drug, doxorubicin for 24 h provided by Maria Condello, CNRVF, Istituto
Superiore di Sanità, Rome, Italy*
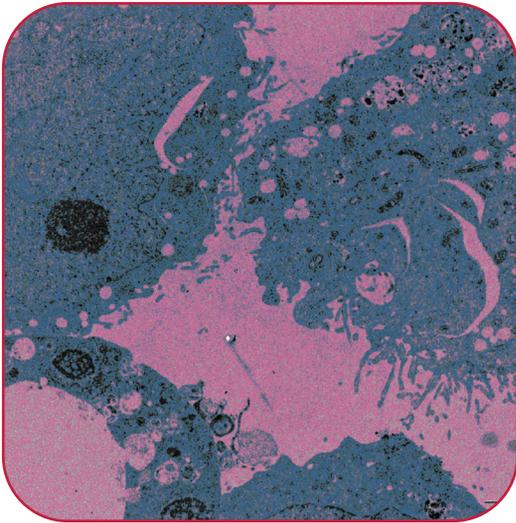
ANNALI
dell'Istituto Superiore di Sanità

Vol. 57, No. 4 2021

Contents

Anti-SARS-CoV-2 antibodies persistence after natural infection: a repeated serosurvey in Northern Italy

Giorgio Fedele[1*], **Paola Stefanelli**[1*], **Antonino Bella**[1*], **Stefano Fiore**[1], **Serena Pancheri**[2], **Eleonora Benedetti**[1], **Concetta Fabiani**[1], **Pasqualina Leone**[1], **Paola Vacca**[1], **Ilaria Schiavoni**[1], **Arianna Neri**[1], **Anna Carannante**[1], **Maurizio Simmaco**[3], **Iolanda Santino**[3], **Maria Grazia Zuccali**[2], **Giancarlo Bizzarri**[2], **Rosa Magnoni**[2], **Pier Paolo Benetollo**[2], **Silvio Brusaferro**[4], **Giovanni Rezza**[5] and **Antonio Ferro**[2]

[1]*Dipartimento Malattie Infettive, Istituto Superiore di Sanità, Rome, Italy*
[2]*Azienda Provinciale per i Servizi Sanitari (APSS), Trento, Italy*
[3]*Dipartimento di Neuroscienze, Salute Mentale e Organi di Senso, Facoltà di Medicina e Psicologia,
Sapienza Università di Roma, Azienda Ospedaliero-Universitaria Sant'Andrea, Rome Italy*
[4]*President, Istituto Superiore di Sanità, Rome, Italy*
[5]*Direzione Generale della Prevenzione Sanitaria, Ministero della Salute, Rome, Italy*
*Equal contributors

Abstract
Introduction. To evaluate the decline of antibodies induced by SARS-CoV-2 infection, the individuals resident in 5 municipalities of the Autonomous Province of Trento, Northern Italy, who resulted IgG positive for anti-SARS-CoV-2 nucleocapsid (NC) in May 2020, were tested four months later.
Methods. Anti-SARS-CoV-2 NC antibodies were detected using the Abbott SARS-CoV-2 IgG assay. Samples that gave a negative result were re-tested using the Liaison SARS-CoV-2 IgG assay to assess anti-spike (S) S1/S2 antibodies. The fifty-percent tissue culture infective dose ($TCID_{50}$) neutralizing assay was performed on a subgroup of formerly positive sera. Statistical analysis was performed by STATA version 16.1 (STATA Corp., College Station, Texas, USA).
Results. Overall, 480 out of 1159 participants became seronegative for anti-NC IgG antibodies. Age above 70 years and cough were associated with persistent anti-NC IgG levels. Most anti-NC IgG negative sera were positive for anti-S IgG (77.9%). The neutralization assay showed high concordance with anti-S antibodies positivity.
Conclusion. In conclusion, a decline of anti-NC IgG values was recorded four months after the first evaluation. A high proportion of anti-NC seronegative individuals were positive for anti-spike IgG antibodies, which appear to persist longer and to better correlate with neutralization activity.

INTRODUCTION

Although cases of reinfection by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have been sporadically reported [1], a previous history of SARS-CoV-2 infection is associated with a greatly reduced risk of reinfection [2, 3]. As suggested by a recent modelling study, the presence of neutralizing antibodies induced by natural infection or by an effective vaccine is likely to be predictive of protection [4]. The duration of protection against infection with common human coronaviruses appears to be rather short [5, 6], however in the previous SARS epidemic, SARS-CoV specific IgG were shown to remain detectable for at least two years [7]. Earlier data on the kinetics of IgG antibodies against SARS-CoV-2 among both symptomatic and asymptomatic individuals showed a rapid decline [8, 9]. More recent studies, however, tend to confirm that infection elicits durable serum antibody titers for several months [10-12]. Whether memory-B-cell and T-cell responses may still confer protection in individuals experiencing antibody decline to undetectable levels is unknown [12, 13].

The type of antibody response may also play a role. Experimental vaccination against SARS-CoV with nu-

Address for correspondence: Giorgio Fedele, Dipartimento Malattie Infettive, Istituto Superiore di Sanità, Viale Regina Elena 299, 00162 Rome, Italy.
E-mail: giorgio.fedele@iss.it.

cleocapsid protein (NC) can induce strong antibody responses that were found to be non-neutralizing [14]. While non-neutralizing antibodies might still exert anti-viral activity, for example via the Fc-Fc receptor-based effector function, non-neutralizing NC antibodies may lead to enhanced disease for some vaccine candidates in animal models when neutralizing antibodies are absent [14]. Studies conducted on SARS-CoV-2 have shown that the spike (S) protein is the main target for neutralizing antibodies [15-17].

To evaluate the persistence of SARS-CoV-2 antibodies, we repeated a serosurvey in five municipalities of the Autonomous Province (AP) of Trento, North of Italy, recruiting those individuals who had resulted positive in a large population-based seroprevalence study conducted four months earlier [18]. In a subsample of seropositive participants, the antibody neutralizing titer was also evaluated.

METHODS

Study population and design

As already reported [18], the study was conducted in 5 municipalities of the AP of Trento with the highest incidence of COVID-19 confirmed cases.

The Department of Prevention of the Azienda Provinciale per i Servizi Sanitari (APSS) sent a letter of invitation to participate in a second study to all the citizens who tested positive for anti-SARS-CoV-2 antibodies in the serosurvey conducted 4 months before, between May 5 and 15, 2020.

Serum preparation and storage

Blood samples (5 ml) were collected in Serum Separator Tubes (BD Diagnostic Systems, Franklin Lakes, NJ, USA) and centrifuged at room temperature at 1600 rpm for 10 min. Aliquots were transferred to 2ml polypropylene, screw cap cryotubes (Sorfa, Zhejiang, China) and immediately frozen at -20 °C. Frozen sera were then shipped to the Istituto Superiore di Sanità (ISS) as national reference laboratory for COVID-19, in dry ice following biosafety shipment condition. Upon arrival serum samples were immediately stored at -80 °C [18].

SARS-CoV-2 IgG immunoassays for nucleocapsid (NC) and spike (S)

Two commercial chemiluminescent immunoassays (CLIA), employing either NC or S antigens and designed for high throughput in healthcare settings, were used. All the serum samples were evaluated by the Abbott SARS-CoV-2 IgG assay (Abbott Diagnostics, Chicago, IL, USA), using the NC antigen; sera resulting negative were retested with the DiaSorin Liaison SARS-CoV-2 IgG assay (DiaSorin, Italy), which uses S1/S2 antigen. The Abbott Diagnostics anti-NC IgG assay was performed on the Architect i2000SR automated analyser. The analyser automatically calculates SARS-CoV-2 NC IgG antibody concentration expressed as an index value. According to the manufacturer's instructions, the results were interpreted considering as positive an index of ≥1.4 and as negative an index of <1.4.

The DiaSorin SARS-CoV-2 IgG was performed on the LIAISON® XL fully automated chemiluminescence analyzer. The analyser automatically calculates SARS-CoV-2 S1/S2 IgG antibody concentrations expressed as arbitrary units (AU/mL). The assay range is up to 400 AU/mL. According to manufacturer's instructions, values ≥15 AU/mL were interpreted as positive, and values ≤12 AU/mL as negative, according to manufacturer's instructions; in case of results falling within an equivocal zone in between 12 AU/mL and 15 AU/mL, the test was repeated.

SARS-CoV-2 neutralizing antibody assay

In vitro neutralizing activity provides quantitative results as a measure of a functional humoral immune response against SARS-CoV-2. A known amount of SARS-CoV-2 (code 77III, isolated and cultivated at ISS, titer $1 \times 10^{5.4}$; GISAID accession ID: EPI_ISL_412973) was incubated with different dilutions of the serum sample to determine the dilution at which cytopathic effect on Vero E6 cells (ATCC® CRL-1586) is observed in 50% of infected wells (MN 50%). The detailed protocol is described below: two-fold serial dilutions of serum samples starting at 1:8 dilution up to 1:512 in cell culture medium EMEM (Sigma) supplemented with 1X pen/strep and 2% fetal bovine serum (FBS; Corning) were added to 96-well plates. The mixture of virus (100 $TCID_{50}$) and serum was incubated at 37 °C for 1 hour for a total volume of 100 µl. After this incubation period, a solution of 22,000 cells per well in a total volume of 100 µl was added and incubated at 37 °C for 5 days.

Finally, the neutralization titer was calculated and expressed as the serum dilution capable of reducing the cytopathic effect to 50% (MN 50%). Positive and negative sera samples and cell culture control together with the virus were added in each test.

Statistical analysis

The IgG levels were summarized by the median and by centiles (25th; 75th). The differences among IgG levels between the first and the second survey were evaluated by the Wilcoxon test. The differences among IgG levels between groups (positive versus negative in the second survey) in the first survey were assessed by Mann-Whitney test. The IgG levels observed in the first survey were categorised in tree classes: "*weak positive*" (between 1.4 and 3.0), "*medium positive*" (between 3.0 and 5.0), and "*high positive*" (>5). The McNemar's test was used to compare frequency on paired data. The concordance between anti-NC, anti-S, and TICD50 was evaluated using the Kappa test [19] (K <0.20 = "poor", 0.20-0.40 = "fair", 0.40-0.60 = "moderate", 0.60-0.80 = "good", and 0.80-1.00 = "very good").

A multivariable logistic regression model was used to determine the relationship between persistent anti-NC IgG in the second serosurvey (positive versus negative) and a set of explanatory variables. The following variables that were significantly associated (p<0.01) at the univariate analysis were included in the multivariable model: gender, age group, geographical area, presence of symptoms, working in contact with the public and household size, IgG positivity group (weak, medium,

high) olfactory and gustatory dysfunctions, fever, weakness, cough, dyspnea, arthralgia, diarrhoea, and abdominal pain and vomit. The likelihood ratio test was used to compare different models.

A subset of anti-NC IgG positive samples was tested with the neutralization test. Assuming a positive proportion of 95% and precision of 4%, 106 samples are required with an alpha error of 5%.

In all the analyses a two-sided p-value < 0.05 was considered statistically significant. Statistical analysis was performed by the STATA version 16.1 (STATA Corp., College Station, Texas, USA).

Ethical approval

Informed consent for blood collection was obtained from all the participants. The study was approved by the Ethical Committee of the ISS (Prot. PRE BIO CE n.15997, 04.05.2020).

RESULTS

Participation in the second survey

Overall, 1159 individuals of the 1402 individuals who resulted seropositive in the first survey (82.7%) were enrolled in the study *(Figure 1)*. All age groups were well represented. The proportion of those who were retested ranged between 72.6% in the age group 20-29 years and 93.1% in the age group 60-69 years.

Changes in antibody levels against NC

Of the 1159 individuals who resulted initially seropositive, 480 (41.4%) seroreverted at the second evaluation. As shown in *Figure 2*, a statistically significant reduction in the median value was observed in the second survey, from a median of 5.7 (25th centile = 3.9; 75th centile = 7.4) to 1.9 (25th centile = 0.8; 75th centile = 3.6) (p-value <0.0001 using the non-parametric Wilcoxon signed-rank test).

Comparing the median values in the positive and negative groups, those who seroreverted started from a lower average value (median = 3.6; 25th centile = 2.7;
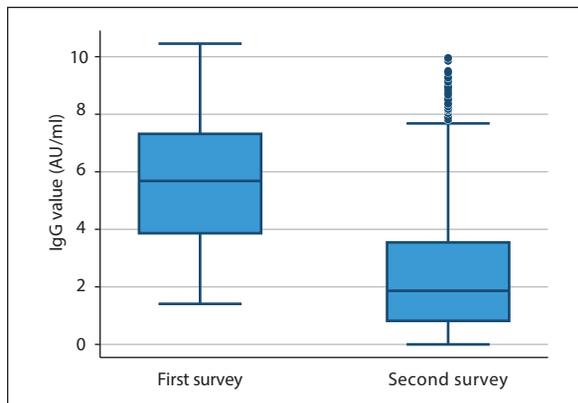
Figure 2
Distribution of the IgG values against SARS-CoV-2 nucleocapsid in the first and in the second serosurvey.

75th centile = 4.6) compared with those who remained positive (median = 7.0; 25th centile = 5.9; 75th centile = 8.2) at the second survey; the difference was statistically significant (Mann-Whitney test; p <0.0001).

As shown in *Figure 3*, when the participants were stratified into three groups in accordance with their anti-NC IgG level at the baseline (i.e., weak positive, with a value between 1.4 and 3; moderate positive, between 3 and 5; and high positive, greater than 5), the median value of the weakly and moderately positive groups decreased below the assay cut-off after 4 months, while the median of the highly positive remained above the cut-off.

Correlation between anti-SARS-CoV-2 IgG against NC and S proteins and neutralization activity

The samples resulting negative for antibodies against NC in the second study were tested to evaluate the presence of antibodies against the S protein. Since for one sample the available amount of serum was not sufficient for the analysis, 479 available serum samples
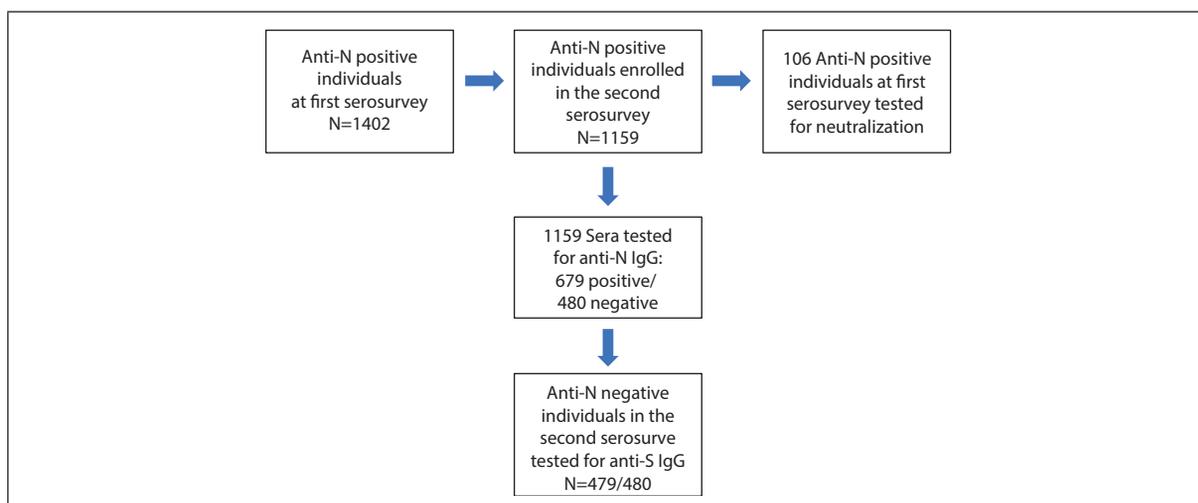
Figure 1
Study flow diagram of the sample collection and testing process.
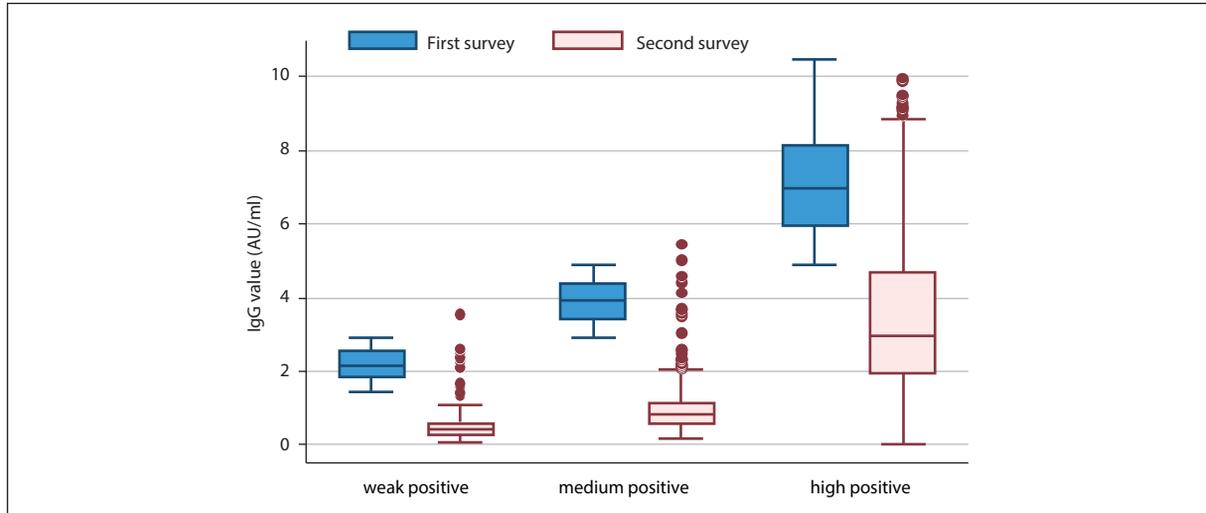
Figure 3
Median of the IgG values against SARS-CoV-2 nucleocapsid in the first and second survey by IgG positivity groups.

were tested, and 373 of them (77.9%) resulted positive *(Figure 4)*.

Comparison between serology and functional neutralization assay

One-hundred-six sera from a subgroup of individuals who tested positive in the initial study were selected for testing anti-NC IgG, anti-S IgG, and *in vitro* neutralizing activity 4 months after the baseline. Comparable numbers of weak, moderate, and high positive sera were selected. Of the 106 sera, 97 (91.5%) showed neutralizing activity (TCID50 ≥1/8), and 9 sera (8.5%) had a TCID50 titer <1/8; 57 (53.8%) were anti-NC positive and 93 (87.7%) were anti-S positive.

As shown in *Table 1*, only 53 sera showing neutralizing activity were anti-NC IgG positive (54.6%) versus 92 (94.8%) which were anti-S IgG positive. Most of the anti-NC IgG negative sera (41 out of 49) were anti-S positive (83.7%) and 44 had neutralizing activity (89.8%). Of the 57 anti-NC IgG positive sera, 52 were also anti-S positive (91.2%). Of 93 anti-S positive sera, 92 showed neutralizing activity. Overall, these data confirmed that despite a decline in anti-NC IgG levels below the positivity threshold, most of the sera are positive for anti-S IgG. A high concordance between anti-S positivity and neutralization activity, as calculated by

McNemar's test was found, showing that neutralizing activity relied on anti-S positivity.

High and significative agreement (94,3%) was found between anti-S and TCID$_{50}$ (k = 0.70; p <0.0001) *(Table 1)*. To further confirm the concordance, when IgG levels were considered, a good correlation between anti-S and TICD50 was observed (rho-Spearman: 0.84, p <0.0001) compared with anti-NC/anti-S (rho-Spearman: 0.61, p <0.0001) and anti-NC/TICD50 (rho-Spearman: 0.56, p <0.0001).

Factors associated with persistent anti-NC IgG after 4 months

The multivariable logistic regression model showed that age group, gender, anti-NC IgG level in the first serosurvey, and cough were factors associated with the persistence of anti-NC seropositivity *(Table 2)*. In particular, the individuals with high anti-NC IgG levels in the first serosurvey had the highest probability to be seropositive after four months (OR = 69.2). Age above 70 years and cough, as reported during the first survey, were also strongly associated with persistent anti-NC IgG levels, this association may be explained since those above 70 years of age and those with cough included a greater proportion of highly positive individuals.

DISCUSSION

Hereby, we report the results of a repeated serosurvey conducted in five municipalities in the AP of Trento, located in Northern Italy [18]. One of the main findings of the second survey, conducted on a large population of initially seropositive individuals, consisted in the rapid decrease of antibodies against the SARS-CoV-2 nucleocapsid. Of the 1159 participants, 41.1% resulted seronegative by 4 months after the first evaluation. Surprisingly, when we tested the NC-negative serum samples for antibodies directed against the S protein, we found different results, with most patients still showing seropositivity. To better understand and explain these findings, we evaluated the presence of neutralizing
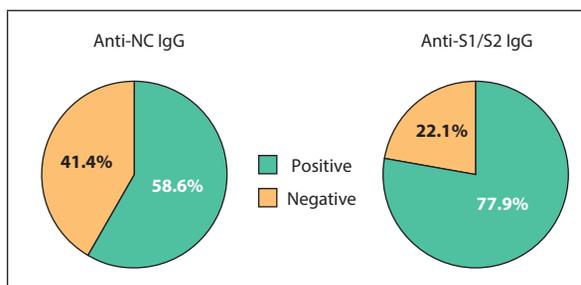
Figure 4
Percentage of anti-spike (S1/S2) IgG antibodies on retested anti-NC IgG negative sera.

Table 1
Concordance between IgG against NC and S proteins and neutralization activity

	Anti-S +	Anti-S -	p-value*	Agreement Kappa; p-value	TCID50≥1/8	TCID50<1/8	p-value*	Agreement Kappa; p-value
Anti-NC +	52	5	<0.0001	56.6%	53	4	<0.0001	54.7%
Anti-NC -	41	8		K=0.08; p=0.1186	44	5		K=0.03; p=0.2787
Anti-S +					92	1	0.1025	94.3%
Anti-S -					5	8		K=0.70; p<0.0001

* McNemar test.

antibodies in a subgroup of previously anti-NC seropositive individuals and found that almost all the sera positives for antibodies against the S protein were able to neutralize the virus entry into cell lines *in vitro*. The key role played by neutralizing antibodies in recipients of anti-SARS-CoV2 vaccines has been recently highlighted [20].

Correlates of protection have been identified for many viral infections. These correlates are usually based on a specific level of antibodies induced by vaccination or natural infection that significantly reduces the risk of (re-)infection. For some viral infections and vaccines, the kinetic of the antibody response is also known, allowing for a prediction of how long protection will persist [21]. Studies on SARS-CoV2 had shown con-

flicting results. Studies conducted on a smaller number of individuals and/or clinical series reported a decay of neutralizing antibody levels 2 months after infection [8, 9]. These results appear to be consistent with those obtained for other human coronaviruses, such as NL63, 229E, OC43, and HKU1, showing a rapid decay of antibodies directed against the nucleocapsid protein [22]. However, other studies showed different results, with high IgG levels after several months [10-12, 23]. The inconsistency in the results of previous studies could be explained by differences in the study populations (i.e., patients with mild vs moderate or severe disease) or by methodological heterogeneity across different studies (i.e., detection of antibodies directed against the NC vs whole S or the receptor binding domain of the spike) [8].

The rapid decay of anti-NC IgG observed in the present study is likely related to the fact that individuals with severe disease or who were institutionalized in nursing homes were excluded from the serosurvey, hence only asymptomatic and paucisymptomatic infections were evaluated. In a longitudinal study of RT-PCR confirmed COVID-19 cases, the participants showed a wide range of antibody responses, and a decline in antibodies levels and virus neutralization was observed within three months of the onset of symptoms [24]. For those who developed a low neutralizing antibody response the titers could return to baseline over a relatively short period, whereas those who developed a robust neutralizing antibody response maintained high titers despite the initial decline [24]. Although the persistence of protective antibodies might be explained, to some extent, by the sporadic COVID-19 reinfection that have been reported [25-27], a consensus is growing on a slow waning of antibody responses in the late convalescent period. In this regard, recent data show that SARS-CoV-2 infection protect from reinfection up to one year [3, 4].

The type of antibody response to infection or vaccination may also play a role. Atyeo *et al.* [28], showed that a predominant humoral response to NC protein is associated with poor outcome in patients admitted to hospital, compared to response to S protein. Accordingly, Wajnberg *et al.* showed that antibody responses to the S protein correlate significantly with SARS-CoV-2 neutralization [22], a finding confirmed by the present study.

Table 2
Factors associated with seropositivity (multivariable logistic regression model)

Variables	OR	95% CI	
Gender			
Female	Ref		
Male	1.80	1.26	2.56
Age group (years)			
<20	Ref		
20-29	0.61	0.30	1.26
30-39	0.70	0.33	1.47
40-49	0.99	0.52	1.90
50-59	1.29	0.68	2.44
60-69	1.30	0.68	2.48
70+	5.09	2.30	11.24
Anti-NC IgG positivity group in the first serosurvey			
Weak	Ref		
Moderate	2.29	1.16	4.55
High	69.23	35.84	133.72
Cough			
No	Ref		
Yes	2.05	1.16	3.63

The observation that anti-NC IgG persist less than anti-S IgG has an important practical implication, in fact the use of anti-NC assays in seroepidemiological studies may cause an underestimation of the real prevalence. On the other hand, anti-NC IgG assays are best candidates for distinction between natural infection and vaccination, as current vaccines are S-based. In this context, it is important to keep in mind that a negative anti-NC IgG result months after infection should consider the possibility of seroreversion rather than the lack of evidence for natural infection.

Before drawing conclusions, strengths and limits should be mentioned. Firstly, only 17.3% of individuals did not participate in the survey, thus the refusal rate was low, and the possibility of a selection bias was minimized. Secondly, the study was repeated approximately 4 months after the first test; however, a proportion of the participants was apparently asymptomatic and others reported having had symptoms suggestive of COVID-19 sometime before the survey. Thus, the 4 months represent the minimum interval of time elapsed between the virtual date of infection and the second test. Thirdly, although the serological assay we used is assumed to have high sensitivity and specificity, the occurrence of some false positive or false negative results influencing the reliability and consistency of the results could not be completely ruled out.

In conclusion, we found a general antibody decay over time, with a relatively high proportion of initially SARS-CoV-2 seropositive individuals losing their anti-NC antibodies by 4 months after the first positive test. However, most of these individuals still had neutralizing anti-S IgG antibodies, suggesting a potential long-term duration of protective immune response even in those individuals with an asymptomatic of paucisymptomatic infection. This finding may have important implications in the choice of the target for antibodies persistence over the time together with the potential effectiveness and long-term protection of immune responses induced by vaccines and on herd immunity. Further studies are needed to understand whether persistence of anti-S, potentially neutralizing antibodies, is actual correlate of long-term protection.

REFERENCES

1. Gidari A, Nofri M, Saccarelli L, et al. Is recurrence possible in coronavirus disease 2019 (COVID-19)? Case series and systematic review of literature. Eur J Clin Microbiol Infect Dis. 2021:40:1-12. doi: 10.1007/s10096-020-04057-6

2. Hall VJ, Foulkes S, Charlett A, et al. SARS-CoV-2 infection rates of antibody-positive compared with antibody-negative health-care workers in England: a large, multicentre, prospective cohort study (SIREN). Lancet 2021;397(10283):1459-69. doi: 10.1016/S0140-6736(21)00675-9

3. Vitale J, Mumoli N, Clerici P, et al. Assessment of SARS-CoV-2 reinfection 1 year after primary infection in a population in Lombardy, Italy. JAMA Intern Med. Published online May 28 2021. doi: 10.1001/jamainternmed.2021.2959

4. Khoury DS, Cromer D, Reynaldi A, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nat Med. 2021;27:1205-11. doi: 10.1038/s41591-021-01377-8

5. Channappanavar R, Zhao J, Perlman S. T cell-mediated immune response to respiratory coronaviruses. Immunol Res. 2014;59(1):118-28. doi: 10.1007/s12026-014-8534-z

6. Li-Ping Wu, Nai-Chang Wang, Yi-Hua Chang, Xiang-Yi Tian, Dan-Yu Na, Li-Yuan Zhang, Lei Zheng, Tao Lan, Lin-Fa Wang, Guo-Dong Liang. Duration of antibody responses after severe acute respiratory syndrome. Emerg Infect Dis. 2007;13(10):1562-4. doi: 10.3201/eid1310.070576

7. Kim DS, Rowland-Jones S, Gea-Mallorquí E. Will SARS-CoV-2 infection elicit long-lasting protective or sterilising immunity? Implications for vaccine strategies (2020). Front Immunol. 2020;11:571481. doi: 10.3389/fimmu.2020.571481

8. Ibarrondo FJ, Fulcher JA, Goodman-Meza D, et al. Rapid decay of anti-SARS-CoV-2 antibodies in persons with

mild COVID-19. N Engl J Med. 2020;383:1085-7. doi: 10.1056/NEJMc2025179

9. Long QX, Tang XJ, Shi QL, et al. Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. Nat Med. 2020;26:1002-1204. doi: 10.1038/s41591-020-0965-6

10. Dan JM, Mateus J, Kato Y, et al. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. Science. 2021;371(6529).

11. Chia WN, Zhu F, Ong SWX, et al. Dynamics of SARS-CoV-2 neutralising antibody responses and duration of immunity: a longitudinal study. Lancet Microbe. 2021;2(6):e240-e249. doi: 10.1016/S2666-5247(21)00025-2

12. Turner JS, Kim W, Kalaidina E, et al. SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans. Nature 2021;595:421-5. doi: 10.1038/s41586-021-03647-4

13. Grifoni A, Weiskopf D, Ramirez SI, et al. Targets of T-cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell. 2020;181:1489-1501.e15. doi: 10.1016/j.cell.2020.05.015

14. Deming D, Sheahan T, Heise M, et al. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med. 2006;3:e525. doi: 10.1371/journal.pmed.0030525

15. Duan L, Zheng Q, Zhang H, et al. The SARS-CoV-2 spike glycoprotein biosynthesis, structure, function, and antigenicity: implications for the design of spike-based vaccine immunogens. Front Immunol. 2020;11:576622. doi: 10.3389/fimmu.2020.576622

16. Wørzner K, Sheward DJ, Schmidt ST, et al. Adjuvanted SARS-CoV-2 spike protein elicits neutralizing antibodies and CD4 T cell responses after a single immunization in mice. EBioMedicine. 2021;63:103197. doi: 10.1016/j.ebiom.2020.103197

17. Shi Xiaojie, Li Yu, Yan lei, Yang Guang, Qiang Min. Neutralizing antibodies targeting SARS-CoV-2 spike protein, Stem Cell Res. 2021;50:102125. doi: https://doi.org/10.1016/j.scr.2020.102125

18. Stefanelli P, Bella A, Fedele G, et al. Prevalence of SARS-CoV-2 IgG antibodies in an area of North-eastern Italy with a high incidence of COVID-19 cases: a population-based study. Clin Microbiol Infect. 2021;27(4):633.e1-633.e7. doi: 10.1016/j.cmi.2020.11.013

19. McHugh ML. Interrater reliability: the kappa statistic. Biochem Med (Zagreb). 2012;22(3):276-82.

20. Bates TA, Leier HC, Lyski ZL, et al. Age-dependent neutralization of SARS-CoV-2 and P.1 variant by vaccine immune serum samples. JAMA. 2021;326(9):868-9. doi: 10.1001/jama.2021.11656

21. Jeyanathan M, Afkhami S, Smaill F, Miller MS, Lichty BD, Xing Z. Immunological considerations for COVID-19 vaccine strategies. Nat Rev Immunol, 2020;20:615-632. doi: 10.1038/s41577-020-00434-6

22. Edrige AWD, Kaczorowska J, Hoste ACR, et al. Seasonal coronavirus protective immunity is short-lasting. Nat Med. 2020;2:1691-3. doi: 10.1038/s41591-020-1083-1

23. Wajnberg A, Amanat F, Firpo A, et al. Robust neutralizing antibodies to SARS-CoV-2 infection persist for months. Science. 2020;370:1227-30. doi: 10.1126/science.abd7728

24. Seow J, Graham C, Merrick B, et al. Longitudinal observation and decline of neutralizing antibody responses in the three months following SARS-CoV-2 infection in humans. Nat Microbiol. 2020;5: 1598-607. doi: 10.1038/s41564-020-00813-8

25. To KK, Hung IF, Chan KH, et al. Serum antibody profile of a patient with COVID-19 reinfection. Clin Infect Dis. 2021;72(10):e659-e662. doi: 10.1093/cid/ciaa1368

26. Gupta V, Bhoyar RC, Jain A, et al. Asymptomatic reinfection in two healthcare workers from India with genetically distinct SARS-CoV-2. Clin Infect Dis. 2020;ciaa1451. doi: 10.1093/cid/ciaa1451

27. Tillett RL, Sevinsky JR, Hartley PD, et al. Genomic evidence for reinfection with SARS-CoV-2: a case study. Lancet Infect Dis. 2021;21(1):528. doi: 10.1016/S1473-3099(20)30764-7

28. Atyeo C, Fischinger S, Zohar T, et al. Distinct early serological signatures track with SARS-CoV-2 survival. Immunity. 2020;53:524-32.e4. doi: 10.1016/j.immuni.2020.07.020

ORIGINAL ARTICLES AND REVIEWS

ORIGINAL ARTICLES AND REVIEWS

The first SARS-CoV-2 wave among pregnant women in Italy: results from a prospective population-based study

Serena Donati[1], **Edoardo Corsi**[1,2], **Alice Maraschini**[3], **Michele Antonio Salvatore**[1] and the ItOSS COVID-19 Working Group*

[1]*Centro Nazionale per la Prevenzione delle Malattie e Promozione della Salute, Istituto Superiore di Sanità, Rome, Italy*
[2]*Dipartimento di Biomedicina e Prevenzione, Università degli Studi Tor Vergata, Rome, Italy*
[3]*Servizio Tecnico Scientifico di Statistica, Istituto Superiore di Sanità, Rome, Italy*
The members of the ItOSS COVID-19 Working Group are reported before the References

Abstract
Introduction. This study aimed to estimate the incidence of SARS-CoV-2 infection among pregnant women during the first pandemic wave in Italy, and to describe COVID-19 disease characteristics and maternal and perinatal outcomes.
Materials and methods. National population-based prospective cohort study collecting information on women with SARS-CoV-2 diagnosis, confirmed within 7 days from hospital admission.
Results. The national SARS-CoV-2 rate was 6.04 per 1,000 births (95% CI 5.62-6.49) among pregnant women and 7.54 (95% CI 7.47-7.61) among women in reproductive age. 72.1% of the cohort developed mild COVID-19 disease without pneumonia nor need for ventilatory support. Severe disease was significantly associated with women's previous comorbidities (OR 2.55; 95% CI 0.98-6.90), obesity (OR 4.76; 95% CI 1.79-12.66) and citizenship from High Migration Pressure Countries (OR 3.43; 95% CI 1.27-9.25).
Conclusions. During the first pandemic wave in Italy, the SARS-CoV-2 rate among pregnant women was lower compared to that detected among women of reproductive age, and risks of severe COVID-19 disease and adverse maternal and perinatal outcomes were rare.

INTRODUCTION

At the beginning of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic, it was feared that the virus could cause the same dramatic maternal and perinatal outcomes observed during the outbreak of other respiratory viruses, such as SARS-CoV-1, Middle East Respiratory Syndrome (MERS) and H1N1 flu [1].

Research capable of gathering sound information in support of public health recommendations and clinical practice was therefore urgently needed. Several publications of single case reports and case series [2] were meta-analysed in a living systematic review by WHO [3]. International multicentre registries [4-6] recruiting from many different countries, have been established, with frequent data overlapping influencing the quality of successive systematic reviews [7-11]. Because of the lack of population-based studies, the proportion of ascertained cases were unclear due to unknown underlying denominators. Therefore, interpreting the findings from these studies in order to give a confident estimate of the true rate of complications for women infected during pregnancy and for their newborns was challenging.

Few countries, participating in the International Network of Obstetric Survey System (INOSS) [12] including Italy with the Italian Obstetric Surveillance System (ItOSS), launched prospective population-based cohort studies able to reliably estimate the prevalence of SARS-CoV-2 infection, investigate COVID-19 disease characteristics and describe adverse maternal and perinatal outcomes. Preliminary results published by the UK Obstetric Surveillance System (UKOSS) [13], ItOSS [14-16] and Nordic Obstetric Surveillance System (NOSS) – that includes Sweden, Denmark, Finland and Norway – [17], showed an absolute low risk of severe COVID-19 disease and rare adverse maternal

Address for correspondence: Serena Donati, Centro Nazionale per la Prevenzione delle Malattie e Promozione della Salute, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: serena.donati@iss.it.

and perinatal outcomes during the first pandemic wave.

The aim of this paper, which is an extension of a previous published series [14], was to estimate the incidence rate of SARS-CoV-2 infection among pregnant women during the first pandemic wave in Italy, and to describe COVID-19 disease characteristics and maternal and perinatal outcomes.

MATERIALS AND METHODS

This national population-based prospective cohort study collected information on women with confirmed SARS-CoV-2 infection admitted to any Italian hospital during pregnancy and within 42 days from its outcome.

Trained reference clinicians in each of the 315 participating maternity hospitals (*Appendix 1*) entered the requested information in a web-based secure system. The online form investigating women's socio-demographic characteristics, medical and obstetric history, disease management, mode of delivery and maternal and perinatal outcomes was revised and pre-tested by a multidisciplinary group of experts. Complete data reporting was ensured by weekly email reminders and phone contacts with the reference clinicians.

Confirmed SARS-CoV-2 infection was defined as the detection of viral RNA on reverse transcriptase-polymerase chain reaction (RT-PCR) testing of nasopharyngeal swab and/or blood and/or the radiological diagnosis of COVID-19 pneumonia. Neonatal SARS-CoV-2 infection was defined as the detection of viral RNA on RT-PCR testing of a nasopharyngeal swab.

In Italy, until the end of March 2020 only symptomatic pregnant women and those defined as close contacts of a SARS-CoV-2 infected person were tested. In April, the Regions progressively adopted universal screening policies, and from May all pregnant women admitted to hospital were tested, regardless of symptoms or exposure.

The present analysis refers to the first pandemic wave, defined as the period between February 25 and August 31, 2020 and includes hospitalized pregnant women with SARS-CoV-2 diagnosis confirmed within 7 days from hospital admission.

ETHICS AND CONSENT

The Ethics Committee of the Istituto Superiore di Sanità (Italian National Institute of Health) approved the project (Prot. 0010482 CE 01.00, Rome 24/03/2020). The study protocol is available at https://www.epicentro.iss.it/en/coronavirus/sars-cov-2-pregnancy-childbirth-breastfeeding-prospective-study-itoss (Italian).

An informed consent to participate in the study was acquired from any woman at study enrolment.

OUTCOMES

The main outcome measures included in the study are: COVID-19 pneumonia confirmed by chest imaging, mechanical ventilatory support (non-invasive mechanical ventilation, orotracheal intubation, extracorporeal membrane oxygenation – ECMO), intensive care unit (ICU) admission. COVID-19 disease severity was defined as follows:

a. mild disease: absence of COVID-19 pneumonia;
b. moderate disease: confirmed COVID-19 pneumonia requiring at most oxygen therapy;
c. severe disease: confirmed COVID-19 pneumonia requiring mechanical ventilatory support and/or ICU admission.

Secondary outcomes include: maternal mortality (maternal death during pregnancy or within 42 days from any pregnancy outcome), maternal severe morbidity, preterm birth (22-31 and 32-36 gestational weeks), mode of delivery (vaginal, elective caesarean section (CS), urgent/emergency CS due to COVID-19, urgent/emergency CS due to maternal/foetal indications), stillbirth (intrauterine foetal death ≥22 completed weeks of gestation), low birth weight (<2,500g), neonatal intensive care unit (NICU) admission, neonatal mortality (death of a live-born infant <7 days of life) and neonatal severe morbidity.

COVARIATES

Covariates include the following socio-demographic and medical characteristics: women's age (<30, 30-34, ≥35 years), citizenship (Italian, High Migration Pressure Countries – HMPCs, not HMPCs) [18], educational level (low: primary school or lower; medium: high school; high: bachelor's degree or higher), previous comorbidities (at least one of the following: diabetes, asthma requiring medical treatment, hypertension, cardiovascular diseases, lung diseases, HIV/AIDS, other morbidities), obesity (body mass index [BMI]>30 kg/m^2).

STATISTICAL ANALYSIS

Statistical analyses were performed using the Statistical Package STATA/MP version 14.2. Frequency distributions, prevalence and odds ratios (ORs) with their 95% confidence intervals (CI) were used to describe data. Missing data were excluded when their proportion was lower than 5%, otherwise included as a modality in the frequency distributions.

The national SARS-CoV-2 incidence rate with 95% CI was estimated among pregnant women. All the hospitalized and outpatient women, with ongoing pregnancy or who gave birth during the study period, irrespective of time of diagnosis, were included in the numerator. Latest available (2019) data on deliveries from the national Birth Registry were used as denominator [19], applying a 3.6% reduction in accordance with the Italian National Statistics Institute (ISTAT) estimate for births variation between 2019 and 2020 [20]. Deliveries were weighted with an estimate of the time of exposure to the risk of infection during pregnancy. The incidence rate among pregnant women has been compared with the rate among the background population of women of reproductive age (15-49 years), calculated considering the SARS-CoV-2 positive cases notified to the national surveillance system during the exact study period. [21].

Percentage distributions of socio-demographic, medical and obstetric characteristics stratified by severity of COVID-19 disease were calculated.

The association between infection severity and potential risk factors (woman's age, citizenship, educational

level, presence/absence of previous comorbidities, and presence/absence of obesity) was assessed by estimating mutually adjusted ORs and their 95% CI, through a multinomial logistic regression model. Plausible interactions (corresponding to all pairwise interactions between the variables included in the model) were tested using the Likelihood Ratio Test (p<0.05). The model was performed on complete cases defined as cases without missing data for any variable of interest (see *Appendix 2* for details about handling of missing data).

Prevalence of mode of delivery and maternal and neonatal outcomes were stratified by infection severity. CS, preterm birth, and neonatal birthweight were compared with data retrieved from the 2019 national Birth Register, and unadjusted risk ratios (RRs) were estimated.

In this observational study, no formal power calculation was performed because the sample size was governed by the disease incidence.

RESULTS

From February 25 to August 31, 2020, the trained clinicians of the 315 Italian participating maternity units (*Appendix 1*) notified 786 women with current or previous confirmed SARS-CoV-2 infection during pregnancy and up to 42 days after childbirth (*Figure 1*). Most of the cases (84.6%) occurred in northern Italy, 10.3% in the Centre and 5.1% in the South. As described in *Figure 1*, this study includes 548 women with ongoing pregnancy or who gave birth, admitted to hospital with a positive SARS-CoV-2 test within 7 days from admission.

The national SARS-CoV-2 incidence rate among pregnant women was 6.04 per 1,000 births (95% CI 5.62-6.49), slightly lower than the rate of 7.54 per 1,000 women (95% CI 7.47-7.61) estimated among the background population of Italian women of reproductive age. The incidence rate among pregnant women ranged between 11.15/1,000 (95% CI 10.31-12.07) in the North, 3.25/1,000 (95% CI 2.59-4.08) in the Centre, and 0.87/1,000 (95% CI 0.63-1.20) in the South of the country. The corresponding figures among women of reproductive age were respectively 12.52/1,000 (95% CI 12.39-12.65), 5.19/1,000 (95% CI 5.07-5.32) and 2.50/1,000 (95% CI 2.43-2.57).

Table 1 describes women's socio-demographic, medical and obstetric characteristics, stratified by COVID-19 disease severity. The vast majority of the cohort (72.1%; n = 395) developed a mild disease, 22.4% (n = 123) a moderate and 5.5% (n = 30) a severe disease. Women's mean age was 31.9 years (SD = 5.54); the percentage of women with foreign citizenship was 28.6%, ranging from 26.1% in the mild disease group to 46.7% in the severe group. Pre-existing comorbidities and obesity concerned respectively 17.8% and 12.1% of the entire cohort and 34.5% and 44.8% of the women with severe disease. Information on educational level was missing for 28.5% of the cases.

At first positive SARS-CoV-2 test, 85.8% of the women were at ≥28 weeks of gestation, 11.8% between 15 and 27 weeks, and 2.4% ≤14 weeks. The vast majority (95.2%) has been diagnosed through a RT-PCR of nasopharyngeal swab specimen, 2.4% respectively through chest imaging and through blood antibodies detection (data not shown). Overall, during the hospital stay, 22.6% (n = 124) of women was with ongoing pregnancy and 77.4% (n = 424) gave birth (*Table 1*). Women with ongoing pregnancy were admitted to hospital mostly for COVID-19 disease (75.0%) while other obstetric reasons or delivery were the main causes for hospitalization of those who gave birth (85.8%) (*Table 1S*, available online as Supplementary material).
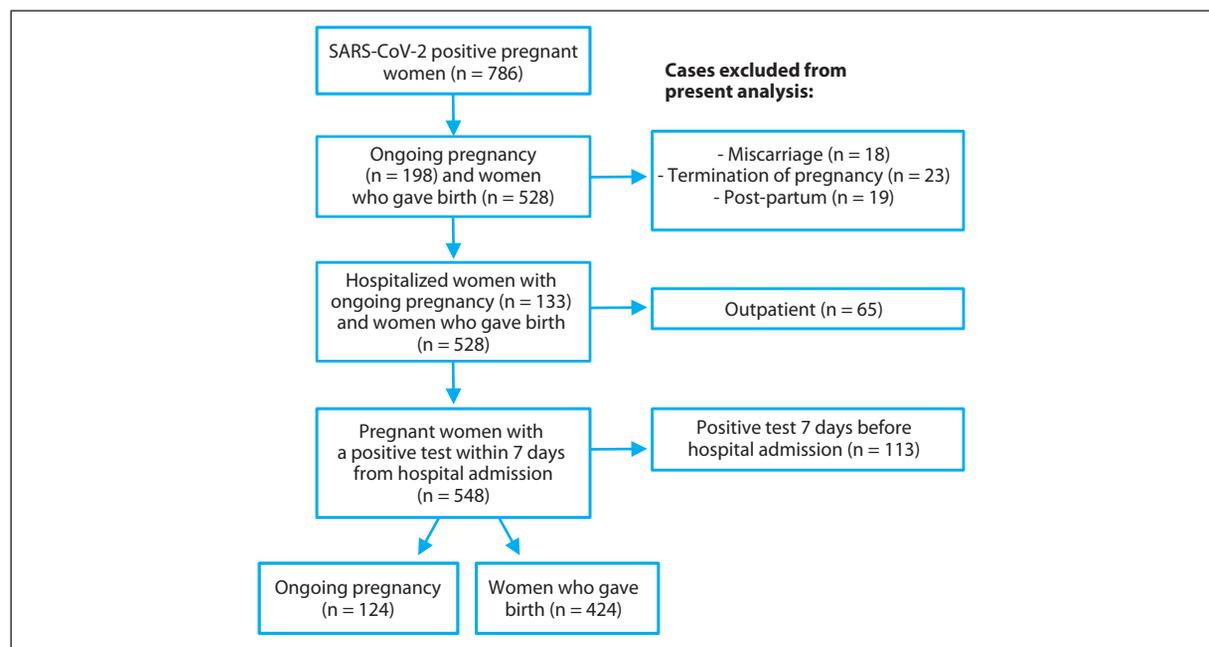
Figure 1
Women enrolled in the ItOSS cohort from February 25 to August 31, 2020.

Table 1
Women's characteristics by COVID-19 disease severity

	Mild[a] (n = 395)		Moderate[b] (n = 123)		Severe[c] (n = 30)		Total (N = 548)	
	n	%	n	%	n	%	N	%
Age, years (7 missing)								
<30	126	32.5	41	33.3	9	30.0	176	32.5
30-34	140	36.1	37	30.1	10	33.3	187	34.6
≥35	122	31.4	45	36.6	11	36.7	178	32.9
Citizenship								
Italian	292	73.9	83	67.5	16	53.3	391	71.4
HMPCs	101	25.6	40	32.5	14	46.7	155	28.3
Not HMPCs	2	0.5	0	0.0	0	0.0	2	0.4
Country of birth								
Italy and western Europe	262	66.3	75	61.0	12	40.0	349	63.7
East Europe	27	6.8	9	7.3	3	10.0	39	7.1
Africa	46	11.6	15	12.2	9	30.0	70	12.8
South/Central America	29	7.3	13	10.6	4	13.3	46	8.4
Asia	31	7.8	11	8.9	2	6.7	44	8.0
Level of education*								
Low	78	19.7	20	16.3	8	26.7	106	19.3
Medium	121	30.6	42	34.1	10	33.3	173	31.6
High	81	20.5	28	22.8	4	13.3	113	20.6
Missing	115	29.1	33	26.8	8	26.7	156	28.5
Previous comorbidities (10 missing)	56	14.5	30	24.6	10	34.5	96	17.8
Pre-gestational diabetes	4	1.0	2	1.6	3	10.3	9	1.7
Autoimmune diseases	7	1.8	5	4.1	0	0.0	12	2.2
Chronic hypertension	3	0.8	3	2.5	5	17.2	11	2.0
BMI >30 kg/m² (10 missing)	36	9.3	16	13.1	13	44.8	65	12.1
Multiparous (2 missing)	215	54.6	79	64.2	16	55.2	310	56.8
Multiple pregnancy (1 missing)	8	2.0	3	2.4	1	3.3	12	2.2
Gestational age at diagnosis, weeks (14 missing)								
≤14	8	2.1	4	3.4	1	3.3	13	2.4
15-27	25	6.5	27	23.1	11	36.7	63	11.8
≥28	354	91.5	86	73.5	18	60.0	458	85.8
Ongoing pregnancy	57	14.4	56	45.5	11	36.7	124	22.6

[a]Absence of COVID-19 pneumonia.
[b]Confirmed COVID-19 pneumonia requiring at most oxygen therapy.
[c]Confirmed COVID-19 pneumonia requiring mechanical ventilatory support and/or ICU admission.
HMPCs: high migration pressure countries.
*Low: primary school or lower; medium: high school; high: bachelor's degree or higher.

Figure 2 describes the weekly trend of the number of positive pregnant women enrolled during the study period, stratified by COVID-19 disease severity. The majority of cases (61.3%), including all severe and most moderate cases, occurred between March and April 2020.

At time of diagnosis, 45.5% of the women was asymptomatic with an increasing trend ranging from 10.8% in March to 74.2% in July - August 2020 (*Figure 3*). Fever (36.3%), cough (33.7%) and tiredness (21.0%) were the most frequently reported symptoms. Dyspnoea was re-

ported by 14.7% of the women, 5.4% among those with mild disease and 76.7% among those with severe COVID-19 disease (*Table 2S*, available online as Supplementary material).

Table 2 shows the ORs of developing moderate and severe disease *vs* mild disease, mutually adjusted for women's age, citizenship, educational level, previous comorbidities and obesity. Women with at least one previous comorbidity were more likely to develop a moderate (OR = 1.87; 95% CI 1.03-3.41) and a severe
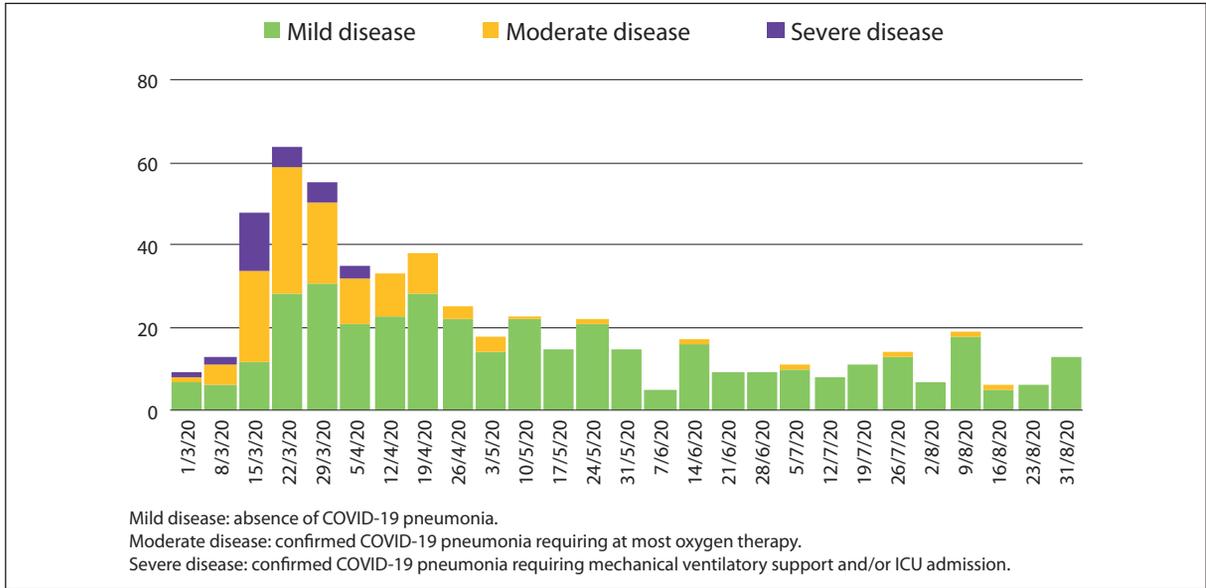
Figure 2
Weekly trend of the number of enrolled women by COVID-19 disease severity, between February 25 and August 31, 2020 (n = 548).

(OR = 2.55; 95% CI 0.98-6.90) COVID-19 disease. The occurrence of severe illness was significantly higher among obese women (OR = 4.76; 95% CI 1.79-12.66) and those with citizenship from HMPCs (OR = 3.43; 95% CI 1.27-9.25). No statistically significant association was found with educational level. None of the tested plausible interactions was statistically significant.

Table 3 describes women's and perinatal outcomes stratified by disease severity. Overall, 29 women (5.3%) received non-invasive ventilatory support, six (1.1%) underwent orotracheal intubation and two (0.4%) received ECMO. Eighteen women (3.3%) were admitted

to ICU and 23 (4.2%) developed severe morbidity. No maternal deaths occurred.

Overall, ten of the 438 livebirths (2.3%) developed severe morbidity and 63 (14.7%) were admitted to NICU (*Table 3*). The percentage of neonates with a birthweight <2,500 grams was 13.6%, higher than the 7.1% national proportion in 2019 [19] (RR = 1.91; 95% CI 1.50-2.43). As shown in *Table 3* neonatal morbidity, access to NICU and low birthweight were more common among mothers with more severe conditions. Four stillbirths (0.9% of total births) were recorded and no neonatal deaths occurred. Overall, 4% of the livebirths
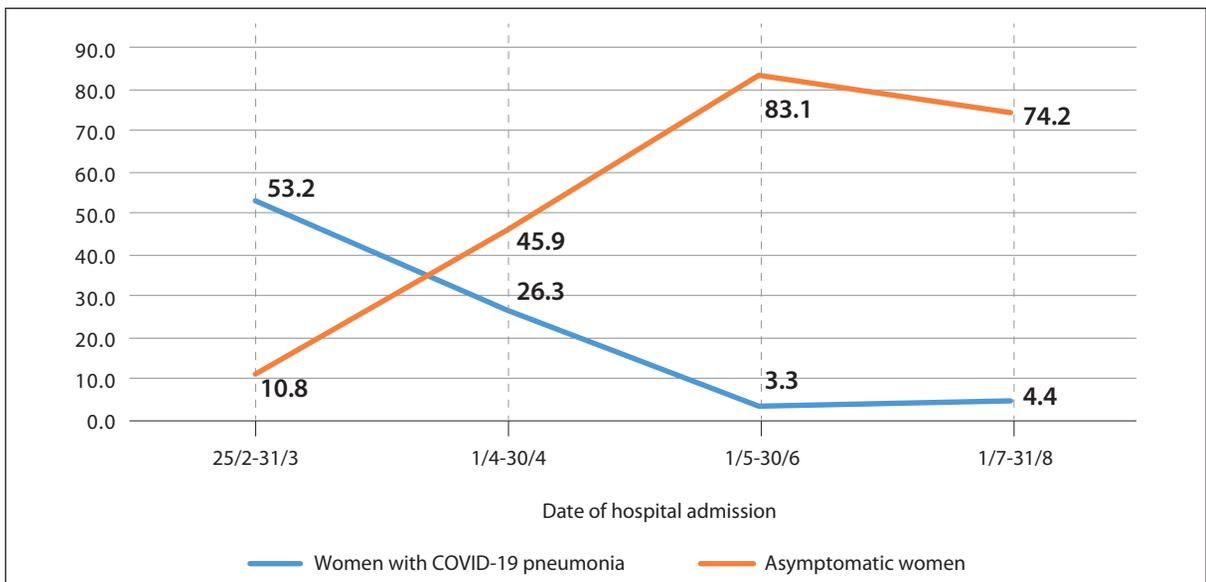
Figure 3
Temporal trend of proportions of women with COVID-19 pneumonia and asymptomatic women.

Table 2
Mutually adjusted odds ratios of moderate and severe disease for the selected variables

Variable	OR (95% CI)	
	Moderate *vs* mild	Severe *vs* mild
Age		
<34	1.00	1.00
≥35	1.20 (0.72-1.99)	1.26 (0.49-3.26)
Citizenship		
Italian + no HMPCs	1.00	1.00
HMPCs	1.47 (0.82-2.62)	3.43 (1.27-9.25)
Level of education[a]		
Low	1.00	1.00
Medium-high	1.65 (0.89-3.06)	1.41 (0.50-3.99)
Previous comorbidities		
No	1.00	1.00
Yes	1.87 (1.03-3.41)	2.55 (0.98-6.90)
Obesity		
No	1.00	1.00
Yes	1.15 (0.55-2.39)	4.76 (1.79-12.66)

Mild disease: absence of COVID-19 pneumonia.
Moderate disease: confirmed COVID-19 pneumonia requiring at most oxygen therapy.
Severe disease: confirmed COVID-19 pneumonia requiring mechanical ventilatory support and/or ICU admission.
HMPCs: high migration pressure countries.
[a]Low: primary school or lower; medium-high: high school or higher.

had a positive SARS-CoV-2 test, 10 within and 7 after 24 hours from birth. Among positive babies, eight were delivered vaginally and nine by CS.

Table 4 shows mode of delivery and gestational age at birth among the 424 SARS-CoV-2 positive women who gave birth. The CS rate was 33.6%, close to the national figure of 31.8% recorded in 2019 [19] (RR = 1.06; 95% CI 0.93-1.21). Urgent and emergency CS were significantly more frequent among mothers with severe COVID-19 disease. CS was performed under general anaesthesia in 7.3% of the cases and in 31.3% of the women with severe COVID-19 illness. The proportion of preterm delivery (13.7%) was higher compared to the 6.7% national average [19] (RR = 2.05; 95% CI 1.60-2.61), especially among mothers with severe disease (63.2%). Iatrogenic indications – defined as elective CS or induction of labour – were responsible for 22.6% of the recorded preterm births.

DISCUSSION
Principal findings
The national incidence rate of SARS-CoV-2 infection in pregnancy during the first pandemic wave in Italy (6.04 per 1,000 births; 95% CI 5.62-6.49) was lower compared to the rate estimated among the background population of women of reproductive age [21] (7.54 per 1,000 women; 95% CI 7.47-7.61).

The vast majority of SARS-CoV-2 positive pregnant

women (72.1%) developed a mild disease without COVID-19 pneumonia and no need for ventilatory support, 22.4% a moderate illness with confirmed pneumonia not requiring any mechanical ventilatory support (at most oxygen therapy), and only 5.5% a severe disease requiring mechanical ventilatory support and/or ICU admission. Previous comorbidities, obesity and foreign citizenship from HMPCs were significantly associated to a higher occurrence of severe disease. Overall, the enrolled pregnant women had an absolute low risk of severe maternal (4.2%) and perinatal (2.3%) morbidity.

Strengths and weakness of the study
A strength of the present study is the nationwide prospective population-based cohort design. The offer of routine screening tests at hospital admission from May 2020, provided a complete denominator assuring reliably ascertainment of incident cases and robust estimates of COVID-19 severe disease among positive pregnant women. Stratifying the cohort by COVID-19 disease severity, instead of presence/absence of SARS-CoV-2 symptoms, represents a further strength of the study, allowing a better portray of women's clinical conditions. The accuracy of the collected data has been monitored and assured by the durable network of trained clinicians in each participating maternity unit and by the weekly email reminders and phone contacts, in order to solicit case notification and recovery of essential missing information.

The study limitations include the absence of a control group of pregnant women without SARS-CoV-2 infection and the small number of women diagnosed during the first two trimesters of pregnancy (14.2%), which requires further analysis to investigate possible effects of early infection. The lack of information on the pregnancy status of women notified to the national SARS-CoV-2 surveillance did not allow a crosscheck of the cases detected through the ItOSS study. In addition, in Italy universal testing for hospitalized pregnant women was implemented from May 2020, we might therefore have missed cases occurred during the first two months of the study. Failure to identify these cases leads to a possible underestimation of the phenomenon among pregnant women, and to a greater extent among the background population of women of reproductive age for whom the screening offer was partial and delayed.

Moreover, due to the restrained circulation of the virus in centre and southern Italy during the first pandemic wave, we cannot generalize the findings of this paper to the whole country.

Comparison with other studies
Similarly to the UKOSS cohort [22], women with ongoing pregnancy compared to those who gave birth were hospitalized more often due to COVID-19 disease. As reported by previous studies [10, 13, 17, 22-24], women with previous comorbidities, obese, and foreigners from HMPCs showed a significantly higher occurrence of more severe forms of COVID-19 disease. A pattern of disadvantaged social conditions affecting ethnic minorities [25, 26] may be linked to worse clinical conditions observed in migrant women.

Table 3
Women's and perinatal outcomes

Women's outcome	Mild[a] (n = 395)		Moderate[b] (n = 123)		Severe[c] (n = 30)		Total (N = 548)	
	n	%	n	%	n	%	N	%
Respiratory support								
Oxygen therapy	8	2.0	51	41.5	30	100.0	89	16.2
Non-invasive ventilatory support	0	0.0	0	0.0	29	96.7	29	5.3
Orotracheal intubation	0	0.0	0	0.0	6	20.0	6	1.1
ECMO	0	0.0	0	0.0	2	6.7	2	0.4
ICU admission	0	0.0	0	0.0	18	60.0	18	3.3
Severe maternal morbidity*	5	1.3	7	5.7	11	36.7	23	4.2
Maternal death	0	0.0	0	0.0	0	0.0	0	0.0
Perinatal outcome	(n = 343)		(n = 69)		(n = 20)		(n = 432)	
Stillbirth	3	0.9	1	1.4	0	0.0	4	0.9
Livebirth	340	99.1	68	98.6	20	100.0	428	99.1
Severe neonatal morbidity**	3	0.9	5	7.4	2	10.0	10	2.3
NICU admission	41	12.1	12	17.6	10	50.0	63	14.7
Neonatal death	0	0.0	0	0.0	0	0.0	0	0.0
Birthweight <2500 grams (3 missing)	38	11.3	10	14.7	10	50.0	58	13.6
5-min Apgar score								
<7	1	0.3	0	0.0	1	5.0	2	0.5
≥7	311	91.5	63	92.6	15	75.0	389	90.9
Missing	28	8.2	5	7.4	4	20.0	37	8.6
Neonatal positive SARS-CoV-2 test:								
<24 hours from delivery	7	2.1	3	4.4	0	0.0	10	2.3
≥24 hours from delivery	4	1.2	1	1.5	2	10.0	7	1.6

[a]Absence of COVID-19 pneumonia.
[b]Confirmed COVID-19 pneumonia requiring at most oxygen therapy.
[c] Confirmed COVID-19 pneumonia requiring mechanical ventilatory support and/or ICU admission.
*Shock, acute respiratory stress syndrome, kidney failure, other.
**Acute respiratory distress syndrome, interstitial pneumonia, intraventricular haemorrhage, necrotizing enterocolitis, neonatal encephalopathy, sepsis, other.
ECMO - extracorporeal membrane oxygenation; ICU - intensive care unit; NICU - neonatal intensive care unit.

Table 4
Mode of delivery and gestational age at birth by COVID-19 disease severity

Outcome	Mild[a] (n = 338)		Moderate[b] (n = 67)		Severe[c] (n = 19)		Total (N = 424)	
	n	%	n	%	n	%	N	%
Mode of delivery (2 missing)								
Vaginal	237	70.1	41	62.1	2	11.1	280	66.4
Elective CS	55	16.3	10	15.2	0	0.0	65	15.4
Urgent/emergency CS due to maternal/foetal indication	43	12.7	10	15.2	6	33.3	59	14.0
Urgent/emergency CS due to COVID-19	3	0.9	5	7.6	10	55.6	18	4.3
Gestational age at birth*, weeks (16 missing)								
≤31	4	1.2	3	4.5	5	26.3	12	2.9
32-36	26	7.9	11	16.4	7	36.8	44	10.8
≥37	299	90.9	46	68.7	7	36.8	352	86.3
Missing	9	-	7	10.4	0	-	16	-

[a]Absence of COVID-19 pneumonia.
[b]Confirmed COVID-19 pneumonia requiring at most oxygen therapy..
[c]Confirmed COVID-19 pneumonia requiring mechanical ventilatory support and/or ICU admission.
CS: caesarean section.
*Missing data were not ignored among the Moderate group because higher than 5%.

Although Italy holds one of the highest CS rates in the world [27], during the pandemic it was close to the 2019 national rate [19] (33.6% *vs* 31.8%), and significantly lower compared to the figure reported in two systematic reviews [10, 28] and by other European countries [13, 17, 22] that usually record lower rates than Italy. The prompt and wide dissemination among Italian obstetricians of the evidence of lack of indication to CS in case of SARS-CoV-2 infection [29, 30] probably helped in limiting it to women in critical conditions, which in fact underwent urgent/emergency CS due to COVID-19 in 55.6% of the cases.

Preterm delivery has been an issue of concern during the pandemic. To date, most studies confirm a higher risk of preterm birth among SARS-CoV-2 positive women, especially in case of severe disease [10, 22, 24]. We detected a two-fold rate (13.7%) compared to the 2019 national figure (6.7%), with significant differences between women affected by severe COVID-19 disease (63.2%) and those with mild disease (9.1%). Moreover, the estimated preterm birth rate could be underestimated due to the missed identification of SARS-CoV-2 positive women not hospitalized before the due date, responsible for a possible deflated denominator. Excluding the proportion of cases with iatrogenic indications (22.6% of preterm births), spontaneous preterm birth rate was equal to 10.6%, mostly due to late preterm births. Although newborns were more likely to be admitted to NICU, no increase in stillbirths and neonatal deaths compared to previous national data was observed, in accordance with the UKOSS data. Given the possibility of a deflated denominator due to the missed identification of SARS-CoV-2 positive women during the first two months of the study, also the reported estimate of low birthweight should be interpreted with caution.

As for maternal outcomes, 153 women (27.9%) was affected by COVID-19 pneumonia but only 23 (4.2%) developed severe morbidity. The prevalence of pneumonia (27.9%; 95% CI 24.3-31.8) was lower compared to that reported by Northern Europe (57.1%) [17], in line with the Spanish data (30.8%) [31], and higher compared to Allotey's systematic review (17.5%) [10] and UKOSS data (15%) [22].

Consistently with other studies [17, 22], poor neonatal outcomes were rare, no neonatal deaths occurred and four stillbirths (0.9%) were notified. During the first pandemic wave, 17 neonates (4.0%; 95% CI 2.5-6.3) had a SARS-CoV-2 positive test at birth. Data from UKOSS and US showed similar percentages, respectively 2% [22] and 2.5% [32]. Our findings cannot confirm or deny the hypothesis of a transplacental virus transmission [33], but reassure on the good outcomes of these positive babies [34].

CONCLUSIONS

The Italian ob-gyn health professionals have shown to be able to manage the emergency context, despite initial fear and uncertainty. Differently from international retrospective non population-based studies [2,4-6], and similarly to the prospective population-based results of the European INOSS cohorts [13,17,22], in Italy the SARS-CoV-2 incidence rate among women was comparable to the one detected among the background population of women of reproductive age [21], and the vast majority of pregnant women and newborns had mild disease and good outcomes. Except for the higher risk of preterm birth, that concerned mainly women with severe COVID-19 disease, the results of this study should reassure women, health professionals and decision makers about the impact of the SARS-CoV-2 infection in pregnancy during the first pandemic wave.

Conflict of interest statement

The Authors and the Working Group members report no conflict of interest.

Individual contribution to the manuscript

Serena Donati: conceptualization, methodology, formal analysis, writing original draft, project administration, supervision; Edoardo Corsi: software, investigation, data curation, writing-review and editing, project administration; Alice Maraschini: methodology, formal analysis; Michele Antonio Salvatore: methodology, software, formal analysis, writing-review and editing; Federica Baltaro: investigation, data curation; Rosaria Boldrini: cooperation in formal analysis; Silvia Bonassisa: investigation, data curation; Roberto Brunelli: investigation, data curation; Angelo Cagnacci: investigation, data curation; Paola Casucci: investigation, data curation, supervision; Ilaria Cataneo: investigation, data curation; Irene Cetin: investigation, data curation, supervision; Elena De Ambrosi: investigation, data curation; Martina Del Manso: cooperation in formal analysis; Massimo Fabiani: cooperation in formal analysis; Stefania Fieni: investigation, data curation; Massimo Piergiuseppe Franchi: investigation, data curation, supervision; Enrico Iurlaro: investigation, data curation; Livio Leo: investigation, data curation, supervision; Marco Liberati: investigation, data curation, supervision; Lucia Li Sacchi: investigation, data curation; Stefania Livio: investigation, data curation; Mariavittoria Locci: investigation, data curation, supervision; Luca Marozio: investigation, data curation; Claudio Martini: investigation, data curation, supervision; Gianpaolo Maso: investigation, data curation; Federico Mecacci: investigation, data curation, supervision; Alessandra Meloni: investigation, data curation, supervision; Anna Domenica Mignuoli: investigation, data curation, supervision; Sascia Moresi: investigation, data curation; Luisa Patanè: investigation, data curation; Francesca

Perotti: investigation, data curation; Enrica Perrone: investigation, data curation, supervision; Federico Prefumo: investigation, data curation; Luca Ramenghi: investigation, data curation, supervision; Raffaella Rusciani: investigation, data curation, supervision; Valeria Savasi: investigation, data curation; Sergio Crescenzo Antonio Schettini: investigation, data curation, supervision; Daniela Simeone: investigation, data curation, supervision; Marco Soligo: investigation, data curation; Martin Steinkasserer: investigation, data curation, supervision; Saverio Tateo: investigation, data curation, supervision; Giliana Ternelli: investigation, data curation; Roberta Tironi: investigation, data curation; Vito Trojano: investigation, data curation, supervision; Patrizia Vergani: investigation, data curation.

THE ItOSS COVID-19 WORKING GROUP

Serena Donati[1], Edoardo Corsi[1,2], Alice Maraschini[3], Michele Antonio Salvatore[1], Federica Baltaro[4], Rosaria Boldrini[5], Silvia Bonassissa[6], Roberto Brunelli[7], Angelo Cagnacci[8], Paola Casucci[9], Ilaria Cataneo[10], Irene Cetin[11], Elena De Ambrosi[12], Martina Del Manso[13], Massimo Fabiani[13], Stefania Fieni[14], Massimo Piergiuseppe Franchi[15], Enrico Iurlaro[16], Livio Leo[17], Marco Liberati[18], Lucia Li Sacchi[19], Stefania Livio[11], Mariavittoria Locci[20], Luca Marozio[21], Claudio Martini[22], Gianpaolo Maso[23], Federico Mecacci[24], Alessandra Meloni[25], Anna Domenica Mignuoli[26], Sascia Moresi[27], Luisa Patanè[28], Francesca Perotti[29], Enrica Perrone[30], Federico Prefumo[31], Luca Ramenghi[32], Raffaella Rusciani[33], Valeria Savasi[34], Sergio Crescenzo Antonio Schettini[35], Daniela Simeone[36], Marco Soligo[37], Martin Steinkasserer[38], Saverio Tateo[39], Giliana Ternelli[40], Roberta Tironi[41], Vito Trojano[42], Patrizia Vergani[43]

[1]Centro Nazionale di Prevenzione delle Malattie e Promozione della Salute, Istituto Superiore di Sanità, Rome, Italy

[2]Dipartimento di Biomedicina e Prevenzione, Università di Roma Tor Vergata, Rome, Italy

[3]Servizio Tecnico Scientifico di Statistica, Istituto Superiore di Sanità, Rome, Italy

[4]ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy

[5]Direzione generale della digitalizzazione, del sistema informativo sanitario e della statistica, Ministero della Salute, Rome, Italy

[6]Ospedale Maggiore della Carità, Novara, Italy

[7]Università Sapienza di Roma - Policlinico Umberto I, Rome, Italy

[8]IRCCS San Martino, Genoa, Italy

[9]Sistema Informativo e Mobilità Sanitaria, Regione Umbria, Perugia, Italy

[10]Ospedale Maggiore, Bologna, Italy

[11]Universita degli Studi di Milano, Milan, Italy

[12]Ospedale Infermi, Rimini, Italy

[13]Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Rome, Italy

[14]Azienda Ospedaliero-Universitaria di Parma, Parma, Italy

[15]Università degli Studi di Verona, Verona, Italy

[16]IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[17]Ospedale Beauregard Valle D'Aosta, Aosta, Italy

[18]Universita degli Studi Gabriele d'Annunzio, Chieti-Pescara, Italy

[19]Regione Sicilia, Palermo, Italy

[20]Universita degli Studi di Napoli Federico II, Naples, Italy

[21]Università di Torino, Turin, Italy

[22]Regione Marche, Ancona, Italy

[23]IRCCS Burlo Garofolo, Trieste, Italy

[24]Universita degli Studi di Firenze, Florence, Italy

[25]Regione Autonoma della Sardegna, Cagliari, Italy

[26]Regione Calabria, Reggio Calabria, Italy

[27]Fondazione Policlinico Universitario A. Gemelli IRCCS - Università Cattolica del Sacro Cuore, Rome, Italy

[28]ASST Papa Giovanni XXII, Bergamo, Italy

[29]Fondazione IRCCS Policlinico San Matteo di Pavia, Pavia, Italy

[30]Servizio Assistenza Territoriale, Direzione Generale Cura Della Persona, Salute e Welfare, Regione Emilia-Romagna, Bologna, Italy

[31]ASST Spedali Civili di Brescia, Brescia, Italy

[32]IRCSS Giannina Gaslini, Genoa, Italy

[33]Dipartimento di Epidemiologia ASL TO3, Turin, Italy

[34]ASST Fatebenefratelli Sacco-Università di Milano, Milan, Italy

[35]Azienda Ospedaliera S. Carlo, Potenza, Italy

[36]Ospedale Civile Antonio Cardarelli, Campobasso, Italy

[37]Ospedale Maggiore di Lodi – Distretti e Presidi Lodigiani, Lodi, Italy

[38]Ospedale Centrale di Bolzano, Bolzano, Italy

[39]Ospedale Santa Chiara, Trento, Italy

[40]Azienda Ospedaliero-Universitaria di Modena, Modena, Italy

[41]Ospedale Manzoni, Lecco, Italy

[42]Mater Dei Hospital, Bari, Italy

[43]Fondazione MBBM/Ospedale San Gerardo, Monza, Italy

APPENDIX 1. ItOSS NATIONAL NETWORK OF MATERNITY UNITS

Piedmont Region

Elena Amoruso *Ospedale Sant'Andrea Vercelli;* Alberto Arnulfo, Enrico Finale *Stabilimento Ospedaliero Castelli Verbania;* Rossella Attini, Marisa Biasio, Luca Marozio, Clara Monzeglio OIRM *Sant'Anna - AOU Città della Salute e della Scienza di Torino;* Maria Bertolino, Andrea Guala *Ospedale San Biagio Domodossola;* Silvia Bonassisa, Alberto De Pedrini *Ospedale Maggiore della Carità Novara;* Mario Canesi, Sara Cantoira *Ospedale Maria Vittoria Torino;* Paola Capelli *Istituto SS. Trinità Borgomanero;* Ilaria Careri, *Ospedale Martini Torino;* Luigi Carratta *Ospedale S. Spirito Casale Monferrato;* Ilaria Costag-

gini *Ospedale degli Infermi Rivoli*; Tania Cunzolo *Presidio Osp. Cardinal G. MASSAIA Asti*; Enza De Fabiani, Andrea Villasco *Azienda Ospedaliera Ordine Mauriziano Torino*; Cinzia Diano *Ospedale Maggiore Chieri*; Fiorenza Droghini, Paola Rota *Ospedale Santa Croce Moncalieri*; Daniela Kozel, Vittorio Aguggia *Ospedale Civile SS. Antonio e Biagio Alessandria*; Francesca Maraucci *Ospedale degli infermi Biella*; Gisella Martinotti *Ospedale SS. Pietro e Paolo Borgosesia*; Maria Milano, Antonia Novelli *Ospedale Civile Mondovì*; Giovanna Oggè *Ospedale maggiore SS. Annunziata Savigliano*; Simona Pelissetto *Ospedale Civile di Ivrea*; Pasqualina Russo *Presidio Osp. riunito Ciriè*; Manuela Scatà *Ospedale Michele e Pietro Ferrero di Verduno*; Federico Tuo, Valentina Casagrande *Ospedale San Giacomo Novi Ligure/Tortona*; Concetta Vardè *Ospedale Agnelli Pinerolo*; Elena Vasario *Azienda Ospedaliera S. Croce e Carle Cuneo*; Daniela Ventrella *Ospedale Civico Chivasso*

Valle D'Aosta Region

Livio Leo *Ospedale Umberto Parini Aosta*

Liguria Region

Silvia Andrietti *ASL1 Imperiese*; Federica Baldi *Ospedale San Paolo Savona*; Angelo Cagnacci, Federica Laraud *IRCCS AOU San Martino*; Franco Camandona, Domenico Grimaldi *Ospedale Galliera di Genova*; Maria Franca Corona, Massimiliano Leoni *Ospedale Civile Sant'Andrea La Spezia*; Paolo Massirio, Luca Ramenghi *IRCCS Giannina Gaslini*

Lombardy Region

Debora Balestrieri *Ospedale di Cittiglio*; Federica Baltaro *Ospedale Niguarda di Milano*; Pietro Barbacini, Elisabetta Venegoni *Ospedale di Magenta*; Michele Barbato *Ospedale di Melegnano*; Lorena Barbetti *Ospedale di Esine*; Paolo Beretta *Ospedale di Como*; Bruno Bersellini *Ospedale di Sondrio*; Stefano Bianchi *Ospedale San Giuseppe di Milano*; Antonia Botrugno *Ospedale di Casalmaggiore*; Donatella Bresciani *Ospedale di Desenzano*; Alessandro Bulfoni *Pio X Humanitas di Milano*; Carlo Bulgheroni *Ospedale di Gallarate*; Orlando Caruso, Elena Pinton *Ospedale di Chiari*; Massimo Ciammella *Ospedale di Seriate*; Elena Crestani, Giulia Pellizzari *Ospedale di Pieve di Coriano*; Antonella Cromi *Ospedale di Varese*; Serena Dalzero, Nikita Alfieri *Ospedale San Paolo di Milano*; Rosa Di Lauro, Carla Foppoli *Ospedale di Sondalo*; Patrizia D'Oria, *Ospedale di Alzano*; Santina Ermito *Ospedale di Piario*; Massimo Ferdico *Ospedale di Vimercate*; Maria Fogliani, Guido Stevanazzi *Ospedale di Legnano - Cuggiono*; Roberto Fogliani *Ospedale di Sesto San Giovanni*; Ambrogio Frigerio *Ospedale di Rho*; Eleonora Fumagalli *Ospedale Macedonio Melloni ASST FBF - Sacco di Milano*; Roberto Garbelli *Brescia Istituto Clinico S. Anna*; Daniela Gatti *Ospedale di Manerbio*; Giampaolo Grisolia, Serena Varalta *Ospedale di Mantova*; Paolo Guarnerio *Ospedale San Carlo di Milano*; Enrico Iurlaro, Marta Tondo *IRCCS Cà Granda Ospedale Maggiore Policlinico - Mangiagalli Milano*; Stefano Landi *Ospedale di Gravedona*; Mario Leonardi *Ospedale di Iseo*; Stefania Livio, Chiara Tasca *Ospedale Buzzi ASST FBF-Sacco di Milano*; Anna Locatelli

Ospedale di Carate; Giuseppe Losa *Ospedale di Melzo*; Massimo Lovotti *Como Valduce*; Anna Minelli *Ospedale di Gavardo*; Luisa Muggiasca *Ospedale di Garbagnate*; Giuseppe Nucera *Ospedale di Busto Arsizio*; Alessandra Ornati *Ospedale di Vigevano*; Luisa Patanè *ASST Papa Giovanni XXIII Bergamo*; Antonio Pellegrino *Ospedale di Lecco*; Francesca Perotti, Arsenio Spinillo *Fondazione IRCCS Policlinico San Matteo di Pavia*; Armando Pintucci *Ospedale di Desio*; Ezio Pozzi *Ospedale di Broni Stradella - Ospedale di Voghera*, Federico Prefumo *Spedali Civili di Brescia*; Anna Catalano *Brescia Fondazione Poliambulanza*; Aldo Riccardi *Ospedale di Cremona*; Alessia Chiesa *Ospedale di Ponte San Pietro*; Tazio Sacconi *Ospedale di Asola*; Valeria Savasi, Silvia Corti *Ospedale Sacco di Milano*; Ubaldo Seghezzi *Ospedale di Saronno*; Vincenzo Siliprandi *Ospedale di Crema*; Marco Soligo, Beatrice Negri *Ospedale di Lodi*; Paolo Valsecchi *Ospedale San Raffaele*; Laura Vassena *Ospedale di Merate*; Federica Brunetti, Patrizia Vergani *Fondazione MBBM Ospedale San Gerardo Monza*; Antonella Villa *Ospedale di Treviglio*; Matteo Zanfrà *Ospedale di Tradate*; Alberto Zanini *Ospedale di Erba*

Autonomous province of Bozen

Martin Steinkasser, Micaela Veneziano *Ospedale Centrale di Bolzano*

Autonomous province of Trento

Pietro Dal Rì, Fabrizio Taddei *UO Rovereto*; Roberto Luzietti *UO Cles*; Saverio Tateo *UO Trento*; Fabrizia Tenaglia *UO Cavalese*

Veneto Region

Giuseppe Angeloni *Ospedale di Piove di Sacco*; Antonio Azzena *Ospedale di Vittorio Veneto*; Gianluca Babbo *Ospedale di Portogruaro*; Roberto Baccichet, Cristina Napolitano *Ospedale di Oderzo*; Valentino Bergamini *Ospedale Borgo Trento*; Luca Bergamini *Ospedale di Chioggia*; Enrico Busato, Monica Zannol *Ospedale di Treviso*; Pietro Catapano, Marco Gentile *Ospedale Mater Salutis - Legnago*; Marcello Ceccaroni *Ospedale Sacro Cuore don Calabria Negrar*; Gianluca Cerri *Ospedale SS. Giovanni e Paolo - Venezia*; Andrea Cocco *Ospedale di Asiago*; Carlo Dorizzi *Ospedale di Schiavonia*; Laura Favretti *Ospedale S. Maria del Prato - Feltre*; Riccardo Federle, Antonino Lo Re *Ospedale P. Pederzoli - Casa di cura Privata Spa*; Massimo Franchi, Marina Sangaletti *Azienda Ospedaliera di Verona*; Franco Garbin *Ospedale di Dolo*; Maria Teresa Gervasi, Daniela Truscia *Azienda Ospedaliera di Padova*; Dimosthenis Kaloudis *Ospedale di San Bonifacio*; Domenico Lagamba *Ospedale di Castelfranco Veneto*; Giovanni Martini *Ospedale di Valdagno*; Carlo Maurizio *Ospedale di Mirano*; Yoram J. Meir *Ospedale di Bassano del Grappa*; Alessia Pozzato *Ospedale di Adria - Ospedale di Rovigo*; Marcello Rigano *Ospedale di Camposampiero*; Cesare Romagnolo *Ospedale all'Angelo di Mestre*; Roberto Rulli *Ospedale di Cittadella*; Giuseppe Sacco *Ospedale di San Donà di Piave*; Maria Grazia Salmeri *Ospedale di Montebelluna*; Marcello Scollo *Ospedale di Santorso*; Francesco Sinatra *Ospedale di Conegliano*; Gianluca Straface *Casa di cura Abano*; Fabio Gianpaolo Tandurella *Ospedale di Pieve*

di Cadore e *Ospedale San Martino - Belluno;* Marco Torrazzina *Ospedale di Bussolengo - Ospedale di Villafranca;* Paolo Lucio Tumaini *Ospedale di Arzignano;* Giuliano Zanni *Ospedale di Vicenza*

Friuli-Venezia Giulia Region

Emanuele Ancona Ospedale *S. Giorgio di Pordenone;* Michela De Agostini *Ospedale di Palmanova;* Gianpaolo Maso, Alice Sorz *IRCSS Burlo Garofolo Trieste;* Edlira Muharremi *S. M. degli Angeli Ospedale di Pordenone;* Alessandra Nicoletti *Ospedale S. Daniele di Tolmezzo;* Roberta Pinzano *Ospedale S.Maria dei Battuti - San Vito al Tagliamento;* Alessia Sala *Ospedale Santa Maria della Misericordia - Udine;* Lucia Zanazzo, *Ospedale di Monfalcone*

Emilia-Romagna Region

Lorenzo Aguzzoli, Alice Ferretti *Ospedale S.M. Nuova Reggio Emilia;* Patrizio Antonazzo, Lucrezia Pignatti *Ospedale Bufalini Cesena;* Angela Bandini, Isabella Strada *Ospedale G.B. Morgagni - L. Pierantoni Forlì;* Chiara Belosi *Ospedale degli Infermi Faenza;* Renza Bonini, Maria Cristina Ottoboni *Ospedale Guglielmo Da Saliceto Piacenza;* Fabrizio Corazza, Paola Pennacchioni *Ospedale Ss. Annunziata Cento;* Fabio Facchinetti, Giliana Ternelli *Azienda Ospedaliero-Universitaria Modena;* Alessandro Ferrari, Cristina Pizzi, *Ospedale S.M. Bianca Mirandola;* Tiziana Frusca, Stefania Fieni *Azienda Ospedaliero-Universitaria Parma;* Maria Cristina Galassi, Federica Richieri, *Nuovo Ospedale Civile Di Sassuolo S.P.A.;* Francesco Giambelli, Carlotta Matteucci *Ospedale S.M. Delle Croci Ravenna;* Pantaleo Greco, Danila Morano *Azienda Ospedaliero-Universitaria Ferrara;* Marinella Lenzi, Ilaria Cataneo, *Ospedale Maggiore Bologna;* Gialuigi Pilu, Marisa Bisulli, *Azienda Ospedaliero-Universitaria Bologna;* Maria Cristina Selleri *Ospedale di Bentivoglio;* Federico Spelzini, Elena De Ambrosi, *Ospedale Infermi Rimini;* Paolo Venturini, Francesca Tassinati *Ospedale B. Ramazzini Carpi;* Stefano Zucchini, Barbara Paccaloni, *Ospedale S.M. della Scaletta Imola*

Tuscany Region

Andrea Antonelli, Carlotta Boni *Ospedale Civile Cecina;* Maria Paola Belluomini, *S. Francesco Barga - PO Valle del Serchio* e *Generale Provinciale Lucca - PO San Luca,* Rosalia Bonura, *S. Maria della Gruccia - Ospedale del Valdarno,* Stefano Braccini, *SS. Cosimo e Damiano Pescia - Osp della Valdinievole,* Giacomo Bruscoli e Pasquale Mario Florio, *Nuovo Ospedale San Jacopo di Pistoia,* Giovanna Casilla, *SS. Giacomo e Cristoforo Massa - PO Zona Apuana,* Anna Franca Cavaliere, *Ospedale Santo Stefano Prato,* Marco Cencini, *Ospedali Riuniti della Val di Chiana,* Venere Coppola e Laura Migliavacca, *Ospedale Misericordia Grosseto,* Barbara De Santi, *PO Felice Lotti Pontedera,* Paola Del Carlo, *Ospedale S.Giovanni Di Dio Torregalli,* Carlo Dettori, *Nuovo Ospedale di Borgo S.Lorenzo,* Mariarosaria Di Tommaso e Serena Simeone, *Careggi - CTO Firenze - AOU,* Giuseppe Eremita, *Civile Elbano Portoferraio,* Sara Failli, *Ospedale Area Aretina Nord Arezzo,* Paolo Gacci, *S.M. Annunziata Bagno a Ripoli - Osp Fiorentino Sud Est,* Alessandra Meucci, *Le Scotte Siena - Azienda*

ospedaliera universitaria, Filippo Ninni, *Riuniti Livorno,* Barbara Quirici, *Ospedale Unico Versilia,* Alessia Sacchi, *Ospedale dell'Alta Val d'Elsa Poggibonsi,* Cristina Salvestroni, *Ospedale S. Giuseppe Empoli,* Sara Zullino, *Ospedali Pisani Pisa - Az universitaria*

Umbria Region

Nazzareno Cruciani, Fabrizio Damiani *Ospedale San Giovanni Battista Foligno;* Leonardo Borrello *Azienda Ospedaliera Santa Maria di Terni;* Gian Carlo Di Renzo, Giorgio Epicoco *Azienda Ospedaliera Santa Maria della Misericordia di Perugia;* Ugo Indraccolo, Donatello Torrioli, *Ospedale di Città di Castello*

Marche Region

Andrea Ciavattini, Sara D'Eusanio *AOU - Ospedali Riuniti di Ancona;* Filiberto Di Prospero *Ospedale di Civitanova Marche;* Rebecca Micheletti, Claudio Cicoli *Azienda Ospedaliera Ospedali Riuniti Marche Nord*

Lazio Region

Francesco Antonino Battaglia, Immacolata Marcucci *PO Santa Maria Goretti Latina;* Leonardo Boccuzzi, Patrizia Ruocco *Ospedale De Santis di Genzano;* Marco Bonito *Ospedale San Pietro Fatebenefratelli Roma;* Maria Clara D'Alessio *San Filippo Neri Roma;* Carlo De Angelis *Casa di Cura Fabia Mater Roma;* Donatella Dell'Anna *Ospedale S. Eugenio;* Daniele Di Mascio, Paola Pecilli *Umberto I - Policlinico di Roma;* Sascia Moresi, Sergio Ferrazzani, Silvia Salvi *Policlinico Universitario Fondazione Agostino Gemelli - Roma;* Gregorio Marco Galati *Ospedale Madre Giuseppina Vannini Istituto delle Figlie di S. Camillo Roma;* Maria Grazia Frigo *Fatebenefratelli San Giovanni Calibita - Isola Tiberina;* Paolo Gastaldi *Ospedale Santo Spirito Roma;* Rita Gentile *Presidio Ospedaliero Giovan Battista Grassi Ostia;* Giovanni Grossi *Ospedale Sandro Pertini Roma;* Giorgio Nicolanti, Patrizio Raggi *Ospedale Belcolle Viterbo;* Flavia Pierucci *Azienda Ospedaliera San Camillo Forlanini Roma;* Giancarlo Paradisi, Maria Rita Pecci *Ospedale Fabrizio Spaziani Frosinone;* Giovanni Testa *Casa di cura Città di Aprilia;* Barbara Vasapollo *Policlinico Casilino Roma;* Barbara Villaccio *Ospedale San Pietro Fatebenefratelli Roma*

Abruzzo Region

Fabio Benucci *Sant'Omero;* Paola Caputo *Sulmona;* Sandra Di Fabio, Maurizio Guido *L'Aquila;* Antonio Di Francesco *Lanciano;* Francesca di Sebastiano, Diego Gazzolo, Marco Liberati *Chieti;* Anna Marcozzi *Teramo;* Francesco Matrullo *Vasto;* Maurizio Rosati, Gabriella Scorpio *Pescara;* Giuseppe Ruggeri *Avezzano;* Alessandro Santarelli *Ospedale di Sant'Omero*

Molise Region

Daniela Simeone *Ospedale di Campobasso*

Campania Region

Annalisa Agangi *Ospedale Evangelico Villa Betania;* Salvatore Ercolano *P.O. "S. Leonardo" di Castellammare di Stabia,* Luigi Cobellis, Annunziata Mastrogiacomo *Ospedale di Caserta;* Maria Vittoria Locci *AOU Federico II Napoli*

Puglia Region

Luca Loiudice *Bari - Presidio Mater Dei;* Antonio Belpiede *Barletta 'Mons. Dimiccoli';* Mariano Cantatore *L. Bonomo Andria;* Ettore Cicinelli, Antonella Vimercati *Bari - Policlinico Ginecologia;* Aldo D'Aloia, Sabina Di Biase, Antonio Lacerenza *AOU "OO RR Foggia";* Alessandro Dalfiero *Cerignola;* Gerardo D'Ambrogio *Galatina "Santa Caterina Novella";* Nicola Del Gaudio *Castellaneta;* Paolo Demarzo *San Severo Teresa Masselli Mascia;* Giovanni Di Vagno *Bari - San Paolo;* Giuseppe Laurelli *Casa Sollievo Dalla Sofferenza - S. Giovanni Rotondo;* Roberto Lupo *Gallipoli;* Nicola Macario *Altamura;* Antonio Malvasi, *Bari - Casa Di Cura Santa Maria;* Guido Maurizio, Elisabetta Monteduro, *Acquaviva "Miulli";* Andrea Morciano *Cardinale G. Panico Di Tricase;* Lucio Nichilo *Umberto I Corato;* Anna Maria Nimis *Francavilla Fontana;* Antonio Perrone *Lecce Vito Fazzi;* Elena Rosa Potì *Brindisi "Perrino";* Sabino Santamato *Monopoli Putignano;* Emilio Stola *Taranto;* Antonio Tau *Scorrano;* Mario Vicino *Bari - Di Venere;* Martino Vinci *Martina Franca*

Basilicata Region

Giampiero Adornato *Policoro;* Francesco Bernasconi *Melfi;* Alfonso Chiacchio *Lagonegro;* Sergio Schettini, Rocco Paradiso *Azienda Ospedaliera Regionale San Carlo - Potenza;* Giuseppe Trojano *Matera*

Calabria Region

Carmelina Ermio *Ospedale Jazzolino - Vibo Valentia;* Michele Morelli, Rossella Marzullo *Ospedale Annunziata - AO Cosenza;* Stefano Palomba *Ospedali Riuniti di Reggio Calabria;* Morena Rocca *Azienda Ospedaliera "Pugliese Ciaccio" di Catanzaro*

Sicily Region

Vincenzo Aidala, *Castiglione Prestianni - Bronte;* Luigi Alio, Giuseppina Orlando *ARNAS Civico di Cristina Benfratelli - Palermo;* Maria Grazia Arena, Santo Recupero *S. Marco (ex V. Emanuele S. Bambino) - Catania* e *Osp. Generale - Lentini;* Salvatore Bevilacqua, Fabrizio Quartararo *Casa di cura Candela SPA - Palermo;* Rocco Billone *Civico Partinico* e *Dei Bianchi - Corleone;* Giuseppe Bonanno, *Maria Paternò Arezzo - Ragusa;* Antonio Bucolo, *Umberto I - Siracusa;* Claudio Campione, *Casa di cura prof. Falcidia - Catania;* Giuseppe Canzone, *S. Cimino - Termini Imerese;* Angelo Caradonna, *V. Emanuele II - Castelvetrano;* Sebastiano Caudullo e Cosimo Raffone, *AO Papardo - Messina;* Giovanni Cavallo, *PO Maggiore - Modica;* Antonio Cianci, Michele Fichera *V. Emanuele Rodolico - Catania;* Salvatore Corsello, Sergio Di Salvo *Casa di cura Villa Serena - Palermo;* Gaspare Cucinella, Maria Elena Mugavero *Villa Sofia - Cervello-Palermo;* Rosario D'Anna, *AOU G. Martino - Taormina,* Maria Rosa D'anna, *Buccheri La Ferla - Palermo;* Maria Di Costa, *Basilotta - Catania;* Giuseppe Ettore, *ARNAS Garibaldi Nesima - Catania;* Giovanni Falzone, Marta Fauzia *Umberto I - Nicosia;* Roberto Fazio, *G. Fogliani - Milazzo* e *Lipari;* Matteo Giardina *Ospedale Paolo Borsellino di Marsala;* Michele Gulizzi e Francesco La Mantia, *G. F. Ingrassia - Palermo;* Laura Giambanco, *S. Antonio Abate - Erice* e *B.* *Nagar-Pantelleria;* Salvatore Incandela, *S. Giovanni di Dio - Agrigento* e *Giovanni Paolo II - Sciacca;* Lilli Maria Klein *S. Vincenzo - Enna* e *Barone Romeo - Patti;* Michele La Greca, Venera Mille, *M. SS. Addolorata - Biancavilla;* Luigi Li Calsi *S. Giacomo d'Altopasso - Licata;* Emilio Lo Meo, Paolo Scrollo *Cannizzaro - Catania;* Vincenzo Miceli, *S. Raffaele Giglio - Cefalù;* Maria Pia Militello, *S. Marta e S. Venera - Acireale;* Alfio Mirenna, *Istituto clinico Vidimura (ex Casa di cura Gretter e Lucina) - Catania;* Pietro Musso, *Abele Ajello - Mazara del Vallo;* Michele Palmieri, *V. Emanuele-Gela;* Concetta Remigia Pettinato, Angelo Tarascio *Gravina - Caltagirone - Caltagirone;* Vincenzo Scattarreggia *Barone Lombardo - Canicattì;* Antonio Schifano *R. Guzzardi - Vittoria;* Calogero Selvaggio *S. Elia - Caltanissetta;* Luigi Triolo *Casa di cure Triolo Zancla SPA-Palermo;* Renato Venezia *P. Giaccone - Palermo*

Sardinia Region

Speranza Piredda *Civile Alghero;* Giangavino Peppi *Giovanni Paolo II - Olbia;* Giovanna Pittorra *S. Fracesco - Nuoro;* Gianfranco Depau *Nostra Signora della Mercede - Lanusei;* Gianfranco Puggioni *S. Martino - Oristano;* Loredana Pagliara *Nostra Signora di Bonaria - San Gavino;* Giulietta Ibba *CTO - Iglesias;* Caterina Tronci, *SS Trinità - Cagliari;* Giampiero Capobianco, *AOU Sassari;* Alessandra Meloni *Duilio Casula Monserrato AOU - Cagliari;* Francesca Palla *S. Michele AO Brotzu - Cagliari*

APPENDIX 2. MISSING DATA

Among the variables included in the model, the percentage of missing values was zero for disease severity and citizenship, 1.3% for woman's age, 1.8% for presence/absence of previous comorbidities, 1.8% for presence/absence of obesity, 28.5% for woman's educational level (*Table 1*). Overall, 29.4% of cases had missing data on at least one variable of interest.

The percentage of missing values was negligible for all variables considered except for educational level. The choice of listwise deletion to handle missing data in the model was justified by the condition that missingness for educational level was not significantly associated to the outcome (COVID-19 disease severity). This condition ensures that listwise deletion does not introduce any bias in the coefficients estimates [1, 2] regardless of whether the missing data mechanism was at random (MAR), as assumed in our case, or not at random (MNAR).

Model interactions were tested through Likelihood Ratio Test to verify that no interaction terms were omitted and no bias was introduced.

1. Little RJA, Rubin DB: Statistical analysis with missing data. Wiley series in Probability and Statistics. New York: Wiley; 2020.
2. White IR, Royston P, Wood AM. Multiple imputation using chained equations: Issues and guidance for practice. Stat Med. 2011;30(4):377-99. doi:10.1002/sim.4067

Original articles and reviews

REFERENCES

1. Schwartz DA, Graham AL. Potential maternal and infant outcomes from (Wuhan) Coronavirus 2019-nCoV infecting pregnant women: Lessons from SARS, MERS, and other human Coronavirus infections. Viruses. 2020;12(2):194. doi: 10.3390/v12020194

2. Ripe-tomato.org – Freemarket environmentalism and more. Jim Thornton, Keelin O'Donoghue, Kate Walker. Covid-19 in pregnancy. Running totals. [cited 2021 Aug 17]. Available from: https://ripe-tomato.org/2020/03/31/covid-19-in-pregnancy-running-totals/.

3. World Health Organization Collaborating Centre for Women's Health University of Birmingham. COVID-19 in pregnancy (PregCOV-19 LRS) [cited 2021 Aug 17]. Available from: www.birmingham.ac.uk/research/whocollaborating-centre/pregcov/index.aspx.

4. WAPM (World Association of Perinatal Medicine) Working Group on COVID-19. Maternal and perinatal outcomes of pregnant women with SARS-CoV-2 infection [published correction appears in Ultrasound Obstet Gynecol. 2021 Aug 9;:]. Ultrasound Obstet Gynecol. 2021;57(2):232-241. doi: 10.1002/uog.23107

5. Reagan-Udall Foundation for the FDA [Internet]. International Registry of Coronavirus (COVID-19) Exposure in Pregnancy (IRCEP) [cited 2021 Aug 17]. Available from: https://reaganudall.org/clinical-trial/international-registry-coronavirus-covid-19-exposure-pregnancy

6. Panchaud A, Favre G, Pomar L, et al. An international registry for emergent pathogens and pregnancy. Lancet. 2020;395(10235):1483-4. doi: 10.1016/S0140-6736(20)30981-8

7. Huntley BJF, Huntley ES, Di Mascio D, Chen T, Berghella V, Chauhan SP. Rates of maternal and perinatal mortality and vertical transmission in pregnancies complicated by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-Co-V-2) infection: A systematic review. Obstet Gynecol. 2020;136(2):303-12. doi:10.1097/AOG.0000000000004010

8. Juan J, Gil MM, Rong Z, Zhang Y, Yang H, Poon LC. Effect of coronavirus disease 2019 (COVID-19) on maternal, perinatal and neonatal outcome: systematic review. Ultrasound Obstet Gynecol. 2020;56(1):15-27. doi:10.1002/uog.22088

9. Raschetti R, Vivanti AJ, Vauloup-Fellous C, Loi B, Benachi A, De Luca D. Synthesis and systematic review of reported neonatal SARS-CoV-2 infections. Nat Commun. 2020;11(1):5164. doi:10.1038/s41467-020-18982-9

10. Allotey J, Stallings E, Bonet M, et al. Clinical manifestations, risk factors, and maternal and perinatal outcomes of coronavirus disease 2019 in pregnancy: living systematic review and meta-analysis. BMJ. 2020;370:m3320. doi:10.1136/bmj.m3320

11. Wei SQ, Bilodeau-Bertrand M, Liu S, Auger N. The impact of COVID-19 on pregnancy outcomes: a systematic review and meta-analysis. CMAJ. 2021;193(16):E540-E548. doi:10.1503/cmaj.202604

12. Knight M and INOSS. The International Network of Obstetric Survey Systems (INOSS): benefits of multi-country studies of severe and uncommon maternal morbidities. Acta Obstet Gynecol Scand. 2014;93(2):127-31. doi:10.1111/aogs.12316

13. Knight M, Bunch K, Vousden N, et al. Characteristics and outcomes of pregnant women admitted to hospital with confirmed SARS-CoV-2 infection in UK: national population based cohort study. BMJ. 2020;369:m2107. doi:10.1136/bmj.m2107

14. Maraschini A, Corsi E, Salvatore MA, Donati S and ItOSS COVID-19 Working Group. Coronavirus and birth in Italy: results of a national population-based cohort study. Ann Ist Super Sanità. 2020;56(3):378-89. doi:10.4415/ANN_20_03_17

15. Corsi E, Maraschini A, Perrone E, et al. La preparedness dell'Italian obstetric surveillance system in occasione della pandemia da SARS-CoV-2: aspetti metodologici di uno studio di popolazione [The preparedness of the Italian obstetric surveillance system in the response to the emergency of the SARS-CoV-2 pandemic: methodological aspects of a population-based study]. Epidemiol Prev. 2020;44(5-6 Suppl 2):81-87. doi:10.19191/EP20.5-6.S2.089

16. Donati S, Corsi E, Salvatore MA, et al. Childbirth care among SARS-CoV-2 positive women in Italy. Int J Environ Res Public Health. 2021;18(8):4244. doi:10.3390/ijerph18084244

17. Engjom H, Aabakke AJM, Klungsøyr K, et al. COVID-19 in pregnancy-characteristics and outcomes of pregnant women admitted to hospital because of SARS-CoV-2 infection in the Nordic countries [published online ahead of print, 2021 Apr 22]. Acta Obstet Gynecol Scand. 2021;10.1111/aogs.14160. doi:10.1111/aogs.14160

18. Istituto Nazionale di Statistica (ISTAT). La presenza straniera in Italia: caratteristiche socio-demografiche – Permessi di soggiorno all'1 gennaio degli anni 2001, 2002, 2003 [The foreign population living in Italy: socio-demographic characteristics – years 2001, 2002, 2003 [Italian]. Available from: www.cestim.it/sezioni/dati_statistici/italia/Istat/2004-06%20permessi%20soggiorno%20Italia%202001%202003.pdf.

19. Italia. Ministero della Salute. Directorate-general of digitalization, of health informative system and of statistics, Italian Ministry of Health. Certificato di assistenza al parto (CeDAP). Analisi dell'evento nascita – 2019 [Birth Registry – Year 2019] [cited 2021 Aug 17]. [Italian]. Available from: www.salute.gov.it/imgs/C_17_pubblicazioni_3076_allegato.pdf.

20. Istituto Nazionale di Statistica (ISTAT). La dinamica demografica durante la pandemia COVID-19 – Anno 2020 [The demographic dynamic during the COVID-19 pandemic – Year 2020] [Italian]. Available from: www.istat.it/it/files/2021/03/REPORT-IMPATTO-COVIDDEMOGRAFIA_2020.pdf.

21. Riccardo F, Ajelli M, Andrianou XD, et al. Epidemiological characteristics of COVID-19 cases and estimates of the reproductive numbers 1 month into the epidemic, Italy, 28 January to 31 March 2020. Euro Surveill. 2020;25(49):2000790. doi: 10.2807/1560-7917.ES.2020.25.49.2000790

22. Vousden N, Bunch K, Morris E, et al. The incidence, characteristics and outcomes of pregnant women hospitalized with symptomatic and asymptomatic SARS-CoV-2 infection in the UK from March to September 2020: A national cohort study using the UK Obstetric Surveillance System (UKOSS). PLoS One. 2021;16(5):e0251123. doi: 10.1371/journal.pone.0251123

23. Zambrano LD, Ellington S, Strid P, et al. Update: Characteristics of symptomatic women of reproductive age with laboratory-confirmed SARS-CoV-2 infection by pregnancy status - United States, January 22-October 3, 2020. MMWR Morb Mortal Wkly Rep. 2020;69(44):1641-7. doi:10.15585/mmwr.mm6944e3

24. Villar J, Ariff S, Gunier RB, et al. Maternal and neonatal morbidity and mortality among pregnant women with and without COVID-19 infection: The INTERCOVID Multinational Cohort Study. JAMA Pediatr. 2021;175(8):817-

26. doi:10.1001/jamapediatrics.2021.1050

25. Martin CA, Jenkins DR, Minhas JS, et al. Socio-demographic heterogeneity in the prevalence of COVID-19 during lockdown is associated with ethnicity and household size: Results from an observational cohort study. EClinicalMedicine. 2020;25:100466. doi:10.1016/j.eclinm.2020.100466

26. Gemelas J, Davison J, Keltner C, Ing S. Inequities in employment by race, ethnicity, and sector during COVID-19 [published online ahead of print, 2021 Jan 15]. J Racial Ethn Health Disparities. 2021;1-6. doi:10.1007/s40615-021-00963-3

27. Betran AP, Ye J, Moller AB, Souza JP, Zhang J. Trends and projections of caesarean section rates: global and regional estimates. BMJ Glob Health. 2021;6(6):e005671. doi:10.1136/bmjgh-2021-005671

28. Khalil A, Kalafat E, Benlioglu C, et al. SARS-CoV-2 infection in pregnancy: A systematic review and meta-analysis of clinical features and pregnancy outcomes. EClinicalMedicine. 2020;25:100446. doi:10.1016/j.eclinm.2020.100446

29. EpiCentro – Epidemiology for public health – Istituto Superiore di Sanità (ISS) – Italian National Institute of Health. COVID-19: pregnancy, childbirth and breastfeeding. Available online from: www.epicentro.iss.it/en/coronavirus/sars-cov-2-pregnancy-childbirth-breastfeeding (accessed on 4 August 2021).

30. Giusti A, Zambri F, Marchetti F, et al. COVID-19 and pregnancy, childbirth, and breastfeeding: the interim guidance of the Italian National Institute of Health. COVID-19 e gravidanza, parto e allattamento: le indicazioni ad interim dell'Istituto Superiore di Sanità. Epidemiol Prev. 2021;45(1-2):14-16. doi:10.19191/EP21.1-2.P014.030

31. Carrasco I, Muñoz-Chapuli M, Vigil-Vázquez S, et al. SARS-COV-2 infection in pregnant women and newborns in a Spanish cohort (GESNEO-COVID) during the first wave. BMC Pregnancy Childbirth. 2021;21(1):326. doi:10.1186/s12884-021-03784-8

32. Khoury R, Bernstein PS, Debolt C, et al. Characteristics and outcomes of 241 births to women with severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection at five New York City Medical Centers. Obstet Gynecol. 2020;136(2):273-282. doi: 10.1097/AOG.0000000000004025

33. Kotlyar AM, Grechukhina O, Chen A, et al. Vertical transmission of coronavirus disease 2019: a systematic review and meta-analysis. Am J Obstet Gynecol. 2021;224(1):35-53.e3. doi:10.1016/j.ajog.2020.07.049

34. Trevisanuto D, Cavallin F, Cavicchiolo ME, Borellini M, Calgaro S, Baraldi E. Coronavirus infection in neonates: a systematic review. Arch Dis Child Fetal Neonatal Ed. 2021;106(3):330-335. doi:10.1136/archdischild-2020-319837

COVID-19 in pediatric palliative care: what can we learn from the pandemic and possible future directions

Irene Avagnina[1]**, Anna Zanin**[1]**, Pierina Lazzarin**[1]**, Enrica Grigolon**[1]**, Aashni Shahi**[2]**, Simonetta Papa**[2]**, Luca Giacomelli**[2] **and Franca Benini**[1]

[1]*Centro Regionale di Cure Palliative Pediatriche e Terapia del Dolore, Dipartimento della Salute della Donna e del Bambino, Azienda Ospedale Università Padova, Padua, Italy*
[2]*Polistudium Srl, Milan, Italy*

Abstract

Introduction. Patients in pediatric palliative care (PPC) live with multiple comorbidities which represent a risk factor for severe form of COVID-19.
Methods. This monocentric retrospective study was performed at the PPC Center of Padua (Italy). Testing methodology, prevention strategies and infection characteristics were documented and compared during the first and second peak of SARS-CoV-2 infection.
Results. Between April-June 2020 a population swab screening was performed and a strong reduction of the habitual family support was observed. Between November 2020-January 2021 swab testing was limited to specific cases and the support network for families was partially restored. Incidence of COVID-19 was low, resulting in 0.04% of total pediatic cases in the Veneto Region. No severe forms were observed.
Conclusion. The use of adequate preventive measures by families and support networks associated with testing in specific contests is safe, cost effective and has a minor impact on caregiver's care load.

INTRODUCTION

To date, the pandemic has reached more than 100 million cases globally [1]. The incidence of COVID-19 among children showed an upward trend during this year [2-5]. Although children have a better prognosis compared to adults [6], in Europe 8-10% of infected children developed a severe form of COVID-19 and required intensive care support [7, 8]. The presence of preexisting comorbidities or complex medical needs is a risk factor for developing a severe form of COVID-19 [7, 9]. Moreover, multisystem inflammatory syndrome (MIS-C) has been observed only in children and is associated with an increased need of intensive care support and higher mortality [10].

Children eligible for pediatric palliative care (PPC) have life-threatening and life-limiting diseases. They normally have multiple comorbidities, complex medical needs and the majority of them need life-saving support (60% in our contest). A supportive multi-specialist network (palliative pediatricians, nurses, psychologists, physiotherapists, physiatrists) is crucial for them and their families in order to ensure an adequate quality of life [11].

Consequent to their critical health condition, chil-

dren who require PPC have a high-risk for severe forms of COVID-19.

The aim of the present retrospective study is to assess the incidence of SARS-COV-2 among children in PPC and to suggest the most safe, helpful and cost-effective management strategy.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Padua Hospital (protocol 0037730).

This monocentric retrospective study involves all patients from the PPC Center of Padua (Italy) and their families. A comparison between the first (April-June 2020) and second wave (November 2020-February 2021) was performed to evaluate the screening methodology adopted by our center, the differences in the home care support and the incidence of SARS-CoV-2 infection among children.

From April to June 2020 a population screening was proposed, with previous consent, to all families in charge at our center and nasopharyngeal swab (NPS) for SARS-CoV-2 molecular search was performed at home on PPC patients and all family members in close contact with the child. Results of laboratory analysis

Address for correspondence: Irene Avagnina, Centro Regionale di Cure Palliative Pediatriche e Terapia del Dolore, Dipartimento della Salute della Donna e del Bambino, Azienda Ospedale Università Padova, Via Ospedale Civile 57, 35121 Padua (Italy). E-mail: irene.avagnina@aopd.veneto.it.

were collected at the PPC Center of the Department of Women and Children Health at the Padua Hospital (Italy).

From November 2020-February 2021 the NPS for the SARS-CoV-2 molecular search was proposed only to patients and caregiver before hospitalization in the Pediatric Hospice and to symptomatic or close contact patients or caregivers.

In April 2020 and February 2021, we submitted an anonymous online questionnaire (*Appendix 1*) to all the main caregivers of children in charge at our PPC center. The topics analyzed in the questionnaire were: the application of the World Health Organization recommendations for the prevention of infection, the changes of habits to reduce the risk of contagion, the reduction in practical support received from people outside the family (health providers/friends/family), the flu vaccinations status of both patient and family. In February at the same questionnaire was added an additional focus regarding the personal opinion of parents on COVID-19 vaccine.

The quantitative and qualitative analysis of the survey was performed at the PPC Center of the Department of Women and Children of the Padua Hospital.

Our data were compared with the absolute number of COVID-19 infections documented until February 2021 at the Regional Register of SARS-CoV-2 infections of the Veneto Region.

In our observational study variables associated with patients, screening methodology and questionnaire were expressed as absolute value, mean, percentage. No further statistical analysis was performed.

RESULTS
Incidence

From April 2020 to February 2021 the total number of positive children was 13 out of 170 patients followed by our PPC center in February 2021 (8%). The characteristics of this population are resumed in *Table 1*. Two patients (15.4%) had a hospital-acquired infection, six patients (46.2%) had a domestic-acquired infection, three asymptomatic patients (23.1%) resulted positive at the pre-hospitalization screening, in two patients (15.4%) the source of the infection was unknown.

In addition, eight other families reported positive NPS among parents and siblings. Of these, two caregivers were asymptomatic and were identified by the pre-hospitalization screening.

Once identified, positive patients were closely monitored by our medical-nursing team with telemedicine. Specific pathways in case of severe symptoms were previously defined and shared with families.

Four patients (30.7%) were asymptomatic, nine patients (69.2%) presented mild-moderate symptoms as mild respiratory symptoms as flu, worsening of secretion, pneumonia (7/13), fever (4/13), abdominal pain and diarrhea (2/13). Two patients (15.4%) required hospitalization for pneumonia, none required intensive care support or presented MIS-C. All patients had a complete recovery from COVID-19.

Only one caregiver resulted positive in April 2020 and no children contracted the infection during the first

Table 1

Characteristics of children with documented SARS-CoV-2 infection in PPC up to February 2021

Characteristic	N of patients (%)
Total number of children in PPC	170
Total number of children with SARS-CoV-2 infection	13 (8%)
Number of children with SARS-CoV-2 infection (April-August 2020)	0
Number of children with SARS-CoV-2 infections (September-October 2020)	2 (15%)
Number of children with SARS-CoV-2 infection (November- February 2021)	11 (85%)
Median age (years), range	7.44 (1.3-15.11)
Age group (y):	
<1	0
1-5	6 (46.2%)
6-10	1 (7.6%)
>10	6 (46.2%)
Sex	
Male	6 (46.2%)
Female	7 (53.8%)
Pathology	
Oncologic	2 (15.4%)
Non oncologic	11 (84.6%)
Life support	
Non invasive ventilation	3 (23.1%)
Tracheostomy	2 (15.4%)
Gastrostomy/SNG	7 (53.8%)
Need of frequent access to the hospital (for treatments or follow up)	4 (30.8%)
Need of physiotherapy	7 (53.8%)
Going to school	4 (30.8%)
Origin of infection	
Hospital	2 (15.4%)
Family	6 (46.2%)
Detected by screening	3 (23.1%)
Not known	2 (15.4%)
Main symptoms	
Fever	4 (30.8%)
Flu	2 (15.4%)
Increase of secretions	3 (23.1%)
Pneumonia	2 (15.4%)
Abdominal pain	1 (7.7%)
Diarrhea	1 (7.7%)
Asymptomatic	4 (30.8%)
Need of hospitalization	2 (15.4%)
Need of intense care unit	0

wave. Since September 2020 we have observed a higher incidence of the infection among children and caregivers, with a peak between November and February 2021 (11/13).

Survey results

The adherence to the questionnaire proposed in April 2020 and February 2021 was respectively 52% and 28%.

In both periods the most adopted prevention measures by the families were: avoiding aggregations (92% *vs* 77%;), ventilation of rooms in the house (70% *vs* 57%) and washing hands (56% *vs* 55%). The use of masks at

home was higher in the first questionnaire (40%) compared to the second (27.5%). The distance of at least 1 meter was respected only in 25% of cases. In February 2021 in case of positive cases in families the use of masks at home and the domestic isolation were the two most adopted measures (respectively 20% and 17.5%).

Since the beginning of the pandemic, we observed an overall reduction in the child's outdoor recreational activity (88% *vs* 77%) and routine medical visits (74% *vs* 55.3%).

In April 2020 families reduced or suspended access of health-care providers to their home in 65.4% of cases and those of external people who normally assist the child in 100% of cases for friends or relatives and 57% for other professional care providers (babysitters, educators). In February 2021, the access at home of health care providers was reduced or suspended in only 39.2 % of patients and that of external people who normally assist the child was reduced in 36.8% for professional care providers and in 97.5% for friends or family members.

In April 68% of parents changed their work habits to reduce their outings whereas in February 64.1% of parents referred not to have any variation in work activities.

The use of telehealth consultation was higher in April 2020 (54%) compared to February 2021 (37.5%).

Evaluating the flu vaccination status, we observed in both periods a non-adherence to the flu vaccination in 40% of families. Moreover, in February 2021 40% of caregivers were still doubting about the SARS-CoV-2 vaccination.

Screening strategies

Between April-June 2020, 163 children were followed by our PPC center, of which 146 (89.6%) agreed to be screened for the SARS-CoV-2 infection. A total of 401 NPS were performed:103 children (71%) and 298 family members (63%), with an overall cost of approximately 25,500 euro (64€/swab). 43 patients and 3 families were not tested for the SARS-CoV-2 infection due to organizational reasons.

Between November 2020-January 2021, 68 families were tested for a total of 169 NPS: 104 as pre-hospitalization screening (61.5%) and 65 in 16 symptomatic families (38.5%). The cost was approximately 10,820 euro (64€/swab).

DISCUSSION

Several reports assessed how the presence of pre-existing comorbidities represents an important risk factor for developing severe forms of COVID-19 [7, 9]. Most patients in PPC present multiple comorbidities, making this population especially at risk.

The analysis of data collected at the Padua PPC center suggests a different picture.

The overall incidence of COVID-19 among children in our region reached 4.2% of the total pediatric population, according to the Veneto Regional Register. Children in PPC represented 0.04% of total positive pediatric cases in Veneto (13/35,504) and no severe forms of the infection were observed. During the first observational period, from the screening performed on the pediatric population in charge of PPC, a zero-

incidence rate of COVID-19 emerged. It is likely that the immediate strict adherence to preventive measures adopted by the family had a positive impact on this finding. In fact, the COVID-19 prevention measures that these families adopt every year during the winter season only increased. Furthermore, the caregivers of children eligible for PPC are subjects who normally live in social isolation and the COVID-19 pediatric rates were not that high during the first wave in our region as during the second one. The survey showed that during the first observation period the major change was the reduction of the support from the health network normally guaranteed at home by the pediatrician, the physiotherapist or nurses, as well as that of other family members or friends who concretely help the family in the child's daily management. This implies a major work for the caregivers that already have an important daily care load [12] with a consequent greater burden [13], which deserves further investigation on the quality of life impact in a medium long term perspective.

During the second observational period, the decision of not repeating any population screening was consequent to the very low incidence of infection during the previous observational period. Moreover, seeing the strong impact of the isolation on the caregiver care load and family burden, preventive strategies were still adopted but with the resumption of the clinical and social network that normally helped the families at home. As a result, the second survey showed that in almost 50% of families the support at home from pediatricians or other health care providers was restored.

The reduction of social restriction compared to the first wave, can partially justify the increased number of SARS-CoV-2 infections observed among children in the second wave, which was also in line with the overall increased incidence of SARS-CoV-2 infections among children during the second spread of COVID-19. The origin of the infection was mostly secondary to other family members (46,2%) and only two cases (15,4%) were secondary to an inhospital spread of the infection.

A considerable number of asymptomatic cases (30.8% among children) were observed justifying the need of keeping the pre-hospitalization screening in order to maintain the Pediatric Hospice COVID-free.

The aim of PPC is the global quality of life of children and family [11]. For this reason, considering the minor impact of COVID-19 in this population and the negative effect of isolation on family's burden, we assume it to be a priority to restore the health-related support network at home. As a consequence of this, specific precautions and screening strategies are needed.

Prevention strategies were continuously used by the families based on WHO's recommendations as shown in the surveys. In terms of screening, using NPS for pre-hospitalisation and in case of symptoms or close contact together with the maintenance of home care (if feasible) were the most safe and cost-effective strategies.

Limitations

This study has potential limitations related to the heterogeneity of the sample and the data collection based on caregiver's evaluations. Moreover, other pos-

sible contributing factors may have caused the increase of incidence of COVID-19 during the second wave. A case-control study comparing our population to the general pediatric population would have helped to evaluate those factors related to the increased incidence of COVID-19.

CONCLUSION

In conclusion, considering the minor impact of COVID-19 in our population, we highlight the importance of preserving the family support network, with an adequate use of preventive measures and NPS. These tools resulted safe, cost-effective and helpful for the caregivers. Moreover, the pandemic has not induced a major awareness among families on vaccinations, with the need in the next future to intensify educational campaigns.

Conflict of interest statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors have nothing to disclose.

Appendix 1. Questionnaire
The questionnaire was submitted to the main caregiver in Italian as an e-CRF using surveymonkey in April 2020. The same questionnaire, with the exception for the first and last question was proposed in February 2021.

1. How important is for you the opportunity to be screened for SARS-CoV-2? (only in April 2020)
   Likert scale from 1 to 5 (1= not at all; 5= very much)

2. At home do you follow these preventive measures (using a Likert scale 1-5 where 1 was no and 5 was always):
   ☐ Frequently wash hands with soap or alcohol-based hand sanitizer
   ☐ Avoid touching your eyes, nose or mouth with unwashed hands
   ☐ Cough or sneeze into a tissue or on your elbow
   ☐ Keep safe distance from other people (1 meter)
   ☐ Disinfect surfaces
   ☐ Avoid aggregation or crowded place
   ☐ Keep your home well ventilated
   ☐ Use of masks

3. Do you use other kind of precautions? (open answer)

4. If anyone in family presented possible symptoms of COVID-19, how did you behave? (multiple choice)
   ☐ Use of mask at home
   ☐ Domestic isolation of the sick person
   ☐ Quarantine of the sick person in another home
   ☐ None
   ☐ No one presented symptoms

5. How did your life change in this period? (Likert scale 1-5; 1= for nothing, 5= very much)
   ☐ Reduction of health-related outside activities
   ☐ Reduction of recreational outside activities
   ☐ Reduction of work activity
   ☐ Reduction of mere necessity activities (i.e., grocery shopping)
   ☐ Reduction of help from friends and family member at home
   ☐ Increased use of web/call for communication with the child support network
   ☐ Sharing of the care load with other member of the family

6. In this period how did it change the support from the health care network normally involved in the care of your son at home?
   (Scale 1-4; 1= unchanged, 2= reduced, 3= stopped, 4= never used)
   ☐ From the pediatrician
   ☐ From other health-care providers (physiotherapist, psychologist, nurse...)
   ☐ From other non-professional figures (baby-sitters, educators, volunteers)
   ☐ Other

7. Is your son regularly vaccinated?
   ☐ Yes
   ☐ No
   ☐ Never been vaccinated

8. Are you and your son vaccinated for flu?
   ☐ Both
   ☐ Only my son
   ☐ Only parents
   ☐ No one

9. Would you like to be vaccinated for COVID-19? (question added in February 2021)
   ☐ Yes
   ☐ No
   ☐ I don't know

REFERENCES

1. World Health Organization. COVID-19 Weekly Epidemiological Update 16 February 2021. Available from: www.who.int/publications/m/item/weekly-epidemiological-update---16-february-2021.

2. Garazzino S, Montagnani C, Donà D, et al. Multicentre Italian study of SARS-CoV-2 infection in children and adolescents, preliminary data as at 10 April 2020. *Euro Surveill*. 2020;25(18):2000600. doi: 10.2807/1560-7917.ES.2020.25.18.2000600

3. CDC COVID-19 Response Team. Coronavirus disease 2019 in children – United States, February 12-April 2, 2020. *MMWR Morb Mortal Wkly Rep*. 2020;69(14):422-6. doi: 10.15585/mmwr.mm6914e4

4. Leidman E, Duca LM, Omura JD, et al. COVID-19 trends among persons aged 0-24 years – United States, March 1-December 12, 2020. *MMWR Morb Mortal Wkly Rep*. 2021;70(3):88-94. doi: 10.15585/mmwr.mm7003e1

5. EpiCentro – Istituto Superiore di Sanità. Epidemiology network of the Italian National Institute of Health. Available from: www.epicentro.iss.it/coronavirus/sars-cov-2-sorveglianza-dati.

6. Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. *Acta Paediatr. Int. J. Paediatr.* 1-8 (2020) doi: 10.1111/apa.15270

7. Götzinger F, Santiago-García B, Noguera-Julián A, et al., ptbnet COVID-19 Study Group. COVID-19 in children and adolescents in Europe: a multinational, multicentre cohort study. *Lancet Child Adolesc Health*. 2020;4(9):653-61. doi: 10.1016/S2352-4642(20)30177-2

8. Tagarro A, Epalza C, Santos M, et al. Screening and severity of Coronavirus Disease 2019 (COVID-19) in children in Madrid, Spain. *JAMA Pediatr*. 2020:e201346. doi: 10.1001/jamapediatrics.2020.1346

9. Shekerdemian LS, Mahmood NR, Wolfe KK, et al. International COVID-19 PICU Collaborative. Characteristics and outcomes of children with Coronavirus Disease 2019 (COVID-19) infection admitted to US and Canadian Pediatric Intensive Care Units. *JAMA Pediatr*. 2020;174(9):868-73. doi: 10.1001/jamapediatrics.2020.1948

10. Radia T, Williams N, Agrawal P, et al. Multi-system inflammatory syndrome in children & adolescents (MIS-C): A systematic review of clinical features and presentation. *Paediatr Respir Rev*. 2020:S1526-0542(20)30117-2. doi: 10.1016/j.prrv.2020.08.001

11. Benini F, Spizzichino M, Trapanotto M, et al. Pediatric palliative care. *Ital J Pediatr*. 2008;34(1):4. doi: 10.1186/1824-7288-34-4

12. Lazzarin P, Schiavon B, Brugnaro L, et al. Parents spend an average of nine hours a day providing palliative care for children at home and need to maintain an average of five life-saving devices. *Acta Paediatr*. 2018;107(2):289-93. doi: 10.1111/apa.14098

13. Santini A, Avagnina I, Salamon E, et al. How the COVID-19 lockdown affected the parents of offspring who needed palliative care in the Veneto region of Italy. *Acta Paediatr*. 2020;109(12):2674-6. doi: 10.1111/apa.15560

The healthcare professionals' support towards organ donation. An analysis of current practices, predictors, and consent rates in Apulian hospitals

Federico Ruta[1], Chiara Musajo Somma[2], Maura Lusignani[3], Giuseppe Vitobello[1], Loreto Gesualdo[4], Francesca Dal Mas[5], Andrea Peloso[6] and Lorenzo Cobianchi[7]

[1]Asl Bt, Ospedale L. Bonomo, Andria (BT), Italy
[2]Centro di Coordinamento Regionale Pugliese per i Trapianti, Policlinico di Bari, Bari, Italy
[3]Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan, Italy
[4]Dipartimento di Emergenza e Trapianti d'Organo, Università Aldo Moro, Unità di Nefrologia, Dialisi e Trapianti, Policlinico di Bari, Bari, Italy
[5]Department of Management, Lincoln International Business School, University of Lincoln, Lincoln, UK
[6]Hepatology and Transplantation Laboratory, Department of Surgery, Faculty of Medicine, University of Geneva, Geneva, Switzerland
[7]Dipartimento di Scienze Clinico-Chirurgiche, Diagnostiche e Pediatriche, Università degli Studi di Pavia; Chirurgia Generale 1, Fondazione I.R.C.C.S. Policlinico San Matteo, Pavia, Italy

Abstract

Introduction. The paper investigates the critical care staff's support towards organ donation by analysing how their attitude, knowledge, confidence, engagement, and training can act as predictors of donation consent rates. Our study focused on hospitals in the Apulia Region, Italy.
Material and methods. The study employs a quantitative methodology based on a survey of healthcare professionals. The rate of consent to organ and tissue donation at the hospital level, given as a ratio of the permissions received to the proposals performed, was extracted from GEDON software related to the year 2019 report. For each Apulian participating hospital, we calculated a median score for each of the five predictors (namely, attitude, knowledge, confidence, engagement, and training) and investigated the association with hospital consent rates.
Results. The results highlight that the engagement of the intensive care units' healthcare personnel stands as the only influential predictor of the consent rate.
Discussion. In Italy's Apulia Region, efforts are needed to increase consent rates for organ donation. Strategies should concentrate on continuous support, as well as specific training of hospital staff involved in the donation process.

Key words
• clinical staff
• nurses
• medical doctors
• donation
• consent

INTRODUCTION

Organ donation is a crucial issue in addressing the medical needs of many patients worldwide. The research community is putting efforts into new strategies to expand the availability of organs for transplantation without direct actions on the donor pool through regenerative medicine and organ bioengineering [1-5]. While regenerative medicine technologies aim to repair and regenerate poorly functioning organs [5], these attempts are still far from an actual clinical translation [6-8], and, as of today, there is not a significant impact on

the number of transplants. Therefore, seeking consent from donors still stands as the only effective strategy to address the medical needs of those waiting for an organ.

In Italy, there was an increasing number of organ donations and transplants in 2019. However, the opposition to organ recovery had risen again. The 2019 annual report of the National Transplant Center mentions two sides of the issue: on the one hand, the transplant network showed signs of constant improvement (2019 was the second-best year for the amount of activity, and the donor lists continued to shrink); on the other hand, the

availability to donate remained lower than necessary [9]. The most significant fact is the recent increase in potential donors, that is, the subjects in a state of brain death reported by the Intensive Care Units (ICUs) as possible candidates for organ recovery. In 2019, the potential donors were 2,766 as opposed to 2,665 in 2018, with a 3.8% increase as a key indicator of the system's efficiency. The reported numbers allowed the system to absorb the negative consequences of the rate of opposition to organ recovering, which rose from 29.8% in 2018 to 31.2% in 2019. Out of 863 negative responses, most were expressed by the deceased patients' relatives. In 2019, each donation generated 2.5 transplants on average. Therefore, the 1.4% increase in the opposition rate had a cost of missed transplants for 122 patients [9]. With no opposition, in 2019 alone, about 2,200 more transplants would have been carried out. The data on donations confirmed substantial deviations from North to South Italy. With a national average of 22.8 donors per million of the population (PMP), the range varies from 49.5 donors PMP in Tuscany to 8 donors PMP in Sicily. General data on the southern regions look worrying and highly negative, showing opposition rates 15-20 points above the national average, with the peak in Sicily (49.6%) and Calabria (49.4%, +7.9% compared with 2018) [9].

Until the end of 2020, Italy required the so-called "explicit consent" for the donation of organs and tissues. Therefore, it was essential to verify the existence of a declaration expressed in life or the non-opposition of the family members [10-12]. The Ministry of Health recently amended Law 91/99, regulating the principle of "tacit approval". As of December 2020, the reform introduced tacit consent. Namely, adults will all be considered potential donors if they did not oppose during their life, so, in the absence of explicit refusal. The declaration of relatives who have the right to express the willingness of the donor is always taken into consideration even if there are no declarations of will manifested in life by the donor. Despite the introduction of the new norm, the Italian system has experienced a worrying increase in opposition rates.

The low donation rate in the Apulia region is associated with a high opposition rate [13]. Different factors can influence donation rates. One of the most important barriers to organ donation is the refusal of the family members, namely the custodians of the deceased's will, influenced by the family's cultural characteristics. Therefore, in the donation process, nurses and medical doctors' active participation and support towards donation represent crucial aspects during the identification and reporting phase. In particular, the health professionals' attitude, knowledge, confidence, engagement, and training can impact donation rates. Thus far, few studies have evaluated the connections among these elements. Several studies highlight that the team's attitude and knowledge dedicated to donation can impact the rate of consensus among family members [14, 15].

As some authors highlight [16], the family's decisions are influenced by the training of the healthcare staff, their intervention, and the satisfaction with the relationship and communication carried on with the fam-

ily and caregivers throughout the hospitalization [17]. Other authors state that healthcare professionals need to transfer unequivocal messages to the family members [18] to understand brain death and the irreversible cessation of all vital functions [19]. Moreover, the healthcare professionals' knowledge and attitude [20] about organ donation are essential for planning awareness-raising activities, which impact donation rates [21, 22].

Generally, studies show that healthcare professionals' support towards organ donation can influence their dedication. If healthcare professionals do believe in the value of organ donation, they will be keener on translating their beliefs to the donor's family [23]. The process of organ donation is widely managed by the nursing staff [24], and the identification of potential donors is considered a nursing activity. Given the importance of the nursing staff's role, they need proper training to understand the importance of the process and fully satisfy the donors and their families' health needs [25, 26].

Starting from these premises, in our paper, we aim at evaluating the attitude, knowledge, confidence, engagement, and training of healthcare professionals dedicated to the donation of organs and tissues and correlating these factors with the hospital-level donation consensus rates. Attitude is considered as the participants' will to be organ and tissue donors, the sharing of choice to donate with the closest family members, and the certainty that brain death corresponds to a person's death. Knowledge is measured as the existence of internal hospital processes, the presence of formal guidelines, and standardized procedures for ascertaining brain death and obtaining consent. Confidence analyzes how comfortable the healthcare workers feel in situations of identification and care of a potential donor, explanation about brain death, acquisition of the related consent, and care of the relationship with the relatives during the phase of the grief. Engagement stands as a significant predictor for evaluating the local transplant coordinator's capability to involve the whole ICU team during all the phases of the donation process. Training, intended as the practice of the staff working in ICUs, refers to the training courses attended and detects the needs concerning the phases of identification and care of a potential donor, communication about severe brain damage to the closest family member, explanation of brain death, and acquisition of the consent. Following the differences in donation rates in Italy's various areas, we focus our analysis on the hospitals in a southern region, Apulia, with high opposition rates, involving its Donation and Transplant Network.

MATERIALS AND METHODS

This multicenter study involved physicians and nursing staff in all ICUs in Apulia by completing an online questionnaire using Google Forms. No identifying data were collected (like name, surname, and so on). An invitation was sent to the transplant coordinator of each institution, who later shared the request with the hospital's direction office and later with the ICU staff. The Regional coordinator office followed up the invitation by telephone to ensure that the request was taken

into high consideration. Most institutions replied without the need to be recalled. The survey evaluated the attitude, knowledge, confidence, training, and engagement of healthcare professionals within the donation network. In the survey, the 26 hospitals that operate in the Apulia Region were investigated.

Data collection tool – survey

A literature review allowed choosing the tool of a Swiss multicenter study by Keel *et al.* [14]. The authors employed a survey composed of 40 closed-ended questions and two open-ended questions. Keel's survey was selected since it was used in a country where the hospital organization of donations and the consent system are similar to the Italian ones, with the opposition rates below 50%, like Apulian ones. Moreover, the questionnaire was submitted to both medical doctors and nursing staff devoted to the donation activity. The data obtained were associated with the actual consent rate in the examined hospitals.

The original questionnaire was not validated, still authorized by CNDO, the Swiss National Committee for Organ Donation [14]. Starting from Keel's survey, the Italian version was validated before the investigation. The CVI-I (Content Validity Index of the items) was calculated to evaluate the validity of the content. Construct validity was investigated through an EFA (Exploratory factor analysis). Cronbach's alpha (α) coefficient was used to examine the internal consistency of each factor on the scale, Spearman's rho coefficient to test its stability.

To associate the results of the questionnaire about the critical care staff's attitudes with the consent rates, we used the reports of Apulian Regional Transplant Coordination Center in 2019 [13], extrapolated by GEDON, a web-based application used for the management of the reports on potential organ donors. GEDON provides an advanced tool for the communication of all clinical data related to a potential organ donor, among the CR (Intensive Care Center), CRT (Regional Transplant Coordination Center), CT (Transplant Center), LT (Tissue Typing Laboratory), CNTO (National Transplant Center Operative Italy), and SIT (Transplant Information System) units.

The study was approved by the CRT in Apulia. According to Italian laws, non-interventional studies do not necessarily require approval by an ethics committee. The survey participants were exclusively healthcare professionals, with voluntary participation. No significant identifying information about the participants is possible. The study was conducted following the principles of the Declaration of Helsinki.

Statistical analysis

For each Apulian hospital involved, we first calculated the consent rate as the ratio between the consents received and the proposals made. For each predictor (attitude, knowledge, confidence, engagement, and training), some questions (ranging from a minimum of two to a maximum of four) were formulated, with a score assigned to each answer, as shown in *Table 1*. For each question, the sum of the responses received was

calculated, with the average of these scores representing the value of the predictor. A descriptive analysis was carried out by evaluating the answers based on the variability of the professional category (medical doctor or nurse). Attitude, knowledge, confidence, engagement, and training were investigated through a variable number of questions (from two to four) with dichotomous answers, with n = 1 assigned for each positive response.

Regarding the predictor engagement, depending on the number of cases in which the healthcare professional was involved in the donation activities, a score of 0 was assigned in case of no involvement, 2 if involved 1 to 3 times, 5 if involved 4 to 6 times and 8 for more than 6 times. The overall score of each predictor is represented by the average of the scores obtained. A pool of questions was also created to collect data relating to the socio-demographic characteristics of the sample: hospital and operating unit, role, gender, age, years of professional experience.

Later, the consent rates were associated with the predictors. Then, the hospital differences were compared using an ANOVA. As a homoskedasticity test, Bartlett was used, with a p-value = 0.2218>0.05. Following the test, we could claim that the variances were homogeneous, as the homoskedasticity hypothesis was true. We then applied the ANOVA method with a p-value = 0.02425<0.05, so we rejected the null hypothesis. The averages of the average scores per area were not statistically equal. Finally, we used the post hoc test to look for which areas had statistically different means.

The statistical analysis was performed using the software R [27]. We carried out an association among the predictors to estimate the set association, ranging between -1 and +1. Later, we associated the variables with the consent rate by conducting a regression analysis of the significance rate of the predictors for the consent rate, with a p-value set at <0.005.

The following *Table 2* reports the numbers of Apulia per ICU in 2019. With 102 donation proposals made, 44 refused and 58 accepted, with an opposition rate of 43.14%.

The opposition rate was calculated based on the proc.7 indicator (established by the CNTO), which relates the number of proposals made / the number of consents/oppositions obtained.

Descriptive analysis of the target population

The questionnaire was sent to all Apulian hospitals, equipped with a neurosurgery and stroke-unit neurology department, involved in the regional network of transplants that regularly carry out brain death and organ and tissue removal activities.

553 participants in 22 Apulian hospitals completed the questionnaire correctly. Four hospitals declined to participate. The sample consisted of 189 medical doctors (34.2%) and 364 nurses (65.8%). The medical personnel comprise 49.7% women and 50.3% men, whereas the nursing staff members are represented by 43.7% women and 56.3% men. Of the sample, 35.3% (split into 22.1% nurses and 13.2% medical doctors) belong to the 35-44 age range, followed by 31.1% within the 45-54 age range. 45.6% of the participants claim profes-

sional experience ranging between 11 and 20 years, and 29.1% have more than 20 years of service.

RESULTS

The attitude of the healthcare staff towards organ and tissue donation is overwhelmingly positive (*Figure 1*). Of the sample, 99% of the participants declared in favour of donation. However, only 7.4% (27) of the nursing staff and 7.9% (15) of the medical staff state that they would donate, but with restrictions, their organs after death. Moreover, 5.5% (20) of the nursing staff and 7.9% (15) of the medical staff do not seem to agree that brain death corresponds to the person's death.

Analysis of Apulian territory

The Apulia Region was divided into three macro-areas, distinguished by the geographical criterion and the population density, thus grouping the hospitals accordingly. The number of hospitals in each area is homogeneously distributed for the population hosted. The regional coordination of Transplants for Apulia has carried out these subdivisions based on the neurosurgery and neurology stroke-unit departments' presence, guaranteeing a right and equitable distribution.

As specified in *Table 1*, for each of the analyzed predictors with a favourable answer, we created a score for the dimension and calculated the average for each hospital. Moreover, we calculated the average score of the consent for each area, comparing and describing eventual intra-regional differences.

After aggregating the survey results, we could observe interesting differences among the macro-areas. In particular, we analyzed the predictors for each area singularly.

Regarding *attitude*, generally positive, the Central Area shows the most favourable attitude, with a value of 1, followed by the Southern Area with 0.96, and lastly, the Northern Area with 0.80.

Regarding *knowledge*, we observed that the Central Area has the highest value with 0.85, followed by the Southern Area with 0.78 and the Northern Area with 0.51 (*Figure 2*).

Confidence shows similar results, with no area obtaining significantly higher scores.

Concerning *engagement*, in a significant way, the Central Area (with 1.56) and the Southern Area (with 1.47) prevail over the Northern Area (with 0.75).

Regarding the predictor *training*, the Central Area obtained the highest value (0.74), followed by the Southern Area (0.67) and the Northern Area (0.57).

We can state that knowledge and engagement are the predictors that significantly differ among the three areas.

The Northern Area assumes a significant value compared to the other two areas, as reported in *Table 3*. In our study, such an area ends up being the most virtuous one within the Apulian transplant network.

Even though the Northern Area has earned the lowest scores for all the predictors examined in the present study, it has registered a 76.19% consent on organ donation. This area has 8 ICUs, but only 4 ICUs reported potential donors in 2019, with 21 donation proposals and 16 permissions.

The Central Area registered an increased procurement activity, even with a consent rate of 55.32%. In this area, 10 ICUs exist, but the reporting activity was carried out only in 5 of them. Specifically, 47 donation proposals were made, obtaining 26 consents. Based on the predictors' analysis, this area stands as the most productive in terms of the personnel's engagement and knowledge.

In the Southern Area, with 8 ICUs, a consent rate of 47.06% was registered. Of the 34 donation proposals, 16 consents were obtained, considering that 7 ICUs prepared the reports on potential donors.
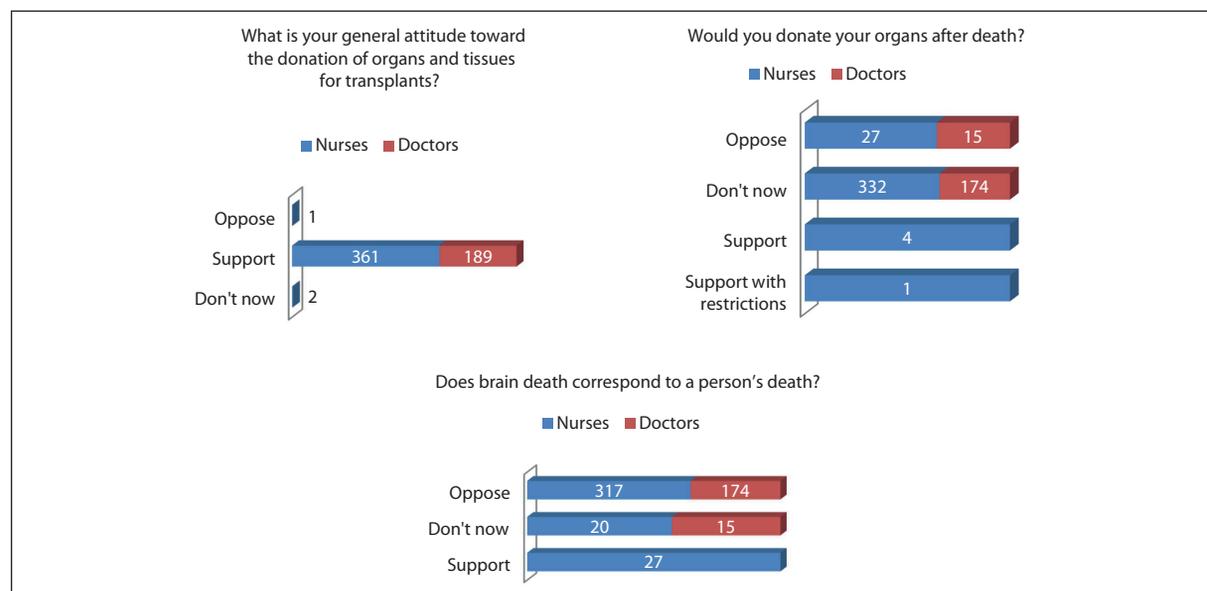

Figure 1
Attitude toward donation healthcare professionals.

Table 1
Survey and predictor cutoff of donation

Dimension	Positive answer	Score
Attitude What is your general attitude towards organ and tissue donation for transplants? Would you donate your organs after death? Does brain death correspond to death?	Yes or Yes with restrictions	0-3
Knowledge Does your hospital have standardized procedures for the donation process? Does your hospital have standardized guidelines for obtaining consent for organ donation?	Yes	0-2
Confidence Do you feel comfortable in the following situation: Explaining to relatives about brain death? Do you feel comfortable in the following situation: Formulating the proposal for organ donation to family members? Do you feel comfortable in the following situation: Accompanying and supporting relatives during their bereavement?	Yes	0-3
Engagement Please indicate the number of cases in which you have been involved in the past year: Reporting of severe brain damage to next of kin Please indicate the number of cases in which you have been involved in the past year: Explaining to relatives about brain death Please indicate the number of cases in which you have been involved in the past year: Formulating the proposal for organ donation to family members In your opinion, what moment do you think is more appropriate to address the issue of organ donation with relatives? (choose one answer)	-	None = 0 1-3 = 2 4-6 = 5 >6 = 8
Training Have you ever received training in the concept of brain death Have you ever received communication skills training (including bereavement management) in the donation process	Yes	0-2

We conducted an analysis of linear association among the single predictors to estimate the set association, ranging between -1 and +1. The associations are displayed in *Figure 3*, and some of them are significant. Attitude has a 43% association with knowledge, 24% with confidence, and 21% with engagement. Confidence has a 59% association with engagement and 25% with training. While engagement assumes a 30% association with training and 18% with consent, the latter predictor is worthy of an in-depth analysis as the only one associated with consent.

We performed a regression analysis by setting a 95% confidence interval for the association among the variables to the consensus rate, with P-value of P<0.005 of the significance rate of the predictors on the consensus.

Next, we analyzed the linear regression model among all the predictors and the consent rate to assess their statistical significance *(Table 2)*. Our analysis of the association between the consent rates for donation and the survey scores has identified the healthcare personnel's
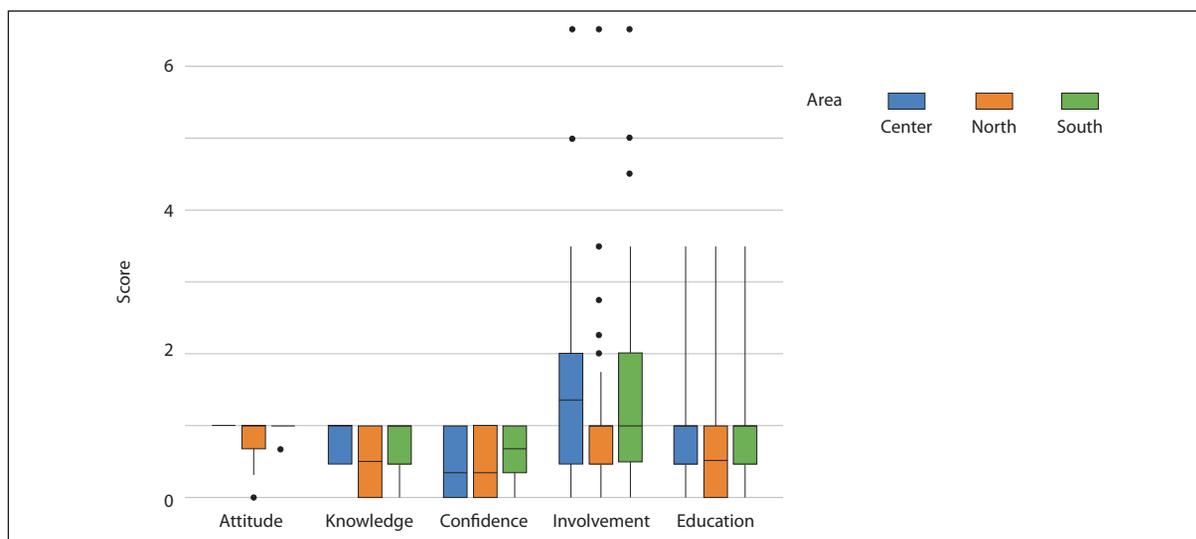

Figure 2
Intra-regional differences in the scores.

Table 2
Activity report on organ donation in 2019 in Apulia Region

Intensive Care Units in Apulia	Proposals made	Oppositions		Consents	Hospital consent = consents/proposals made
	n.	n.	%	n.	%
Foggia	6	2	33.33	4	66.67
San Giovanni Rotondo 1	1	0	0.00	1	0.00
San Giovanni Rotondo 2	2	1	50.00	1	50.00
San Severo	0	0	0.00	0	0.00
Cerignola	0	0	0.00	0	0.00
Andria	12	2	16.67	10	83.33
Barletta	0	0	0.00	0	0.00
Bisceglie	0	0	0.00	0	0.00
Bari Policlinico 1	21	9	942.86	12	57.14
Bari Policlinico 2	14	5	35.71	9	64.29
Bari Di Venere	6	5	83.33	1	16.67
Bari San Paolo	0	0	0.00	0	0.00
Bari Giovanni XII	0	0	0 0.00	0	0.00
Bari Mater Dei	0	0	0.00	0	0.00
Acquaviva	5	1	20.00	4	80.00
Altamura	1	1	100.00	0	0.00
Castellana	0	0	0.00	0	0.00
Monopoli	0	0	0.00	0	0.00
Brindisi	3	2	66.67	1	33.33
Taranto	15	4	26.67	11	73.33
Lecce	11	8	72.73	3	27.27
Casarano	1	1	100.00	0	0.00
Tricase	2	2	100.00	0	0.00
Scorrano	0	0	0.00	0	0.00
Gallipoli	1	0	0.00	1	100.00
Città di Lecce	1	1	100.00	0	0.00
Total	**102**	**44** *M* **43.14**		**58**	*M* **25.09**

engagement in ICUs as the only influential predictive factor of the consent rate, with a statistical significance of p<0.00006. The healthcare personnel's engagement stood as the strongest predictor of the consent rate. An increase in the score of the healthcare professionals' engagement led to the probability of a 5.24% increase in the consent rate *(Table 4)*.

Moreover, training and knowledge obtained positive scores, even if not significant. A previous study highlights that specific training in the context of organ and tissue donation increases the probability of consent [28]. Our study confirms the need to increase the specific training of health professionals who appear sensitive to the topic to create a "culture of donation" among the team members.

In contrast, attitude and confidence have proven to

be negative, in line with previous studies [29, 30], which report that behaving in an impersonal way with the family members and being too self-confident are perceived by the family in a negative way and therefore leads to a low probability of obtaining the family's consent.

DISCUSSION

The healthcare professional who has been appointed to explain brain death and formulate the subsequent donation proposal to the deceased's family must assume an empathetic attitude, dedicating the needed time through simple communication and knowledge translation [20, 31-33]. Several studies showed that the healthcare personnel's sensitive and compassionate approach to the family is associated with higher consent rates [34-36]. Other studies emphasize that obtaining

Table 3
Statistical difference of the predictors to donation between the North, the Center and the South of the Apulia Region

	Difference	p-value	Significance	LCL	UCL
North - Central	-0.37043592	0.0120	*	-0.6546435	-0.08622839
North - South	-0.32096430	0.0279	*	-0.6051718	-0.03675677
Central - South	0.04947163	0.7267		-0.2347359	0.33367915

LCL: lower control limit; UCL: upper control limit.
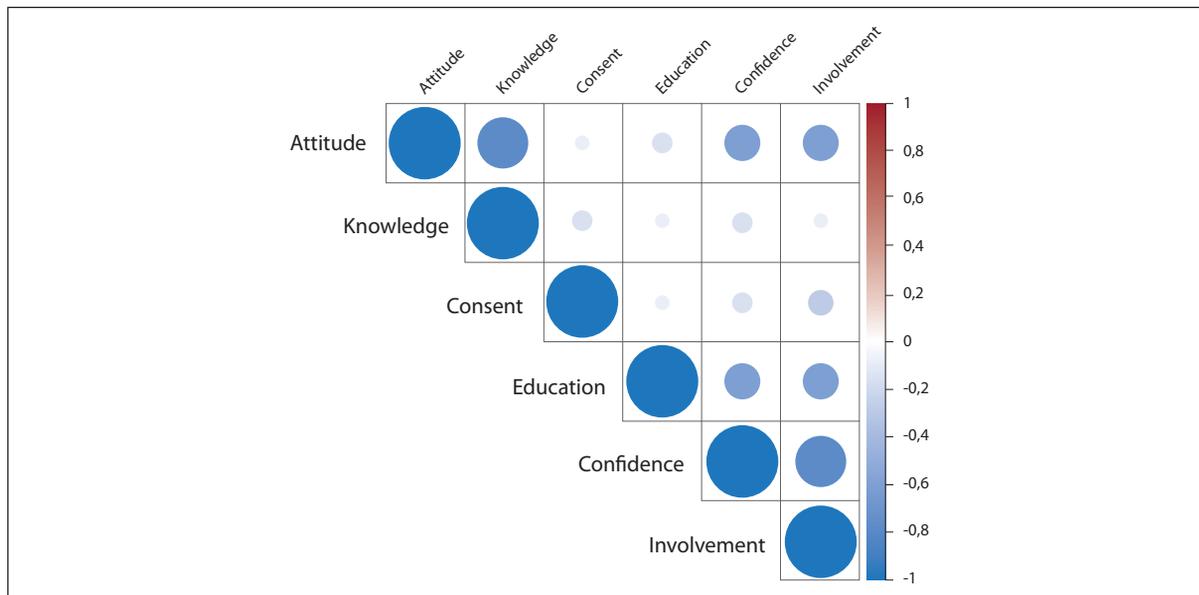
Figure 3
Association of consent-predictor rates.

"non-opposition" is not only influenced by the person who formulates the donation proposal but also by the approach and the sufficient time to understand the information and make a decision, as perceived by the family [37]. Therefore, the combination of these parameters can contribute to predicting consent rates. However, the predictors should be considered collectively to observe how the independent variables (attitude, knowledge, confidence, engagement, and training) are associated with the dependent variable (consent rate).

Ours stands as the first study conducted in the Apulia region in Italy to investigate the association between the consent rates for organ donation and the attitude, knowledge, confidence, engagement, and training of the medical and nursing personnel in ICUs. The survey has highlighted that the majority of healthcare professionals are in favour of organ donation.

The general analysis shows that engagement is the strongest predictor of consent. The differences in the regional areas should be explored to observe the predictors of interest. Despite their positive attitude toward donation, the healthcare personnel in the Northern Area showed lower scores than their colleagues in the other areas in terms of knowledge, confidence, engagement, and training. The reasons may be related to a territorial issue associated with the local Health Agencies' policies. It may be that such Health Agencies had not invested in organ donation as one of the main objectives of the modern healthcare system.

We observed that the Central Area obtained higher scores in all five predictors, particularly in engagement and training. It should be emphasized that the Central Area is the territorial headquarter of the CRT. We may presume that the presence of the Apulian CRT's headquarter offices at the Policlinic University Hospital of Bari may easily affect the professionals who are directly involved. Eventual best practices carried on from an organizational perspective in training or dealing with the

healthcare staff devoted to transplants should then be recognized and shared with the other regional hospitals, especially those with less favourable outcomes.

The analysis of the Southern Area highlights acceptable levels of confidence. However, also in this case, it is not confirmed by the scores of the other predictors that in some way remain similar to those in the Northern Area. Several studies show that where there are many hospitals that are under the jurisdiction of the same Local Health Agency, the coordination of the extended area should be present [14].

In our study, the consent rate is significantly and positively associated with the engagement of healthcare professionals. Moreover, it is observed that the consent rate is higher if the knowledge and training of the medical doctors and the nurses show a favourable inclination toward organ donation. Indeed, those professionals who are already sensitive towards the topic are the ones undergoing training. Nurses and medical doctors play an essential role in the family's final decision. Therefore, being perceived as close to the family, they should provide simple and understandable information [37, 38]. Thus, the professionals' lesser propensity to the donation may influence the family to withhold their consent to donate [39]. It is crucial that the professionals involved in this process are conveniently trained and know the communicative and relational dynamics, being able to use their soft skills by employing a simple language to adequately translate knowledge to the family members in charge of the decision [18, 31, 36]. Moreover, our study confirms that healthcare professionals involved in organ and tissue donation activities should have continuous support in their specific training oriented to acquiring a practical knowledge of the whole process and increase self-confidence. It is essential that the professionals' support towards donation raises the awareness of the population so that the number of oppositions decreases. Further research av-

Table 4
Comparative analysis of predictors to donation

Coefficients			
Estimate Standard Error t value Pr(>|t|)			
(Intercept)	346.519	77.589	4.466	9.68e-06***
Attitude	-82.886	89.451	-0.927	0.3545
Knowledge	107.014	45.971	2.328	0.0203*
Confidence	-50.273	44.453	-1.131	0.2586
Engagement	52.427	13.031	4.023	6.54e-05***
Training	0.1364	39.516	0.035	0.9725

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual Standard Error: 34.67 on 547 degrees of freedom, Multiple R-squared: 0.04239, Adjusted R-squared: 0.03364, F-statistic: 4.843 on 5 and 547 DF, p-value: 0.0002416.

enues include in-depth qualitative studies, for instance, interviewing the family members who consented to the donation to deepen which reasons fostered them to do so, measuring the real impact of the healthcare staff's support and counselling.

Our article has several limitations. One significant limitation is connected to the possible presence of other variables that could influence the consent rates. Such variables may include a strong and hardly influenceable cultural belief of the family, the changing in the common feeling towards donation, the shock following the death of a dear one, especially if unexpected or sudden, like a car accident. Moreover, it would be interesting to include other Italian regions, especially those located in the northern and central areas, to compare results and strategies.

Some practical and policy implications emerge from this study, especially a call for policymakers and hospital managers to provide an extension of training programs for both nursing and medical students but also residents and professionals in their lifelong learning education to improve their expertise to deal with such topic. A collaboration with, for instance, the psychologists of the hospital may be welcome, as it proved to be successful in other cases and clinical disciplines, like oncology [40, 41]. It is necessary to set up adequate training, employing pioneering informative, communicative, soft-skill-based tools, leading healthcare professionals to get precise information and a positive attitude in handling the matter. Moreover, common strategies should be employed in all educational settings. While information should be spread even at very early stages, like primary or secondary schools, such topics should be intensified and deepen at healthcare university degrees, to transfer and share the proper knowledge to the nurses and medical doctors-to-be.

Authorship

FR, CMS, ML, and GV conceived the idea of the study. FR and CMS developed the theory and performed the computations. FR had oversight and leadership responsibility for the research activity planning and execution. FR, CMS, and FDM wrote the first draft of the manuscript. ML, GV, LG, AP, and LC critically reviewed and amended the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest statement

The Authors have no conflict of interests to declare.

REFERENCES

1. Cobianchi L, Zonta S, Vigano J, Dominioni T, Ciccocioppo R, Morbini P, et al. Experimental small bowel transplantation from non-heart-beating donors: a large-animal study. Transplant Proc. 2009;41(1):55-6.
2. Hogan AR, Doni M, Damaris Molano R, Ribeiro MM, Szeto A, Cobianchi L, et al. Beneficial effects of ischemic preconditioning on pancreas cold preservation. Cell Transplant. 2012;21(7):1349-60.
3. Marzorati S, Bocca N, Molano RD, Hogan AR, Doni M, Cobianchi L, et al. Effects of systemic immunosuppression on islet engraftment and function into a subcutaneous biocompatible device. Transplant Proc. 2009;41(1):352-3.
4. Wassmer C-H, Lebreton F, Bellofatto K, Bosco D, Berney T, Berishvili E. Generation of insulin-secreting organoids: a step toward engineering and transplanting the bioartificial pancreas. Transpl Int Off J Eur Soc Organ Transplant. 2020;33(12):1577-88.
5. Edgar L, Pu T, Porter B, Aziz JM, La Pointe C, Asthana A, et al. Regenerative medicine, organ bioengineering and transplantation. Br J Surg. 2020;107(7):793-800.
6. Croce S, Peloso A, Zoro T, Avanzini MA, Cobianchi L. A Hepatic scaffold from decellularized liver tissue: Food for Thought. Biomolecules. 2019;9(12).
7. Orlando G, Soker S, Stratta RJ, Atala A. Will regenerative medicine replace transplantation? Cold Spring Harb Perspect Med. 2013;3(8).
8. Berney T. Liraglutide in islet transplantation: from bench to bedside. Transpl Int Off J Eur Soc Organ Transplant. 2010;23(3):257-8.

9. National Transplant Center Italy. Report 2019. Activity of donation and transplantation: the organs, tissues and hematopoietic stem cells. Rome; 2019.

10. Repubblica Italiana. Legge 1 aprile 1999, n. 91 "Disposizioni in materia di prelievi e di trapianti di organi e di tessuti". Gazzetta Ufficiale n. 87 del 15 aprile 1999. 1. Available from: https://www.gazzettaufficiale.it/eli/gu/1999/04/15/87/sg/pdf.

11. Repubblica Italiana. Legge 29 dicembre 1993, n. 578 "Norme per l'accertamento e la certificazione di morte". Gazzetta Ufficiale Serie Generale n. 5 del 8 gennaio 1994. Available from: https://www.gazzettaufficiale.it/eli/id/1994/01/08/094G0004/sg

12. Ministero della Salute. Decreto 11 aprile 2008. Aggiornamento del decreto 22 agosto 1994, n. 582 relativo al: "Regolamento recante le modalita' per l'accertamento e la certificazione di morte". Gazzetta Ufficiale Serie Generale n. 136 del 12 giugno 2008. Available from: www.gazzettaufficiale.it/atto/serie_generale/caricaDettaglioAtto/originario?atto.dataPubblicazioneGazzetta=2008-06-12&atto.codiceRedazionale=08A04067&elenco30giorni=false

13. Regional Transplantation Coordination Apulia. Annual Report 2019. Donation and transplantation activities. Apulia Region. Bari; 2019.

14. Keel I, Schürch R, Weiss J, Zwahlen M, Immer FF. Is there an association between consent rates in Swiss hospitals and critical care staffs' attitudes towards organ donation, their knowledge and confidence in the donation process? PLoS One. 2019;14(2):e0211614.

15. Rumsey S, Hurford DP, Cole AK. Influence of knowledge and religiousness on attitudes toward organ donation. Transplant Proc. 2003;35(8):2845-50.

16. López JS, Soria-Oliver M, Aramayona B, García-Sánchez R, Martínez JM, Martín MJ. An integrated psychosocial model of relatives' decision about deceased organ donation (IMROD): Joining pieces of the puzzle. Front Psychol. 2018;9:408.

17. Roels L, Spaight C, Smits J, Cohen B. Critical care staffs' attitudes, confidence levels and educational needs correlate with countries' donation rates: data from the Donor Action database. Transpl Int. 2010;23(8):842-50.

18. Dal Mas F, Garcia-Perez A, Sousa MJ, Lopes da Costa R, Cobianchi L. Knowledge translation in the healthcare sector. A structured literature review. Electron J Knowl Manag. 2020;18(3):198-211.

19. Rassin M, Lowenthal M, Silner D. Fear, ambivalence, and liminality: key concepts in refusal to donate an organ after brain death. JONAS Healthc Law Ethics Regul. 2005;7(3):75-9.

20. Dal Mas F, Bagarotto EM, Cobianchi L. Soft skills effects on knowledge translation in healthcare. Evidence from the field. In: Lepeley MT, Beutell N, Abarca N, Majluf N (Eds). Soft skills for human centered management and global sustainability. New York: Routledge; 2021. p. 95-109.

21. Wakefield CE, Watts K., Homewood J, Meiser B, Siminoff LA. Attitudes toward organ donation and donor behavior: a review of the international literature. Prog Transplant. 2010;20(4):380-91.

22. Salmani Nadoushan M, Nozary Heshmati B, Shabanzadeh Pirsaraee A, Salmani Nodoushan I, Jafari Nadoushan R, Yazdi F. Knowledge and attitude of Iranian physicians towards organ and tissue donation. Int J organ Transplant Med. 2014;5(2):66-70.

23. da Silva Knihs N, Santos JD, Schuantes Paim SM, Pestana Magalhães AL, Erbs Pessoa JL, Ramos SF, et al. Communication of death in the context of infant-child donation: Best practices for creating family interview for organ and tissue donation. Transplant Proc. 2020;52(5):1216-22.

24. Ruta F, Montemurro A, Zambello D, Rizzato L. Mappatura delle competenze infermieristiche per lo sviluppo organizzativo: analisi nazionale degli infermieri coinvolti nel processo di donazione e trapianto. Prof Inferm. 2021;74:105-12.

25. McGlade D, McClenahan C, Pierscionek B. Pro-donation behaviours of nursing students from the four countries of the UK. PLoS One. 2014;9(3):e91405.

26. McGlade D, Pierscionek B. Can education alter attitudes, behaviour and knowledge about organ donation? A pretest-post-test study. BMJ Open. 2013;3(12):e003961.

27. R Development Core Team. The R Manuals. 2021. Available from: https://cran.r-project.org/manuals.html

28. Ruta F, Delli Poggi A, Ferrara P, Lusignani M. Analysis of attitude, knowledge and willingness of undergraduate nursing students' toward organ donation. Prof Inferm. 2019;72(4):247-52.

29. Simpkin AL, Robertson LC, Barber VS, Young JD. Modifiable factors influencing relatives' decision to offer organ donation: systematic review. BMJ. 2009;338:b991.

30. Weiss J, Coslovsky M, Keel I, Immer FF, Jüni P. Organ donation in Switzerland: an analysis of factors associated with consent rate. PLoS One. 2014;9(9):e106845.

31. Featherstone RM, Leggett C, Knisley L, Jabbour M, Klassen TP, Scott SD, et al. Creation of an integrated knowledge translation process to improve pediatric emergency care in Canada. Health Commun. [Internet] 2018;33(8):980-7. doi: https://doi.org/10.1080/10410236.2017.1323538

32. Graham ID, Logan J, M.B. H, Straus SE, Tetroe J, Caswell W, et al. Lost in knowledge translation: Time for a map? J Contin Educ Health Prof. 2006;26:13-24.

33. Ruta F, Gallo G, Ferrara P, Terzoni S, Della Monica A, Dal Mas F, et al. Translating knowledge about organs and tissues donation using Webinars. An exploratory study in Italy. Transplant Proc. 2021;53(6):1792-7.

34. Rodrigue J. R, Cornell DL, Howard RJ. Organ donation decision: comparison of donor and nondonor families. Am J Transplant. 2006;6(1):190-8.

35. Jacoby LH, Breitkopf CR, Pease E. A qualitative examination of the needs of families faced with the option of organ donation. Dimens Crit Care Nurs. 2005;24(4):183-9.

36. Jacoby L, Jaccard J. Perceived support among families deciding about organ donation for their loved ones: donor vs nondonor next of kin. Am J Crit Care. 2010;19(5):e52-61.

37. Siminoff LA, Lawrence RH. Knowing patients' preferences about organ donation: does it make a difference? J Trauma. 2002;53(4):754-60.

38. Zambudío AR, Martínez-Alarcón L, Parrilla P, Ramírez P. Attitude of nursing staff toward organ donation in a Spanish hospital with a solid-organ transplant program. Prog Transplant. 2009;19(4):371-7.

39. Ruta F, Lusignani M. Knowledge on the corneas donation in the adult population in a province of Apulia Region. Prof Inferm. 2019;72(4):253-9.

40. Dal Mas F, Biancuzzi H, Massaro M, Barcellini A, Cobianchi L, Miceli L. Knowledge translation in oncology. A case study. Electron J Knowl Manag. 2020;18(3):212-23.

41. Cobianchi L, Dal Mas F, Massaro M, Bednarova R, Biancuzzi H, Filisetti C, et al. Hand in hand: A multistakeholder approach for co-production of surgical care. Am J Surg. 2021.

ORIGINAL ARTICLES AND REVIEWS

Measurable residual disease in multiple myeloma and in acute myeloid leukemia, an evolving topic

Germana Castelli, Elvira Pelosi and Ugo Testa

Dipartimento di Oncologia, Istituto Superiore di Sanità, Rome, Italy

Abstract
Minimal or measurable residual disease (MRD) is a term that refers to the submicroscopic tumor disease persisting after therapy. Sensitive immunophenotypic and molecular techniques are used to detect the small amount of residual tumor cells, conferring a detection capacity clearly more sensitive of common cytomorphologic techniques. MRD evaluation now represents an important tool in the study of solid tumors and of hematological malignancies. Concerning hematological malignancies, MRD evaluation was particularly developed in the study of multiple myeloma and acute myeloid leukemia, representing in these diseases a precious biomarker to quantify response to treatment, to evaluate the chemosensitivity/chemoresistance of the disease and to have a prognostic prediction on disease outcome. The finding that MRD evaluation may have a prognostic value, predicting the risk of relapse, stimulated interest in the introduction of MRD in clinical trials, either as a clinical endpoint or as a tool to guide treatment decisions. However, the clinical use of MRD requires a standardization of the techniques used for its detection, the use of multiple techniques and the development of a consistent accuracy and reproducibility. Finally, prospective clinical trials are required to assess the real clinical benefit potentially deriving from the introduction of MRD evaluation into clinical studies.

INTRODUCTION

Measurable residual disease (MRD, also known as minimal residual disease) in neoplastic diseases can be defined as the amount of residual tumor cells that remains in the body after the end of treatment. The objective of cytoreductive or of new targeted therapies consists in the complete eradication of all tumor cells; however, a significant proportion of patients display a residual number of resistant cells that represent the MRD and that are responsible for disease relapse. Historically, the response to treatment was based on cytologic examination of tumor biopsies with a detection limit of 10^{-1}-10^{-2}. It is evident that using a traditional technology, such as cytology, there is an intrinsic limitation to detect low levels of residual tumors; whole detection, however, is of fundamental importance at clinical level.

The development of new techniques of high-sensitivity able to quantify tumor cells, even when present in low or very low amounts, has revolutionized the detection of residual tumor cells. Techniques such as multiparameter flow cytometry (MFC), reverse transcription quantitative polymerase chain reaction (RQ-PCR), dig-ital droplet polymerase chain reaction (dd-PCR), amplico-based next generation sequencing (NGS), panel directed- NGS and whole-exome or whole-genome NGS have reached sensitivities up to 10^{-6} and allow to detect even a very minor residual tumor cell population, providing a much more accurate definition of the response to therapy.

Dramatic progresses have been made in the last years in the treatment of patients with hematological malignancies. Although these progresses, not all patients respond equally to the treatments due to disease heterogeneity and intrinsic or acquired resistance to antitumor drugs used to treat these patients. In the treatment of these patients, it is particular important to distinguish between patients who really respond to treatment with virtual disease eradication from those responding in only a partial way to these treatments with a residual and variable amount of tumor cells. The consistent progresses made in the definition of the recurrent cellular and molecular abnormalities observed in these tumors offered the unique opportunity to detect and quantify even small amounts of cells surviving to treatments [1]. Particularly, efficient techniques have been developed

Address for correspondence: Ugo Testa, Dipartimento di Oncologia, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: ugo.testa@iss.it.

for evaluation of MRD in seven hematological malignancies, including chronic myeloid leukemia (CML), chronic lymphoid leukemia (CLL), follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), multiple myeloma (MM), acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) [1]. Molecular techniques based on polymerase chain reaction were developed and standardized for all these diseases, while MFC techniques were used for MRD detection in MM, ALL, AML and CML.

In some of these diseases, the evaluation of MRD was of fundamental importance at clinical level. Thus, the monitoring of MRD in CML patients based on the quantification of the BCR-ABL1 transcript was essential for the definition of the best individual algorithm treatment and for a selection of patients who may discontinue tyrosine kinase inhibitors [2].

In ALL, MRD was evaluated by different techniques, ranging from MFC, allele-specific and mutation specific RQ-PCR and NGS techniques; these studies unequivocally supported the clinical utility of MRD evaluation as a parameter predicting clinical outcome, providing criteria for the selection of patients for intensified treatments and for MRD-targeted therapy [3].

CLL is a disease whose therapy was in continuous evolution during the last years, a condition that required the support of an assay, such as MRD, providing fast information on therapeutic efficacy. In CLL, MRD can be evaluated with a high level of sensitivity by MFC, RQ-PCR and NGS; MRD status was adopted in numerous clinical trials in CLL patients and showed that a MRD-negative status was associated with a better PFS and OS [4, 5]. Undetectable MRD was considered a main objective in some clinical studies [6].

Although is undoubted that MRD evaluation represents a precious tool for oncology clinical studies, it is also evident that MRD assays require not only a good sensitivity, but also careful procedure of standardization and the formulation of international scientific guidelines generated by experts in the specific field and institutional guidelines formulated by regulatory agencies.

In this review we analyze the progress made in the clinical use of MRD evaluation in MM and AML, considered as paradigmatic for an understanding of the contribution of MRD to clinical progress in both the understanding and treatment of these diseases.

DETECTION OF MRD IN MULTIPLE MYELOMA

Dramatic progresses have been made in the last years in the therapy of multiple myeloma (MM), leading to a significant improvement of the outcome of these patients (*Table 1*). Thus, many therapeutic strategies are capable of inducing a significant rate of complete responses. This progress rendered particularly important the accurate definition and the sensitive detection of MRD to better stratify the risk and the need for supplementary treatments of MM patients achieving complete response (CR). In fact, a significant proportion of CR patients' relapse, thus indicating that low, but clinically significant levels of MRD remain in the majority of patients attaining CR. This explains the absolute need

of developing highly sensitive techniques able to detect deeper responses than CR, as recently indicated by the International Myeloma Working Group (IMWG) [7].

The key role of MRD detection in MM patients is strongly supported by a meta-analysis carried out in 14 clinical studies and on a total of 1273 patients: in fact, in these patients an MDR-negative status after treatment for newly diagnosed MM was associated with long-term survival [8]. An updated analysis extended to 8098 MM patients for progression-free survival (PFS) analysis and 4297 patients for overall survival (OS) analysis confirmed these results showing that compared with MRD positivity, the achievement of MRD negativity was associated with a significant improvement of both PFS and OS [9]. Importantly, MRD negativity was associated with improved OS independently of the disease status (newly diagnosed or relapsed disease), MRD sensitivity level, cytogenetic risk, method used for MRD assessment and the level of the clinical response at the time of MRD evaluation [9].

According to Burgos *et al.* techniques used for evaluation of MRD in MM can be divided into those able to detect extramedullary disease (such as positron emission tomography/computed tomography, PET/CT) and those able to detect intramedullary disease (such as molecular detection of immunoglobulin gene rearrangements or multiparameter flow cytometry (MFC) immunophenotyping) [10].

Radioimaging techniques play an important role in the diagnostic procedures of MM to assess both medullary and extramedullary disease. Low-dose whole body computed tomography is a sensitive technique to assess the osteolytic bone disease, superior in its sensitivity to other conventional techniques of skeletal survey in the detection of bone disease [11, 12]. Conventional magnetic resonance imaging (MRI) was shown to be superior to ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET-CT) for the detection of small focal lesions and diffuse marrow infiltration; however, FDG-PET-CT had the advantage to provide more quantitative measures [13]. A peculiar technique of MRI, whole-body diffusion-weighted MRI (WB-DWI), based on a non-ionizing radiation modality is suitable for measurement of disease burden and treatment response in MM [13]. WB-DWI offers the advantage compared to standard MRI to be more sensitive and quantitative; furthermore WB-DWI allows the evaluation of skeletal complications and does not require intravenous contrast [13]. FDG-PET-CT imaging was shown to give 11% of false negative results in MM patients, due to the low expression of the hexokinase-2 gene in PET false-negative cases [14].

Multiparametric flow cytometry (MFC) is one of the techniques that allows to detect the intramedullary extent of MRD in MM patients. This technique is based on the identification of myelomatous plasma cells according to aberrant phenotypic features and to the presence of light-chain clonality. MFC evolved from a phase I technology with a 10^{-4} sensitivity to a more sensitive technique developed by Euro-Flow, next-generation flow cytometry (NGF) with a sensitivity of $2 \times 10^{-6.9}$. MFC technique is based on the labeling of

bone marrow cells with a panel of monoclonal antibodies: the immunophenotype of normal plasma cells was $138^+45^+19^+56^-$, whereas the phenotype of myelomatous plasma cells was $138^+45^-19^-56^+$; this technique allows the detection of both normal and neoplastic plasma cells [15]. Rawstrom et al. have used this first-generation assay of MFC to evaluate the outcome of MM patients undergoing autologous stem cell transplantation (ASCT) and showed that this technique helped to define early after transplantation patients with MRD-positive, needing additional treatment strategies [15].

San Miguel et al. reported the MFC detection of neoplastic plasma cells using a more extended panel of monoclonal antibodies; they defined the phenotype of normal plasma cells as 38^{+++}, 56^-, 45^+, 20^-, 28^-, 33^-, 117^- [16]. Using this first-generation MFC technique they showed that ASCT induced a greater reduction of the number of residual neoplastic plasma cells compared to high-dose chemotherapy alone and that after ASCT the coexistence of normal and neoplastic plasma cells was observed, a condition similar to that observed in monoclonal gammopathies of undetermined significance [16].

At variance with most routine diagnostic tests currently used for the evaluation of response to treatment in MM, MFC suffered from large intra-laboratory variations in terms of sensitivity, sample preparation, data acquisition and analysis. However, a recent study provided evidence that full standardization of interlaboratory MM MRD evaluation is feasible and compatible with the generation of highly concordant and reproducible MRD data [17].

The comparison of the detection of MRD in MM undergoing ASCT using first-generation MFC and allelic-specific real-time PCR showed that the first technique is less sensitive than the second technique; however, in patients with detectable MRD using both techniques, the percentage of tumor cells estimated by the two techniques was similar [18].

The introduction of a second-generation 8-color multiparameter-flow cytometry allowed to improve the sensitivity of MFC technique for MRD detection; the application of this technique to the study of elderly MM patients allowed to define three groups of patients according to MRD levels: i) MRD-negative ($<10^{-5}$); ii) MRD-positive (range from 10^{-5} to 10^{-4}); MRD-positive ($\geq 10^{-4}$) [19]. The standardization of the 8-color flow-cytometry, the so-called Next Generation FLOW (NGF) allowed an additional improvement of both the sensitivity and reproducibility of this technique [20]. The Euro-Flow PCD 8-color panel included the analysis of 12 different markers: CD38, CD138, CD45, CD19, CD27, CD28, CD56, CD81, CD117, Cylgκ, Cylgγ and β2-microglobulin [20]. Using this technique, multicenter analysis of bone marrow samples from 110 MM patients showed that NGF-MRD was significantly more sensitive than conventional 8-color flow-MRD [20].

The possible clinical uses of MRD evaluation in MM patients is reported in *Table 2*.

Terpos et al. have evaluated by NGF cytometry 52 patients with sustained complete remission (≥2 years) after frontline therapy: 45% of patients were MRD-positive at the level of 10^{-5} and 17% at 10^{-6} level [21]. All patients who relapsed during the follow-up were MRD-positive, including those with ultra-low tumor burden [21]. Paiva et al. have recently reported the results observed in a large set of MM patients monitored by NGF for MRD status and treated in the context PET-HEMA trial with high-intensity chemotherapy, ASCT and consolidation chemotherapy [22]. The NGF assay achieved a median limit of detection of 2.9×10^{-6} [22]. 45% of these patients achieved a MRD-negative status after consolidation therapy: 7% of these patients experienced disease progression and 50% of these patients displayed extramedullary disease [22]. Patients MRD-negative by NGF assay displayed a 88% decrease of the risk of death [22]. These findings strongly support the NGF assay of MRD in clinical evaluation of the efficacy of MM treatment.

In MM, as well as in other tumors, tumor cells can be detected in peripheral blood. A recent study showed that circulating plasma cells are detected in MM patients and can be studies by NGF cytometry [23].

Interestingly, combining detection of MRD by DW-MRI and functional imaging by DW-MRI improved prediction of outcome of MM patients, double-negativity defining patients with excellent prognosis and double-positivity patients with dismal prognosis [24].

The study of MRD by NGF was of fundamental importance not only as a prognostic measure of outcome, but also as a tool to better understand the mechanisms of treatment resistance in MM patients. Goicoechea et al. have evaluated MRD with the NGF technique in MM patients with standard and with high-risk cytogenetic abnormalities enrolled in the PETHEMA trial [25]. In patients with MRD-negative, both those pertaining to the standard and to the high-risk groups, progression-free survival and overall survival rates were greater than 90% after 36 months of follow-up [25]. MRD-positivity was associated with a median time of progression-free survival of two and three years in high-risk and standard risk patients, respectively [25]. The NGF technology was used also to explore the whole-exome sequencing of paired diagnostic and MRD tumor cells, showing remarkable difference between the two groups of patients: standard-risk MM patients showed greater clonal selection, whereas high-risk MM patients showed acquisition of new mutations [25]. The characterization of clones of MRD tumor cells may represent an important tool to understand the molecular mechanisms of MRD resistance.

The other fundamental technique used for the evaluation of intramedullary MM disease consists in the molecular assessment of immunoglobulin gene rearrangements. As observed for flow cytometry, there was a similar evolution for molecular studies of detection of immunoglobulin gene rearrangements, moving from an initial allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) more complex and less sensitive technique to a more sensitive next generation sequencing techniques with a sensitivity in the order of $10^{-6.9}$. The ASO-PCR detects rearranged B-cell receptor genes on the basis of the identification of clonotypic sequences; this technique is specific and sensitive, but has the

considerable disadvantage of being technically complex and of limited applicability. The development of high throughput sequencing technologies, using amplification and sequencing of immunoglobulin gene segments using consensus primers, improved of about 1 log the sensitivity of detection of immunoglobulin gene rearrangements and showed a good applicability, greater than 90%. MM patients who were MRD-negative by NGS displayed a significantly better survival than those who were MRD-positive [26].

Using this deep-sequencing technology, Perrot *et al.* provided evidence that in a large group of MM patients treated with lenalidomide, bortezomib and dexamethasone molecular MRD negativity was a strong prognostic factor predicting a prolonged overall survival, regardless of cytogenetic risk profile and disease stage at diagnosis [27].

In MM, as well as in many other tumors, tumor cells are not only resident in bone marrow but circulate also and release tumor DNA that can be found in peripheral blood. Mazzotti *et al.* have explored whether plasma could replace bone marrow for assessment of MRD in MM using deep sequencing [28]. However, the results of this study failed to show an association between circulating tumor DNA and bone marrow for MRD by NGS using only immunoglobulin gene rearrangements [28].

All the clinical trials that included the evaluation of MRD using a sensitive and standardized technique have reached the conclusion that MM patients achieving a MRD-negative status, either after chemotherapy treatments or ASCT, displayed a better PFS and OS compared to those with MRD-positivity [29, 30]. The available data were sufficiently clear to convince regulatory medicinal agencies, such the European Medicine Agency (EMA) that MRD measured by a standardized method with a quantitative lower limit set of at least 10^{-5} can be used as an intermediate endpoint in randomized controlled trials [31]. In line with this view, some ongoing clinical trials have as main objectives the study of MRD in MM: the trial NCT04108624 aims to assess for MRD in MM at a deeper level by combining novel imaging and laboratory techniques, to determine if patients who are MDR-negative by multiple evaluation and discontinue post-transplant maintenance therapy, and to determine is liquid biopsy is a more accurate and less invasive sampling technique for MM; the trial NCT04140162 aims to determine whether a double duratumamab-based regimen (induction and consolidation) is able to increase the proportion of MM patients reaching a MRD-negative status.

There is now consistent evidence that MRD negativity is a superior prognostic factor than conventional CR for MM patients. However, many questions related to the clinical use of MRD assays remain open.

One of these problems is related to the optimal threshold of MRD detection. Although initial studies have proposed the ideal threshold of MRD detection at 10^{-5}, however, there is now evidence that a more sensitive set-up limit of 10^{-6} is more relevant at clinical level: in fact, Paiva *et al.* using MFC [22] and Perrot *et al.* using NGS [27] showed that patients achieving MRD

negativity at the level of 10^{-6} have longer PFS periods in comparison with those that are MRD negative at 10^{-5}. Future studies will evaluate whether ultra-sensitive techniques with a limit detection in the order of 10^{-7} may further improve the prognostic predictive capacity of MRD.

In spite of the consistent improvements of the sensitivity of MRD assays and the clear clinical impact of achieving MRD negativity at 10^{-6}, disease relapses still occur in a significant proportion of patients. Thus, Paiva *et al.* using NGF reported that 7% of patients with MRD negative status at 10^{-6} displayed disease relapse after a median follow-up period of 40 months post-consolidation therapy; Perrot *et al.* showed that 29% of MM patients with MRD negativity by NGS at 10^{-6} after a follow-up of 38-55 months after randomization [27]. Interestingly, the analysis of MM patients participating to the CASSIPOETH study showed a 61.9% concordance between MRD negativity and PET-CT radioimaging post-consolidation: 6.8% of all patients displayed PET-CT positivity with a MRD negativity [32]. This finding implies the necessity of evaluating treatment responses by both MRD assays and functional radioimaging, particularly in patients with extramedullary disease [32]. Other studies confirmed the need of combining PET-CT radioimaging with MRD assay to provide an accurate prognostic evaluation of these patients [33]. The problem of disease relapse in patients with a MRD-negative status after ASCT is specifically under evaluation in the ongoing REMNANT clinical study, proposing to compare the treatment of these patients either just after MRD positivization or after disease progression [34].

It is important to identify therapies and regimens that drive sustained MRD negativity and can improve long-term outcomes. A sustained negativity of MRD may be operationally defined as a negative MRD status confirmed for one or more than one year. The detection of a sustained MRD negativity is of fundamental importance in clinical studies in newly diagnosed MM patients not eligible for ASCT and in patients with refractory/relapsing disease. The introduction in therapy of the anti-CD38 monoclonal antibody Duratumumab, in association with standard of care drug combinations, allowed in a part of patients sustained clinical responses. Recently, Avet-Loiseau reported the results on the long-term evaluation of MRD status in the POLLUX and in the CASTOR clinical trials involving the treatment of refractory/relapsing MM patients with Daratumumab/ Lenalidomide/Dexamathasone and Duratumumab/ Bortezomib/Dexamethasone, respectively [35]. After a follow-up of more than 50 months, the MRD negativity status was 32.5% in the POLLUX trial and 15.1% in the CASTOR trial; in these two studies, patients who achieved a MRD negative condition displayed improved PFS compared with patients who achieved MRD negative status but did not maintain MRD durability [35]. These observations support the view that achieving sustained MRD negativity predicts long-term outcomes in refractory-relapsing MM patients [35].

MM treatment has considerably changed and improved during the last two decades. The current par-

Table 1
Sentivity of the various techniques that can be used to detect the presence of multiple myeloma or acute myeloid leukemia cells

Technique	Multiple myeloma	Acute myeloid leukemia
Morphology	$1\text{-}5\times10^{-2}$	$1\text{-}5\times10^{-2}$
Cytogenetics	$1\text{-}5\times10^{-2}$	$1\text{-}5\times10^{-2}$
FISH	1×10^{-2}	1×10^{-2}
MFC	$1\times10^{-4}\text{-}2\times10^{-6}$	$1\times10^{-4}\text{-}1\times10^{-5}$
RT-PCR	$1\times10^{-5}\text{-}1\times10^{-6}$	$1\times10^{-3}\text{-}1\times10^{-6}$
NGS	$1\times10^{-5}\text{-}1\times10^{-6}$	$1\times10^{-3}\text{-}1\times10^{-5}$

adigm for transplant-eligible newly diagnosed MM patients implies chemotherapy induction treatments [VRd (Bortezomib, Lenalidomide, Dexamethasone) or DuraVTD (Duratumumab, Bortezomid, Thalidomide, Dexamethasone)], induction of stem cell mobilization and autologous stem cell transplantation [ASCT], followed by consolidation and maintenance [36]. Among patients achieving a CR, patients exhibiting a MRD-positive status were associated with a reduced PFS and OS compared to those with a MRD-negative condition and outcomes similar to those observed in patients exhibiting a partial response [8].

The study of MRD status in MM patients eligible for ASCTC may provide important information at various stages of the whole treatment procedure. The evaluation of MRD status may help to define the optimal chemotherapy induction regimen preceding ASCT; thus, the results of IFM/DFC/2009 study have provided that MM patients undergoing upfront ASCT after 3 cycle of RVd induction displayed a better response compared to the patients undergoing delayed ASCT after 8 cycles of RVd induction; however, in spite this finding, patients achieving a MRD negativity at $\leq10^{-6}$ in both arms of the study showed a similar OS, thus, suggesting that early ASCT did not provide additional benefit in cases achieving a MRD negativity status [37]. The study by Paiva *et al.* [22] provided evidence that MM patients achieving a MRD-negativity either before or after ASCT display a similar OS. The FORTE trial compared various induction pre-transplantation regimens (KRd 4 cycles, KRd 12 cycles, KCD) and two maintenance regimens (KR *vs* R) [34]. The results of this study showed that: KR maintenance induced a higher rate of conversion from MRD-positivity to MRD-negativity; the outcomes of patients that were MRD-negative at 10^{-5} by MFC and NGS were similar; MRD-negative patients receiving 4d KRd-ASCT exhibited a longer PFS than patients receiving 12d KRd-ASCT; KR compared to R in the maintenance regimen significantly prolonged PFS in patients achieving a MRD-negative condition before maintenance [38]. The analysis of a large phase III clinical trial (EMN02/H095 MM) showed that MRD negativity was associated with reduced risk of disease progression or disease-related death in all subgroups treated, including also patients at high-risk; in the 1-year MRD

maintenance population, 42% of patients MRD-positive at pre-maintenance became MRD-negative after lenalidomide treatment [39].

The current standard of care for MM patients not eligible for ASCT implies three therapeutic options based on three different regimens: VRd, DaraRd (Daratumumab, Lenalidomide, Dexamethasone) or DaraVMP (Duratumumab, Bortezomib, Melphalan, Prednisone). In these MM patients ineligible for transplantation the introduction of the anti-CD38 monoclonal antibody Duratumumab elicited a clear benefit in terms of reduced risk of disease progression or death. In these patients, durable MRD negativity (i.e., lasting for at least 12 months) was associated with improved PFS and clinical outcomes [40]. A similar conclusion was reached in the MANHATTAN clinical study carried out to assess the safety and effectiveness of Duratumumab, Carfilzomib, Lenalidomide and Deaxamethasone combination therapy for newly diagnosed MM patients, in the absence of high-dose melphalan chemotherapy and ASCT [41]. In this nonrandomized clinical study, the primary endpoint consisted in the achievement of MRD negativity, an objective reached in 29 of 41 patients, with a median time to MRD negativity corresponding to 6 cycles [41].

MRD is currently evaluated by NGS or by NFC in bone marrow and, therefore, it is not surprising that it is influenced by the quality of bone marrow samples. Bone marrow tissue may be obtained either by needle aspiration or by biopsy; bone marrow biopsy is better than bone marrow aspiration because it allows to obtain a higher number of tumor cells, less diluted by blood than bone marrow aspirates [42, 43]. Ideally, bone marrow aspirates should be obtained at multiple sites [42, 43]. To ensure a better detection of tumor cells some studies have used the immune magnetic CD138+ cells enrichment, a procedure commonly used in MM patients for baseline FISH analysis to obtain a concentrated source of neoplastic cells [42, 43].

Another important problem is related to the definition of optimal time points of MRD detection. These time points have not been yet standardized. In this context, most information derives from clinical trials carried out in transplantation-eligible MM patients, where MRD was evaluated after high-dose melphalan therapy and ASCT. Since MRD is a measure of the tumor cells present after the end of the therapeutic effects of a treatment, it is evident that the optimal time for MRD evaluation at the level of bone marrow during the course of treatment is directly related to the dynamics of response to a particular therapeutic regimen [44].

MRD IN THE PROGNOSIS AND TREATMENT ASSESSMENT OF AMLs

AMLs are a heterogeneous group of hematological malignancies, characterized by a complexity of molecular alterations and clonal development. In the last years, considerable progresses have been made in the characterization of the molecular abnormalities underlying AMLs, with the identification of recurrent chromosomal alterations and of gene mutations, allowing the classification of these leukemias in various subgroups,

characterized by different genetic alterations and response to current treatments [45, 46]. This molecular classification identified some major molecular subtypes: i) AMLs characterized by peculiar translocation events leading to the formation of fusion genes and correspondent fusion proteins, including inv(6), t(15;17), t(8;21), inv(3); ii) AMLs exhibiting chromatin-spliceosome gene abnormalities, including mutations of genes involved in RNA splicing, chromatin and transcription; iii) AMLs characterized by *TP53* mutations, complex karyotype alterations and copy-number alterations; iv) AMLs displaying mutations of the nucleophosmin 1 (NPM1) gene; v) AMLs characterized by double CEBPA mutation [45, 46]. The genes most frequently mutated in AMLs are represented by: mutations of the tyrosine kinase membrane receptor Flt3, more frequently (about 30% of adult AMLs) with Flt3-Internal Tandem Duplication (FLT3-ITD) and less frequently (about 10%) with FLT3-Tyrosine Kinase Domain (FLT3-TKD) mutations; mutations of the *NPM1* gene observed in 30-35% of cases; mutations of the methyltransferase *DNMT3A* gene (20-30% of AMLs); *NRAS* (15-20% of cases); mutations of the transcription factor *RUNX1* (15% of AMLs); the methylcytosine dioxygenase 2 *TET2* gene (15-20% of AMLs); the isocitrate dehydrogenase 2 (*IDH2*) gene (10-15% of AMLs) and *IDH1* gene (5-10%) [46]. The identification of genetic abnormalities in AMLs was of fundamental importance for the understanding of leukemia pathogenesis, for the identification of new therapeutic targets and for the identification of biomarkers suitable to monitor the response to anti-leukemia therapy [47].

According to various molecular criteria the European Leukemia Net stratified AMLs into three risk subgroups, with favorable prognosis (comprising t(15;17), t(8;21), inv(6), biallelic mutated *CEBPA* and *NPM1* mutant without *FLT3-ITD*), intermediate prognosis (encompassing *NPM1* mutant with *FLT3-ITD*low, t(9;21) and various cytogenetic abnormalities not classified as favorable or adverse) and adverse prognosis (comprising monosomy 7 and 5, deletion of long arm (q) chromosome 7, abnormalities of 3q, 17p and 11q, multiple cytogenetic abnormalities, *NPM1* wt and *FLT3-ITD*high, *TP53* mutations associated with complex karyotype, *ASXL1* mutations, t(6;9) and t(3;3) groups [48]. Prognostic stratification of AML patients at diagnosis had strong clinical implications in that it allows to allocate after remission patients with high-risk and intermediate-risk AMLs to allogeneic stem cell transplantation. The standard therapy for adult AML patients involves treatment with induction chemotherapy with cytarabine and an anthracycline; the majority of patients achieve a morphological remission with this treatment, but their prognosis remains poor in that more than 50% of these patients relapse [49, 50].

For a long time, the evaluation of "complete response" (CR) to therapy was based on the morphological evaluation of bone marrow and a threshold of 5% or less was required for assessment of CR [50] (*Table 1*). Progresses in the techniques of multicolor flow-cytometry allowing the identification of leukemia-associated immune phenotypes and in the molecular detection of leukemia-specific molecular alterations by quantitative PCR, next generation sequencing and digital droplet PCR have allowed the detection of MRD, a measure of response to therapy much more stringent than CR [51].

A consensus document from the European Leukemia Network MRD working party reported the key clinical and scientific issues in the measurement and application of MRD in AML, stressing the need of a qualitative and quantitative standardization of the flow cytometry and molecular protocols used to evaluate MRD in AML [50] For flow cytometry blood evaluation of MRD a value of 0.1% as the threshold to distinguish MRD-positive from MRD-negative patients was recommended [52].

Voso *et al.* have analyzed the applications, the advantages and the limitations of molecular methods used for AML MRD detection [51]. Quantitative RQ-PCR methods are sensitive and specific and are used in the detection of the fusion genes observed in some AMLs and NPM1-mutant AMLs; NGS offers the advantage to be potentially applicable to all leukemic patients and detect the potential of combined mutations assessment

Table 2

Possible clinical use of MRD evaluation during the clinical course of multiple myeloma

Clinical phase	Potential clinical utility
Patients with newly diagnosed MM eligible for ASCT	Evaluation of MRD status at various times after ASCT
Transplant-inelegible newly diagnosed MM patients	Evaluation of the percentage of patients with post-therapy and sustained MRD negativity during and after maintenance therapy
Elderly frail MM patients with newly diagnosed disease not-eligible for high-dose chemotherapy or ASCT	Evaluation of MRD negativity rate following various treatments
Previously diagnosed patients with MM on lenalidomide maintenance post SCT	Evaluation of the conversion rate to MRD negativity
Relapsed/refractory MM patients treated with multiple lines of therapy	MRD negativity before and after experimental treatments
Relapsed MM patients previously treated with ASCT	Evaluation of MRD negativity rate at various time points after experimental treatments
Patients with relapsed and lenalidomide refractory MM	Evaluation of MRD negativity ratre in patients who achieved CR with experimental treatments

for MRD evaluation; digital droplet PCR is a technique based on amplification of target genes without a reference standard curve, providing an absolute quantification: this technique is highly sensitive and specific and is increasingly used for MRD evaluation [51].

Several studies have strongly supported the clinical utility of MRD detection in AMLs (*Table 3*). In this context, particularly instructive were two studies. Freeman *et al.* evaluated the CR and MRD status (as assessed by MFC) in a large set of adult AML patients undergoing standard induction chemotherapy: about 31% of these patients (and 42% of those with good and intermediate risk) achieved a CR/MRD-negative status and their survival was significantly longer than that observed for CR/MRD-positive patients [53].

A second fundamental study carried out by Jongen-Lavrencic *et al.* implied the analysis of 482 AML patients with targeted next generation sequencing: i) 89% of these patients displayed at least one mutation; ii) mutations persisted in 51.4% of these patients; iii) the detection of mutations associated with clonal hematopoiesis, such as mutations of *DNMT3A, TET2* and *ASXL1* genes was not correlated with disease relapse; iv) after exclusion of these clonal-related mutations the presence of a MRD positivity was clearly associated with a reduced relapse rate, relapse-free survival and overall survival compared to the presence of MDR negativity; these differences remained statistically significant in both univariate and multivariate analysis [54].

Very recently, Tsai *et al.* have investigated the prognostic impact of NGS MRD detection in a cohort of 335 *de novo* AML patients at two time points after chemotherapy: after induction chemotherapy and after the first consolidation cycle [55]. Excluding *DNMT3A, TET2* and *ASXL1* mutations, MRD was detected in 46% of patients at the first time point and in 29% at the second time point [55]. Patients with detectable NGS MRD at either time point had a higher incidence of relapse and a shorter survival; however, NGS MRD evaluation after consolidation therapy was more predictive of outcomes than the one after induction chemotherapy [55]. Thus, the evaluation of NGS MRD after first consolidation therapy can help to individually predict the clinical outcome of AML patients [55].

Other fundamental studies have shown the predictive value of outcome of MDR status measured prior to myeloablative allogeneic stem cell transplantation (ASCT): MRD negativity before ASCT was associated with a clearly better survival compared to that observed in MRD-positive patients [56, 57]. Promising targets of MRD prior to allogeneic stem cell transplantation are represented by *NPM1, FLT3-ITD* and *IDH1/IDH2* mutations [58].

AML is a very heterogeneous disease and this requires to analyze the strategy for the measurement of MRD in the different AML subsets. Here we will analyze the evidence supporting *NPM1* mutations as a suitable biomarker for evaluation of MRD status in *NPM1*-mutant AMLs.

NPM1-mutant AMLs represent about 30% of adult AML and are now recognized as a distinct entity in the 2017 World Health Organization (WHO) classification of hematopoietic neoplasms [59]. NPM1 is a multifunctional nucleolar protein with shuttling properties, delocalized in the cytoplasm following the mutations observed in AMLs, usually involving the exon 12 of the gene [60]. *NPM1* mutations are always heterozygous and frequently co-occur with other mutations. In animal models *NPM1* mutations cooperate with *DNMT3A* and *FLT3-ITD* mutations to promote leukemia development [47]. The prognostic impact of *NPM1* mutations in AML is dependent upon the co-mutation pattern and the allelic ratio of *NPM1* mutations [60]. Concerning the co-mutation, the presence of *NPM1* confers a relatively favorable prognosis in the absence of *FLT3-ITD* co-mutations; it was proposed that only *NPM1-mutant/FLT3-ITD*[high], but not *NPM1-mutant/FLT3-ITD*[low] double mutants AMLs are associated with a negative prognosis, but this point remains controversial [60]. Concerning the variant allelic ratio (VAF), it was shown that *NPM1*-mutant AMLs with a high allelic ratio (≥0.44) display a shortened overall survival following standard treatment compared to *NPM1*-mutant AML with a low-allelic ratio (≤0.44) [61].

NPM1 mutations represent a good candidate for MRD evaluation for three important properties: a high frequency; the stability at relapse; the absence of clonal hematopoiesis [60]. These properties have triggered numerous studies evaluating MRD in *NPM1*-mutant AMLs. A study by Gorello *et al.* reported the development of a quantitative PCR technique for quantification of *NPM1*-mutations: this technique was both sensitive and quantitative and allowed to define the level of *NPM1* mutations remaining after therapy [62]. Alternative methods for monitoring MRD in *NPM1*-mutant AMLs are based on digital droplet PCR or NGS [63, 64].

Table 3
Possible clinical use of MRD evaluation during the clinical course of acute myeloid leukemia

Clinical phase	Potential clinical utility
After induction therapy	MRD positivity may support therapeutic choices : i) an intensifying treatment at induction therapy ; ii) an extra treatment ; iii) a targeted therapy
At disease relapse	MRD status post-salvage therapy in relapsing patients is fundamental for prognostic stratification and HSCT choice
Before stem cell transplantation	MRD status may provide a fundamental tool for risk stratification and choice of optimal consolidation therapy (consolidation chemotherapy or stem cell transplantation)
After stem cell transplantation	MRD status may provide criteria for post-transplant therapeutic choices, such as targeted therapy or any other possible therapeutic intervention

In an initial study, Kronke *et al.* have evaluated the prognostic value of MRD in a group of AML patients with *NPM1* mutation: after double consolidation therapy, patients achieving a negative MRD status by quantitative RQ-PCR displayed after 4 years a clearly better survival than patients with a positive MRD status [65].

These findings were confirmed and extended by Ivey *et al.* who have explored the persistence of *NPM1*-mutated transcripts in the blood of 346 *NPM1*-mutated AML patients after second cycle of induction chemotherapy: 15% of these patients displayed persistence of *NPM1*-mutated transcripts and exhibited a significantly shorter overall survival than patients without detectable *NPM1*-mutant transcripts in their peripheral blood [66]. Importantly, in this study in 69/70 patients *NPM1* mutations were found at the time of relapse, thus supporting the stability of these mutations during disease evolution [66].

Several studies have all supported the predictive prognostic value of pretransplant NPM1 MRD levels in outcome after allogeneic stem cell transplantation. Thus, Kaiser *at al.* have shown that pre-transplant *NPM1* MRD levels >1%, as evaluated by quantitative RQ-PCR, are an independent prognostic factor for poor survival after allogeneic SCT [67]. Thiol *et al.* showed that pre-transplant MRD for *NPM1* mutations and *FLT3-ITD* mutations, as measured by NGS, are predictive of allogeneic SCT outcome [68]. Lussana *et al.* have reported the study of 89 patients with *NPM1*-mutant AML; after two cycles the MRD status was strongly associated with patient outcome. In MRD-negative patients, post-remission consolidation with allogeneic SCT did not result in an improved survival compared to conventional chemotherapy. In MRD-positive patients, overall survival was improved in patients treated with ASCT, compared to those receiving conventional chemotherapy [69].

Dillon *et al.* have analyzed in peripheral blood and bone marrow of 107 *NPM1*-mutant AML patients undergoing ASCT after standard consolidation chemotherapy for *NPM1*-mutant content using quantitative RQ-PCR [70] Using this approach, they have stratified patients as MRD-negative with an overall survival of 83% after 4.9 years of follow-up, MRD-low (<200 copies/10^5 *ABL* gene) with an overall survival 63% and MRD-high levels with 13% of overall survival [70]. Patients with *FLT3-ITD* co-mutations had poorer outcomes [70].

In addition to allogeneic SCT, in AML patients with good- or intermediate-risk AML autologous SCT is an alternative transplantation-based therapeutic approach; the pre-transplantation status is the most important determinant for eligibility to autologous SCT [71]. A recent study reported the study of 42 AML patients with *NPM1*-mutated AML undergoing autologous SCT: determinants of patient outcome were the *NPM1* MRD status and the CD34+ mobilizing capacity, in those patients MRD-negative have a much better overall survival than MRD-positive patients and low CD34 mobilizer patients have a better survival than highly mobilizer patients [72]. Interestingly, patients MRD-negative and low CD34 mobilizers have a particularly good outcome,

while those MRD-positive and CD34 highly mobilizers have a dismal prognosis [72].

In recent studies MRD was used as a tool to evaluate the efficacy of new drug combinations in *NPM1*-mutant AMLs. Thus, Kapp-Schwoerer *et al.* have evaluated the impact of gemtuzumab ozogamicin on MRD (*NPM1*-mutant transcript levels) and relapse risk in a large group of *NPM1*-mutated AMLs treated in the context of AML SG 09-09 trial [73]. In this study AML patients were treated with induction therapy alone or in combination with gemtuzumab ozogamicin. The achievement of a MRD-negative status in these patients was associated with a reduced relapse rate [73]. *NPM1*-mutant transcription levels were significantly lowered in the arm of patients treated with gemtuzumab ozogamicin, resulting in a significantly reduced rate of relapses [73].

In another study, Tiong *et al.* have evaluated the capacity of treatment based on low-intensity chemotherapy and venetoclax (a Bcl2 inhibitor) to lower *NPM1*-mutant levels in AML patients either with molecular persistence or with molecular relapse/progression after standard induction chemotherapy [74]. All the five patients with molecular persistence achieved durable molecular complete remission and 6/7 patients with molecular relapse/progression achieved a switch from a MRD-positive to a MRD-negative status [74]. In the ongoing phase II PEMAZA clinical trial (NCT 03769532) it is under evaluation the combination therapy of azacitidine and pembrolizumab (anti-PD1) to *NPM1*-mutated AML patients with MRD positivity and impending hematological relapse after conventional induction chemotherapy. Therefore, this is a trial based on MRD-guided treatment in *NPM1*-mutated AML patients.

Bataller *et al.* recently reported the results of a study involving 114 *NPM1*-mutated AML patients achieving CR after induction chemotherapy; in the post-remission phase, patients exhibiting molecular failure (33/114) or hematological relapse (13/114) were treated with MRD-based pre-emptive intervention: two-years OS of patients with molecular failure 86% and of patients with hematological relapse was 42% [75]. These authors showed also that quantitative NPM1 detection was predictive of leukemia-free survival (LFS): patients with an MRD ratio *NPM1*mut/ABL1=<0.05 displayed a two-year LFS of 77%, compared to a LFS of 40% for patients with a MRD *NPM1*mut/ABL1>0.05 [75].

Although the data concerning the *NPM1* mutational status in relapsing patients support a consistent genetic stability of these mutations, a recent study by Hollein *et al.*, based on the study of 104 relapsing *NPM1*-mutant AMLs reported that 14 of these patients relapsed with *NPM1*[WT] AML [76]. Several findings supported the view that *NPM1*-mutated AMLs that relapse with wild-type *NPM1* is a distinct disease compared to the rest of *NPM1*-mutated AMLs: blood counts at diagnosis were very different between patients with *NPM1*[mut] and NPM1[wt] relapse (30 *vs* 3×10^9/L); *NPM1*[mut] relapse occurred earlier than *NPM1*[wt] relapse (14 *vs* 43 months); *DNMT3A* mutations are more frequent in patients with *NPM1*[wt] relapse [76].

The difficulties to use MRD testing in AML clinical studies

There is no doubt that MRD assays have improved our ability to measure the level of response to treatment beyond the limitations of morphological analysis. When introduced in clinical trials, the various techniques of MRD detection in AML, either based on immunophenotypic or molecular parameters, may give a strong contribution by providing: a sensitive measure of effectiveness; a surrogate endpoint in these studies; a clear rationale for their use to guide treatment [77, 78]. These three objectives are of increasing complexity and require not only a high sensitivity and standardization of MRD assays to detect residual neoplastic disease, but also the capacity to predict the outcome of individual patients. Thus, concerning the first objective, it is possible to conclude that MRD assays are able to improve the definition of treatment effectiveness in AML patients.

The evaluation of treatment effectiveness by MRD assays allowed the identification of AML patients displaying MRD-positivity: these patients are considered at a high-risk of relapse. Future studies should develop specific trials aiming to identify specific treatments that could reduce the relapse risk in patients with a positive MRD test. In this context, a number of promising targets for a MRD-directed therapy have been identified and are under current investigation [58, 79].

However, the clinical potential utility of the detection of a MRD-positivity is highly variable for different AML subtypes. In fact, the clinical utility of the detection of MRD-positivity is related to two fundamental variables: the positivity of MRD assay must be precedent to the clinical relapse, giving a sufficient lapse of time for alternative therapies to try to prevent disease relapse; the availability of alternative therapies potentially effective. The first point implies the variability in the kinetics of relapse for different AML subtypes. Thus, Ommen *et al.* reported that the relapse kinetics is remarkably slower in *CBFB-MYH11* AMLs than in *RUNX1-RUNXT1*, *PML-RARA* and *NPM1*-mutated AMLs; this finding implies the need of a different timing of sampling of blood in these leukemias for MRD assay to have chances to detect MRD-positivity with a sufficient lapse of time before clinical relapse [80].

According to these observations it was suggested an individualized follow-up for different AML subtypes in remission. This individualized follow-up implies not only a different timing of sampling but also a different frequency of sampling in different AML subsets [81]. Thus, the European Leukemia Net MRD work group has recommended optimal time points for MRD evaluation by PCR and MFC for different molecular targets according to evidences deriving from specific studies: thus, the most relevant time points for MRD evaluation in *PML-RARA* and *RUNX1-RUNXT1*-positive AMLs using specific PCR assay is at the end of the consolidation treatment, while in *NPM1*-mutant AMLs is after 2 cycles of chemotherapy [52, 82, 83].

In line with these conclusions, a recent study by Puckrin *et al.* evaluated whether monitoring of MRD every 3 months for two years after chemotherapy treatment could predict and prevent morphologic relapse in 114 patients with core-binding factor AMLs [71]. However, the results of this study provided evidence that MRD evaluation was able to detect impending relapse in only 25% of patients [84]. This finding implies the need to develop alternative strategies for monitoring of MRD in these patients [84]. Furthermore, other studies showed that the kinetics of relapse showed heterogeneity within molecular subgroups of AMLs, according to the co-mutation pattern: thus, AML with partial tandem duplications (PTD) within the *MLL* gene displayed a slower relapse kinetics than AMLs with *MLL* translocations; however, *MLL-PTD* showed a consistent heterogeneity in their relapse kinetics, dictated by the presence of *RUNX1* or *FLT3-ITD* mutations accelerating relapse timing [85]. Finally, targeted DNA sequencing for residual disease is clearly more informative after than during initial induction chemotherapy [86].

RUNX1-RUNXT1 transcript levels after treatment represent the best biomarker to monitor MRD in t(8;21) AMLs and are a marker to predict relapse. The combination of *KIT* mutation, the only gene with prognostic significance in t(8;21) AMLs, with MRD status improves risk stratification and treatment guidance [87].

MRD detection was introduced as a major endpoint in some recent clinical studies involving new therapeutic approaches based on immunotherapy. AML patients, compared with normal controls, display increased inhibitory coreceptor expression on CD8 cells, involving molecules such as PD1, TIM3 and LAG3 [88]. High PD1, PDL1 and PDL2 expression in AML was associated with poor overall survival [89] IN *NPM1* and *FLT3*-mutated AMLs, high PDL1 expression predicts a poor outcome [90]. These observations have supported the study of immune check inhibitors in AML patients. Anti-CTLA4 monoclonal antibody elicited a significant clinical response in 4/12 AML patients relapsing after allogeneic SCT; interestingly, all these patients displayed an extramedullary disease [91]. Monotherapy with anti-PD1 antibodies induced only modest clinical responses in AML patients [92].

To improve the response rate to anti-PD1 of AMLs, the anti-PD1 drug Nivolumab was administered in combination with induction chemotherapy or the hypomethylating agent azacitidine. In a phase II study, newly diagnosed AML patients were treated with induction chemotherapy, followed by nivolumab up to 1 year: 77% of patients achieved a CR and 53% displayed a MRD-negative status, as assessed by MFC [93]. In relapsing/refractory AML patients treated with azacitidine, complete responses were observed in 22% of cases [94]. A phase II pilot study evaluated nivolumab as maintenance therapy and not eligible for SCT; the large majority of these 14 AML patients had a MRD-positive status and 1 of these patients switched to MRD-negative status during maintenance therapy [95]. A randomized, phase II clinical trial (NCT02275533) is evaluating a maintenance therapy based on nivolumab to eliminate MRD and to prevent relapse in AML patients in CR after standard chemotherapy.

Given the capacity of azacitidine to stimulate CTLA4

expression in AML patients, another trial evaluated the association of azacitidine, nivolumab and ipilimumab (a monoclonal antibody anti-CTLA4) on refractory/relapsed AML patients [96]. 43% of 20 treated patients showed a CR with an OS at 1 year of 58%; however, this drug association was accompanied by a consistent toxicity [96]. Finally, an ongoing phase II clinical trial (NCT04214249) is evaluating whether blockade of PD1 added to standard chemotherapy is able to target MRD in AML patients; this randomized study treatment with intensive chemotherapy alone or in association with anti-PD1 Pembrolizumab as frontline therapy in AML patients.

One of the most relevant and potentially useful contributions of MRD detection in AML patients would consist in providing a guide for SCT and for the type of SCT in AML patients achieving a CR status after consolidation therapy. Several studies have explored this topic. In a retrospective study, Versluis *et al.* reported the results on 547 AML patients achieving a CR after consolidation therapy and all explored for MRD status by MFC before post-remission therapy: 52% received allo-SCT, 19% auto-SCT and 29% a third cycle of chemotherapy [84]. 19% of these patients were MRD-positive after induction chemotherapy and their OS was poorer after post-remission therapies compared to that of patients with an initial MRD-negative condition [97]. Importantly, allo-SCT significantly reduced the rate of relapse compared with chemotherapy or auto-SCT, an effect similarly observed in MRD-negative and MRD-positive patients [97].

Recently, the results of a prospective study, GIMEMA AML 1310 trial of risk-adapted, MRD-directed therapy for young AML patients were reported. This trial involved the treatment of AML patients with favorable-risk after consolidation therapies with auto-SCT, of AML patients with poor-risk AML with allo-SCT and of AML patients with intermediate-risk AML received either auto-SCT or allo-SCT depending on the post-consolidation levels of MRD [98]. This study involved the analysis of MRD by MFC in 342 AML patients achieving a CR post-consolidation therapy. Two-year OS in the favorable-risk group was 74% and, in the poor-risk group was 42%; in the intermediate-risk AMLs, OS was 79% in the MRD-negative group and 70% in the MRD-positive group [85]. The absence of a significant difference in OS among intermediate-risk AMLs receiving auto-SCT and intermediate-risk AMLs receiving allo-SCT, supports the view that MRD status is a valuable biomarker for risk-stratification of this group of AML patients [98].

The decision to recommend or not a SCT to an AML patient in first remission remains a complex choice. This choice is particularly challenging for AML patients in first remission with a MRD-negative status and is related to the decision to transplant or not, to the type of SCT, auto-SCT or allo-SCT, and to the type of conditioning regimen, myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC). This choice cannot be guided only by the MRD status but must consider criteria related to the risk category of the AML subtype and to several patient-specific clinical features.

Retrospective analysis performed on a very large set of AML patients suggested that allo-SCT with myeloablative conditioning regimens should be the preferred choice for MRD-positive patients and that MRD-negative patients should be treated with transplantation procedures involving reduced-intensity conditioning regimens, avoiding the toxicities of the myeloablative conditioning regimens [99].

The role of conditioning regimen was explored in a recent phase III study in a group of AML patients in morphologic remission after induction chemotherapy, explored by ultradeep NGS sequencing for 13 commonly mutated genes in AML and randomly assigned to allo-SCT after MAC or RIC [87]. In patients with no mutations, the OS of patients undergoing either MAC or RIC was similar; however, in patients MRD-positive MAC compared to RIC resulted in a reduced relapse rate (19% *vs* 67%) and survival (after 3-years, 61% *vs* 43%) [100]. The results of this study supported the view that MAC rather than RIC in MRD-positive AML patients before allo-SCT resulted in an improved survival.

The choice at the level of individual AML patients cannot be based only on the MRD status as a predictor of relapse risk but must be based on a number of co-variates, including white blood cell counts at diagnosis, number of chemotherapy cycles to achieve first remission, cytogenetic and mutation profiles and global risk evaluation according to ELN [101]. This type of approach is strongly justified by the observation that the accuracy and precision of MRD in predicting outcomes of therapy in AML is limited and must be carefully evaluated using standardized methods and using more than one technique (i.e., MFC and RQ-PCR) [101]. Furthermore, in clinical studies MRD results are reduced in terms of negative and positive, where the positivity may correspond from few to many AML cells. Finally, the impact of MRD-testing in the context of SCT must be evaluated in prospective studies.

It is important to note that a recent systematic review and meta-analysis based on 81 studies involving a total of 11,151 AML patients provided evidence that the estimated 5-year disease-free survival was 64% for patients with a negative MRD status, compared to 25% for those with a positive MRD status; the estimated overall survival was 68% for patients without MRD and 34% for those with MRD [102]. The findings of this meta-analysis support the view that achievement of MRD negativity represents a fundamental therapeutic objective and is associated with a better disease-free survival and overall survival in patients with AML. These observations also support the evaluation of MRD status as a fundamental end-point for evaluation of new drugs or treatments for the therapy of AMLs.

In conclusion, the studies on MRD have shown a clear association between MRD positivity and adverse outcomes, thus supporting the role of MRD as a routine biomarker in both current clinical practice and clinical trials [103]. The use of MRD as a surrogate efficacy-response biomarker is a potentially important strategy to accelerate drug development/approval [103]. The assessment of MDR after induction inten-

sive chemotherapy represents an important prognostic factor for risk stratification of patients and for guiding therapeutic choices in some AML subsets, such as intermediate-risk patients: MRD-negative patients are selected to receive autologous stem cell transplantation, whereas MRD-positive patients are selected for allogeneic stem cell transplantation. Future studies will be required to demonstrate whether treatment effects on MRD, such as the timing of therapeutic intervention with respect to MRD assessment (at the moment of MRD detection or at overt disease recurrence) may improve outcomes.

CONCLUSION

In conclusion, the studies in MM and AML patients strongly support the clinical utility of MRD detection as a tool to obtain an evaluation on the quality of response to treatment. However, MRD assessment is challenging and requires MRD assay optimization and standardization. Different sensitive techniques for MRD assess-

ment are currently evaluated and a combination of different techniques seems to provide the accurate results. There is no doubt in these pathological conditions that patients in histological complete remission with an MRD-test-negative have better outcomes than those with an MRD-test-positive. These results have justified the inclusion of MRD evaluation in clinical trials involving new therapeutic approaches in MM and AML. However, it is evident that when MRD role in clinical studies moves from a passive role (i.e., a measure of the extent of treatment effectiveness) to an active role (i.e., a tool to guide treatment choices), a careful standardization, a consistent sensitivity and reproducibility of MRD assays are strictly required.

Conflict of interest statement
The Authors declare no conflict of interest.

REFERENCES

1. Jones CF, Wieffer HM, Oudard M. The application of minimal residual disease in hemato-oncology: a review of its current utility in trials, regulatory decisions and clinical practice. Blood. 2017;130(suppl. 1):5630.
2. Cumbo C, Anelli L, Specchia G, Albano G. Monitoring of minimal residual disease (MRD) in chronic myeloid leukemia: recent advances. Cancer Manag Res. 2020;12:3175-89.
3. Berry DA, Zhou S, Higley H, et al. association of minimal residual disease with clinical outcome in pediatric and adult acute lymphoblastic leukemia. JAMA Oncol. 2017;3:e170580.
4. Letestu R, Dehmani A, Boubaya M, Baseggio L, Campos L, Chatelain B, et al. Prognostic value of high-sensitivity measurable residual disease assessment after front-line chemoimmunotherapy in chronic lymphocytic leukemia. Leukemia. 2021;35(6):1597-1609.
5. Thompson PA, Srivastava J, Peterson C, Strati P, Jorgensen JL, Hether T, et al. Minimal residual disease undetectable by next-generation sequencing predicts improved outcome in CLL after chemoimmunotherapy. Blood. 2019;134:1951-9.
6. Lew TE, Anderson MA, Lin VS, Handunnetti S, Came NA, Blembery P, et al. Undetectable peripheral blood MRD should be the goal of venetoclax in CLL, but attainment plateaus after 24 months. Blood Adv. 2020;4:165-74.
7. Kumar S, Paiva B, Anderson KC, Durie B, Landgren O, Moreau P, et al. International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. Lancet Oncol. 2016;17:e328-e346.
8. Munshi NC, Avet-Loiseau H, Rawstron AC, Owen RG, Child A, Thakurta A, et al. Minimal residual disease predicts superior survival in patients with multiple myeloma : a meta-analysis. JAMA Oncol. 2017;3:28-35.
9. Munshi NC, Avet-Loiseau H, Anderson KC, Neri P, Paiva B, Samur M, et al. A large meta-analysis establishes the role of MRD negativity in long-term survival outcomes in patients with multiple myeloma. Blood Adv. 2020;4:5988-99.
10. Burgos L, Puig N, Cedema MT, Mateos MV, Lahuerta JJ, Piaba B, San-Miguel JF. Measurable residual disease in multiple myeloma : ready for clinical practice? J Hematol Oncol. 2020;13:82.
11. Baffour FI, Glazebrook KN, Kumar SK, Broski SB. Role of imaging in multiple myeloma. Am J Hematol. 2020;95:966-77.
12. Hillengass J, Usmani S, Rajkumar SV, Durie B, Mateos MV, Louial S, et al. International myeloma working group group consensus recommendations on imaging in monoclonal plasma cell disorders. Lancet Oncol. 2019;20:e302-e312.
13. Pawlyn C, Fowkes L, Otero S, Jones JR, Boyd KD, Davies FE, et al. Whole-body diffusion-weighted MRI : a new gold standard for assessing disease burden in patients with multiple myeloma? Luekemia. 2016;30:1446-8.
14. Rasche L, Angtuaco E, McDonald JE, Buros A, Stein C, Pawlyb C, et al. Low expression of hexokinase-2 is associated with false-negative FDG-positron emission tomography in multiple myeloma. Blood. 2017;130:30-4.
15. Rawstrom AC, Davies FE, DasGupta R, Ashcroft J, Patmore R, Drayson MT, et al. Flow cytometric disease monitoring in multiple myeloma: the relationship between normal and neoplastic plasma cells predicts outcome after transplantation. Blood. 2002;100:3095-100.
16. San Miguel JF, Almeida J, Mateo G, Bladé J, Lopeéz-Berges C, Caballero D, et al. Immunophenotypic evaluation of the plasma cell compartment in multiple myeloma : a tool for comparing the efficacy of different treatment strategies and predicting outcome. Blood. 2002;99:1853-6.
17. Bruinink DH, Oliver S, Rihova L, Schmitz A, Gilestro M, Marvelde J, et al. Standardization of flow cytometric minimal residual disease assessment in international clinical trials. A feasibility study from the European Myeloma Network. Haematologica. 2021;106:1496-9.
18. Sarasquete ME, Garcia-Sanz R, Gonzalez D, Martinez J, Mateo G, Martinez P, et al. Minimal residual disease monitoring in multiple myeloma: a comparison between allelic-specific oligonucleotide real-time quantitative polymerase chain reaction and flow cytometry. Haemato-

logica. 2005;90 :1365-72.

19. Paiva B, Cedena MT, Puig N, Arana P, Vidriales MB, Cordon L, et al. Minimal residual disease monitoring and immune profiling in multiple myeloma in elderly patients. Blood. 2016;127:3165-74.

20. Flores-Montero J, Sanoja-Flores L, Paiva B, Puig N, Garcia-Sanchez O, Bottcher S, et al. Next generation flow for highly sensitive and standardized detection of m inimal residual disease in multiple myeloma. Leukemia. 2017;31:2094-301.

21. Terpos E, Kostopoulos IVC, Kastritis E, Ntanasis-Statho-poulos I, Migkou M, Rousakis P, et al. Impact of mini-mal residual disease detection of next-generation-flow cytometry in multiple myeloma patients with sustained complete remission after frontline therapy. Hemasphere. 2019;3:e300.

22. Paiva B, Puig N, Cedena MT, Rosinol L, Cordòn L, Vidriales MB, et al. Measurable residual disease by next-generation flow cytometry in multiple myeloma. J Clin Oncol. 2020;38:784-92.

23. Sanoja-Flores L, Flores-Montero J, Puig N, Conreras-Sanfeliciano T, Pontes R, Corral-Mateos A, et al. Blood monitoring of circulating tumor plasma cells by next gen-eration flow in multiple myeloma after therapy. Blood. 2019;134:2218-22.

24. Rasche L, Alapat D, Kumar M, Gershner G, McDonald J, Wardell JP, et al. Combination of flow cytometry and functional imaging for monitoring of residual disease in myeloma. Leukemia. 2019;33:1713-22.

25. Goicochea I, Puig N, Cedena MT, Burgos L, Cordòn L, Vidriales MB, et. Deep MRD profiling defines outcome and unveils different modes of treatment resistance in standard and high risk myeloma. Blood. 2021;137:49-60.

26. Martinez-Lopez J, Lahuerta JJ, Pepin F, Gonzalez M, Barrio S, Ayala R, et al. Prognostic value of deep sequenc-ing method for minimal residual disease detection in mul-tiple myeloma. Blood. 2014;123:3073-9.

27. Perrot A, Lauwers-Cances V, Corre J, Robillard N, Hulin C, Chretien ML, et al. Minimal residual negativity using deep sequencing is a major prognostic factor in multiple myeloma. Blood. 2018;132:2456-64.

28. Mazzotti C, Buisson L, Maheo S, Perrot A, Chetien MR, Leleu X, et al. Myeloma MRD by deep sequencing from circulating tumor DNA does not correlate with results obtained in the bone marrow. Blood Adv. 2018;2:2811-3.

29. Gozzetti A, Raspadori D, Bacchiari F, Sicuranza A, Pacelli P, Ferrigno I, Tocci D, Bocchia M. Minimal re-sidual disease in multiple myeloma: state of the art and applications in clinical practice. J Pers Med. 2020;10:120.

30. Avet-Loiseau H, Ludwig H, Landgren O, Paiva B, Morris C, Yang H, et al. Minimal residual disease status as a sur-rogate endpoint for progression-free survival in newly di-agnosed multiple myeloma studies: a meta-analysis. Clin Lymphoma Myeloma Leuk. 2020;20:e30-e37.

31. European Medicinal Agency Guideline on the use of minimal residual disease as a clinical endpoint in multiple myeloma studies. 2018; EMA/CHMP/459559/2018.

32. Moreau P, Zweegman S, Perrot A, Hulin C, Caillot D, Falcon T, et al. Evaluation of the prognostic value of positron emission tomography-computed tomography / PET-CT) at diagnosis and follow-up in transplant-eligible newly diagnosed multiple myeloma (TE NDMM) pa-tients treated in the phase 3 Cassiopea study results of the cassiopet companion study. Blood. 2019;134(Suppl. 1):692.

33. Muronova L, Havel M, Popkova T, Plonkova H, Hjek R, Jelinek T. Prognostic value of PET/CT performed day +100 post autologous stem cell transplantation in multi-

ple myeloma: real-world single center experience. Blood. 2020;136(Suppl. 1):6-7.

34. Askeland FB, Rosmussen AM, Schjesvold F. Relapse from MRD negativity as indication for treatment in multiple myeloma - the REMNANT study. Blood. 2020;136(Suppl. 1):21-2.

35. Avet-Loiseau H, San-Miguel J, Casneuf T, Lida S, Lonial S, Usmani SZ, et al. Evaluation of sustained minimal re-sidual disease negativity with duratumumb-combination regimens in relapsed and/or refractory multiple myelo-ma: analysis of POLLUX and CASTOR. J Clin Oncol. 2021;39:1139-49.

36. Dimopoulos MA, Moreau P, Terpos E, Moleos MV, Zwe-gman V, Cook G, et al. Multiple myeloma: EHA-ESMO clinical practice guidelines for diagnosis, treatment and follow-up. HemaSphere. 2021;2:e528.

37. Attal M, Lauwers-Cances V, Hulin C, Leleu X, Caillot D, Escoffre M, et al. Lenalidomide, bortezomib, and dexa-methasone with transplantation for myeloma. N Engl J Med. 2017;376:1311-20.

38. Oliva S, Genuardi E, Petrucci MT, D'Agostino M, Au-clair D, Spadano A, et al. Impact of minimal residual dis-ease (MRD) by multiparameter flow cytometry (MFC) and next-generation sequencing (NGS) on outcome: results of newly diagnosed transplant-eligible mul-tiple myeloma (MM) patients in the Forte trial. Blood. 2020;136(Suppl.1):abst. 491.

39. Oliva S, Bruinink DH, Rihova L, D'Agostino M, Pantani L, Capra A, et al. Minimal residual disease assessment by multiparameter flow cytometry in transplant-eligible myeloma in the EMN02/HOVON 95 MM trial. Blood Cancer J. 2021;11:106.

40. San Miguel JF, Avet-Loiseau H, Paiva B, Kumar SK, Di-mopoulos SA, Facon J, et al. Sustained minimal residual disease negativity with duratumumab in newly diagnosed multiple myeloma: MAIA and ALCYONE. Blood. 2021; in press.

41. Landgren O, Hultcrantz M, Diamond B, Lesokhin A, Mailankody S, Hassoun H, et al. Safety and effectiveness of weekly carfilzomib, lenalidomide, dexamethasone, and duratumumab combination therapy for patients with newly diagnosed multiple myeloma. The MANHATTAN nonrandomized clinical trial. JAMA Oncol. 2021;7:862-8.

42. Romano A, Palumbo GA, Parrinello NL, Conticello C, Martello M, Terragna C. Minimal residual disease as-sessment within the bone marrow of multiple myeloma: a review of caveats, clinical significance and future perspec-tives. Fron Oncol. 2019;9:699.

43. Gozzetti A, Raspadori D, Bacchiarri F, Sicuranza A, Pacelli P,m Ferrigno I, et al. Minimal residual disease in multiple myeloma: state of the art and applications in clinical practice. J Personalized Med. 2020;10:120.

44. Rustand EH, Boyle EM. Monitoring minimal residual disease in the bone marrow using next generation se-quencing. Best Pract Res Clin Hematol. 2020;33:101149.

45. Voigt P, Reinberg D. Genomic and epigenomic land-scapes of adult de novo acute myeloid leukemia. N Engl J Med. 2013;368:2059-74.

46. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic classification and prognosis in acute myeloid leukemia. N Engl J Med. 2016;374:2209-21.

47. Yang F, Anekpuritananag T, Press RD. Clinical utility of next-generation sequencing in acute myeloid leukemia. Mol Diagn Ther. 2020;14:1-13.

48. Dohner H, Estey E, Grimwade D, Amadori S, Appel-baum FR, Buchner T, et al. Diagnosis and management

of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129:424-47.

49. Burnett AK, Goldtone A, Hills RK, Milligan D, Prentice A, Yin J, et al. Curability of patients with acute myeloid leukemia who did not undergo transplantation in first remission. J Clin Oncol. 2013;31:1293-1301.

50. Dohner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. N Engl J Med. 2015;373:1136-52.

51. Voso MT, Ottone T, Lavorgna S, Venditti A, Maurillo L, Lo-Coco F, et al. MRD in AML: the role of new techniques. Front Oncol. 2019;9:655.

52. Schuurhuis GJ, Heuser M, Freeman S, Bené MC, Buccisano F, Clos J, et al. Minimal/measurable residual disease in AML: a consensus document from the European Leukemia Net MRD Working Party. Blood. 2018;131:1275-91.

53. Freeman SD, Hills RK, Virgo P, Khan N, Couzens S, Dillon R, et al. Measurable residual disease at induction redefines partial response in acute myeloid leukemia and stratifies outcomes in patients at standard risk without NPM1 mutations. J Clin Oncol. 2018;36:1486-97.

54. Jongen-Lavrencic M, Grob T, Hanekamp D, Kavelaars FG, Al Hinai A, Zeilemaker A, et al. Molecular minimal residual disease in acute myeloid leukemia. N Engl J Med. 2018;378:1189-99.

55. Tsai CH, Tang JL, Tien FM, Kuo YY, Wu DC, Lin CC, et al. Clinical implications of sequential MRD monitoring by NGS at 2 time points after chemotherapy in patients with AML. Blood Adv. 2021;5:2456-66.

56. Walter RB, Gyurkocza B, Storer BE, Wood BL, Storer BE, Sandmaier BM, et al. Comparison of minimal residual disease as outcome predictor for AML patients in first complete remission undergoing myeloablative or nonmyeloablative allogeneic hematopoietic cell transplantation. J Leukemia. 2015;29:137-44.

57. Araki D, Wood BL, Othus M, Radich JP, Harpern AB, Zhou Y, et al. Allogeneic hematopoietic cell transplantation for acute myeloid leukemia: time to move toward a minimal residual disease-based definition of complete remission? J Clin Oncol. 2016;34:329-36.

58. Bull B, Stein EM. Which are the most promising targets for minimal residual disease-directed therapy in acute myeloid leukemia prior to allogeneic stem cell transplant? Haematologica. 2019;104:1521-31.

59. Arber DA, Brunning RD, Le Beau MM, Jaffe ES, Pileri SA. Thiele J. Acute myeloid leukemia with recurrent genetic abnormalities. In: Sherdlow SH, Campo E, Harris NL (Eds). WHO classification of haematopoietic and lymphoid tissues, revised 4th ed. Lyon, France: International Agency for Research on Cancer; 2017. p. 130-49.

60. Falini B, Brunetti L, Sportoletti P, Martelli MP. NMP1-mutated acute myeloid leukemia: from bench to bedside. Blood. 2020;136:1707-21.

61. Patel SS, Kuo FC, Gibson CJ, Steensma DP, Soiffer RJ, Alyea EP, et al. High NPM1-mutant allele burden at diagnosis predicts unfavorable outcomes in de novo AML. Blood. 2018;131:2816-25.

62. Gorello P, Cazzaniga G, Alberti F, Dell'Oro MG, Gottardi E, Specchia G et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. Leukemia. 2006;20:1103-8.

63. Bacher U, Dicker F, Haferlach C, Alpermann T, Rose D, Kern W, et al. Quantification of rare NPM1 mutation subtypes by digital PCR. Br J Haematol. 2014;167:710-4.

64. Mencia-Trinchant N, Hu Y, Alas MA, et al. Minimal residual disease monitoring of acute myeloid leukemia by massively multiplex digital PCR in patients with NPM1

mutations. J Mol Diagn. 2017;19:537-48.

65. Kronke J, Schlenk RF, Jensen KO, Tschutz F, Corbacioglu A, Gaidzik VI, et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol. 2011;29:2709-16.

66. Ivey A, Hills RK, Simpson MA, Jovanovic JV, Gilkes A, Grech A, et al. Assessment of minimal residual disease in standard-risk AML. N Engl J Med. 2016;374:422-33.

67. Kayser S, Benner A, Thiede C, Martens U, Huber J, Stadtherr P, et al. Pretransplant NPM1 MRD levels predicts outcome after allogeneic hematopoietic stem cell transplantation in patients with acute myeloid leukemia. Blood Cancer J. 2016;29:e449.

68. Thol F, Gabdoulline R, Liebich A, Klement P, Schiller J, Kandiora K, et al. Measurable residual disease monitoring by NGS before allogeneic hematopoietic cell transplantation in AML. Blood. 2018;132:1703-13.

69. Lussana F, Caprioli C, Stefanoni P, Pavoni C, Spinelli O, Buklijas K, et al. Molecular detection of minimal residual disease before allogeneic stem cell transplantation predicts a high incidence of early relapse in adult patients with NPM1 positive acute myeloid leukemia. Cancers. 201911:1455.

70. Dillon R, Hills R, Freeman S, Potter N, Jovanovic J, Ivey A, et al. Molecular MRD status and outcome after transplantation in NPM1-mutated AML. Blood. 2020;135:680-8.

71. Zhao Y, Chen X, Feng S. Autologous hematopoietic stem cell transplantation in acute myelogenous leukemia. Biol Blood Marrow Transpl. 2019;25:e285-e292.

72. De Santiago de Benito A, Jeker B, Gfeller E, Porret N, Banz Y, Novak U, et al. Molecular minimal residual disease negativity and decreased stem cell mobilization potential predict excellent outcome after autologous transplant in NPM1 mutant acute myeloid leukemia. Haematologica. 2020;105:e9-e12.

73. Kapp-Schwoerer S, Weber D, Carbacioglu A, Gaidzik VI, Paschka P, Kronke J, et al. Impact of gemtuzumab ozogamicin on MRD and relapse risk in NPM1 mutated AML patients: results from the AMLSG 09-09 trial. Blood. 2020;136(26):3041-50.

74. Tiong IS, Dillon R, Ivey A, The TC, Nguyen P, Cumming N, et al. Venetoclax induced rapid elimination of NPM1 mutant measurable residual disease in combination with low-intensity chemotherapy in acute myeloid leukaemia. Br J Haematol. 2021;192(6):1026-30.

75. Bataller A, Onate G, Diaz-Beaya M, Guijharro F, Garrido A, Vices S, et al. Acute myeloid leukemia with NPM1 mutation and favorable European Leukemia Net category: outcome after preemptive intervention based on measurable residual disease. Br J Haematol. 2020;191(1):52-61.

76. Hollein A, Meggendorfer M, Dicker F, Jeromin S, Nadarajah N, Kern W, et al. NPM1 mutated AML can relapse with wild-type NPM1 persistent clonal hematopoiesis can drive relapse. Blood Adv. 2018;2:3118-25.

77. Hourigan CS, Gale RP,Gormley NG, Ossenkoppele GJ, Walter RB. Measurable residual disease testing in acute myeloid leukaemia. Leukemia. 2017;31:1482-90.

78. Freeman DS, Hourigan CS. MRD evaluation of AML in clinical practice: are we there get? Am Soc Hematol Educ Program. 2019;1:557-69.

79. Percival ME, Estey EH. Current treatment strategies for a measurable disease in patients with acute myeloid leukemia? Cancer. 2019;125:3121-30.

80. Ommen HB. Schnittger S, Jovanovich JV, Ommen IB, Hasle H, Ostergaard M, et al. Strikingly different molec-

ular relapse kinetics in MPM1c, PML-RARA, RUNX1-RUNXT1, and CBFB-MYH11 acute myeloid leukemias. Blood. 2020;115:198-205.

81. Hokland P, Ommen HB. Towards individualized follow-up in adult acute myeloid leukemia in remission. Blood. 20111;117:2577-84.

82. Grimwade D, Jovanovic JV, Hills RK, Nugent EA, Patel Y, Flora R, et al. Prospective minimal residual disease monitoring to predict relapse of acute promyelocytic leukemia and to direct pre-emptive arsenic trioxide therapy. J Clin Oncol. 2009;27:3650-8.

83. Rucker FG, Agrawal M, Corbacioglu A, Weber D, Kapp-Schwoerer S, Gaidzik VI, et al. Measurable residual disease monitoring in acute myeloid leukemia with t(8;21)(q22;q22.1): results from the AML study group. Blood. 2019;134:1608-18.

84. Puckrin R, Atenafu EG, Claudio JO, Chan S, Gupta V, Maze D, et al. Measurable residual disease monitoring provides insufficient lead-time to prevent morphologic relapse in the majority of patients with core-binding factor acute myeloid leukemia. Haematologica. 2021;106(1):56-63.

85. Ommen HB, Hokland P, Haferlach T, abildgaard L, Alpermann T, Haferlach C. et al. Relapse kinetics in acute myeloid leukemias with MLL translocations of partial tandem duplications within the MLL gene. Br J Haematol. 2014;165:618-28.

86. Wong HY, Sung AD, Lindblad KE, Sheela S, Roloff g, Rizzieri D, et al. Molecular measurable residual disease testing of blood during AML cytotoxic therapy for early prediction of clinical response. Front Oncol. 2019;8:669.

87. Qin YZ, Jiang Q, Wang Y, Jiang H, Xu LP, Zhao XS, et al. The impact of the combination of KIT mutation and minimal residual disease on outcome in t(8;21) acute myeloid leukemia. Blood Cancer J. 2021;11:67.

88. Williams P, Basu S, Garcia-Manero G, Hourigan CS, Oetjen KA, Cortes JE, et al. The distribution of T-cell subsets and the expression of immune checkpoint receptors and ligands in patients with newly diagnosed and relapsed acute myeloid leukemia. Cancer. 2019;125:1470-81.

89. Chen C, Liang C, Wang S, Chio CL, Zhang Y, Zeng C, et al. Expression patterns of immune checkpoints in acute myeloid leukemia. J Hematol Oncol. 2020;13:28.

90. Brodska B, Otevrelova P, Salek C, Fuchs O, Gasova Z, Kuzelova K, et al. High PD-L1 expression predicts for worse outcome of leukemia patients with concomitant NPM1 and FLT3 mutations. Int J Mol Sci. 2019;20:2823.

91. Davids MS, Kim HT, Brachireddy P, Costello C, Liguori R, Savell A, et al. Ipilimumab for patients with relapse after allogeneic transplantation. N Engl J Med. 2016;375:143-53.

92. Berger R, Rotem-Yehudar R, Slama G, Landes S, Knelle A, Leiba A, et al. Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. Clin Cancer Res. 2008;14:3044-51.

93. Assi R, Kantarjian HM, Daver NG, Kadia TM, Pemmaraju N, Jabbour E, et al. Results of a phase 2, open-label study of idarubicin (I), cytarabine (A) and nivolumab (Nivo) in patients with newly diagnosed acute myeloid leukemia (AML) and high-risk myelodysplastic syndrome (MDS). ASH Annual Meeting 2018. Blood. 2018;132(Suppl. 1):abst 905.

94. Daver N, Garcia-Manero G, Basu S, Boddu PC, Alafayez M, Cortes JE, et al. Efficacy, safety, and biomarkers of response to azacytidine and nivolumab in relapsed/refractory acute myeloid leukemia: a nonrandomized, open-label, phase II study. Cancer Discov. 2019;9:370-83.

95. Kadia TM, Cortes JE, Ghorab A, ravandi F, Jabbour E, Daver N, et al. Nivolumab (Nivo) maintenance (maint) in high-risk (HR) acute myeloid leukemia (AML) patients. J Clin Oncol. 2018;36:15_s (Suppl; abstr 7014).

96. Daver N, Garcia-Manero G, Basu S, Boddu PC, Alfayez M, Cortes JE, et al. Safety, efficacy and biomarkers of response to azacitidine (AZA) with nivolumab (Nivo) and AZA with Nivo and Ipilimumab (Ipi) in relapsed/refractory acute myeloid leukemia: a non-randomized, phase 2 study. Blood. 2018;132(Suppl. 1):908.

97. Versluis J, Kalin B, Zeijlemaker W, Passweg J, Graux C, Manz MG, et al. Graft-versus-leukemia effect of allogeneic stem-cell transplantation and minimal residual disease in patients with acute myeloid leukemia in first complete remission. JCO Precis Oncol. 2017;1:1-13.

98. Venditti A, Piciocchi A, Candoni A, Melillo L, Calafiore V, Cairoli R, et al. GIMEMA AML1310 trial of risk-adapted, MRD-directed therapy for young adults with newly diagnosed acute myeloid leukemia. Blood. 2019;134:935-45.

99. Gilleece MH, Labopin M, Yakoub-Agha I, Volin L, Socié G, Ljungman P, et al. Measurable residual disease, conditioning regimen intensity, and age predict outcome of allogeneic hematopoietic cell transplantation for acute myeloid leukemia in first remission: a registry analysis of 2292 patients by the Acute Leukemia Working Party European Society of Blood and Marrow Transplantation. Am J Hematol. 2018;93:1142-52.

100. Hourigan CS, Dillon LW, Gui G, Logan BR, Fei M, Ghannam J, et al. Impact of conditioning intensity of allogeneic transplantation for acute myeloid leukemia with genomic evidence of residual disease. J Clin Oncol. 2019;38:1273-83.

101. Othus M, Gale RP, Hourigan CS, Walter RB. Statistics and measurable residual disease (MRD) testing: uses and abuses in hematopoietic cell transplantation. Bone Marrow Transplant. 2020;55:843-50.

102. Short NJ, Zhou S, Fu C, Berry DA, Walter RB, Freeman SD, et al. Association of measurable residual disease with survival outcomes in patients with acute leukemia: a systematic review and meta-analysis. JAMA Oncol. 2020;6:1890-9.

103. Walter RB, Ofran Y, Wierzbowska A, Ravandi F, Hourigan CS, Ngai LL, et al. Measurable residual disease as a biomarker in acute myeloid leukemia: theoretical and practical considerations. Leukemia. 2021;35(6):1529-38.

Ecological meta-analytic study of kidney disease in Italian contaminated sites

Marta Benedetti[1], Fabrizio Minichilli[2], Maria Eleonora Soggiu[1], Valerio Manno[3] and Lucia Fazzo[1]

[1]*Dipartimento Ambiente e Salute, Istituto Superiore di Sanità, Rome, Italy*
[2]*Unità di Ricerca di Epidemiologia Ambientale e Registri di Patologia, Istituto di Fisiologia Clinica, Consiglio Nazionale delle Ricerche (CNR), Pisa, Italy*
[3]*Servizio di Statistica, Istituto Superiore di Sanità, Rome, Italy*

Abstract

Introduction. Environmental heavy metals exposure has been associated with kidney disease. There is also some evidence that exposure to solvents may be a risk factor for kidney disease. We estimated the risk of hospitalization for kidney diseases (ICD-9 580-586) and chronic kidney disease (CDK, ICD-9 585) in residents in thirty-four Italian National Priority Contaminated Sites (NPCSs) polluted by heavy metals.

Methods. Random-effects model meta-analyses of SHR (Standard Hospitalization Ratio) computed for each NPCS was performed for all the NPCSs together, and separately, according to the presence/absence of selected industrial activities (petrochemical/refinery and steel plants), and the presence/absence of solvents contamination.

Results. Pooled SHRs of overall NPCSs were in excess in both genders. Statistically significant excesses were found for CKD in both genders, and for kidney diseases in females, residing in NPCSs with the combined presence of heavy metals and solvents contamination. The pooled SHRs for CKD and kidney diseases were not statistically significant in excess in NPCSs with petrochemical/refinery and steel plants, and only petrochemical/refinery plants.

Conclusions. The results are suggestive of a possible kidney disease risk in population living in the above-mentioned NPCSs. Epidemiological surveillance and remediation actions in these areas are recommended.

INTRODUCTION

The incidence and prevalence of kidney disease, mainly chronic kidney disease (CKD), is rapidly increasing worldwide. Recent estimates suggest that 8-16% of the global population is affected by some form of CKD, with a projection that CKD will become the fifth most common cause of years of life lost globally by 2040. Despite this, kidney disease is missing from the international agenda for global health [1, 2].

The kidney is particularly vulnerable to toxic effects from environmental pollutants. Occupational and environmental exposure to high levels of some heavy metals, such as cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As), are established risk factors for the development of acute kidney diseases and CKD [3-8]. Cadmium, along with lead, are considered to be the most nephrotoxic heavy metals. At present, there is increasing evidence that chronic environmental exposure to Cd, Pb and Hg at low levels, previously thought to be acceptable, may lead to renal damage [3, 5, 7, 9, 10]. Moreover, there are indications that co-exposure to Cd

and As causes more pronounced human renal damage than exposure to each element alone [8, 11, 12].

Humans are exposed to heavy metals via occupational exposure (e.g., mining, petrochemical, refinery, and metallurgical activities), consumption of contaminated food and water, and inhalation of polluted air in areas with heavy traffic [5, 13]. Other exposures can occur with people who live near industrial sites or hazardous dumping sites [5]. In areas with contaminated soil due to heavy metals, house dust is a possible route of exposure even decades after the cessation of the industrial activity [3, 13, 14]. People living in polluted sites are exposed to heavy metals via atmospheric particulate matter (PM) at a level higher than the level allowed [15, 16]. Cigarette smoke is also an important source of Cd and Pb released as CdO and PbO. Inhaled CdO and PbO are more bioavailable than oral Cd and Pb. On average, Cd concentrations in the blood of smokers are four to five times greater, and in the kidney two to three times greater than non-smokers [3, 17].

Once entered the human body, Cd and Pb, due to

their long half-life, remain in the body for decades. Once adsorbed, Cd is stored mainly in the kidney (approximately 80%) and reaches a biologic half-life of more than 10 to 40 years [18, 19]. The extremely long half-life of Cd in the human body suggests that the majority of Cd that is taken is retained indefinitely in the renal tubular cells [20]. Lead accumulates mainly in bone and teeth (approximately 94%) with a half-life, in bone, of about 30 years. The stored lead may be mobilized from bone under physiological and pathophysiological conditions (e.g., advanced age, hyperthyroidism, broken bones, lactation, menopause, and physiological stress) [19, 21, 22].

Cd levels in men and women appear to differ significantly. In women of reproductive age, the body burden of Cd tends to be greater than in men. This seem to be due to the lower iron stores in women than in men as iron deficiency increases absorption of Cd, rendering women more susceptible to Cd uptake [23]. Moreover, according to Satarug et al. (2020) [24], which evaluated kidney dysfunction associated with chronic low-level exposure to Cd and Pb in a population of residents in Bangkok, women may also be more susceptible than men to nephrotoxicity due to exposure to Pb.

There is also evidence that exposure to solvents (mainly via occupational exposure) may be a risk factor for kidney disease [25-28]. Furthermore, recent studies indicate that exposure to some dibenzo-p-dioxins (PCDDs), chlorinated dibenzofuran (PCDFs), and dioxin-like polychlorinated biphenyls (DL-PCBs) might raise the risk of kidney disease [29, 30].

Although it is well established that occupational exposure to high levels of Cd, Pb, Hg, As, and solvents may be a risk factor for kidney disease, few studies have been carried out to evaluate the occurrence of kidney disease in the general population living in proximity to industrial sites contaminated by these elements/chemicals [31-39].

In addition to occupational or environmental exposure to heavy metals and solvents, other factors may also increase the risk of developing kidney disease. Diabetes and hypertension are considered to be the two leading causes of kidney disease. However, hypertension can be either a cause or a consequence of renal failure. Other additional risk factors are obesity, family history of kidney disease, being over age 60, elevated ambient temperature, being affected by lupus erythematosus, and low socioeconomic status [13, 14, 40].

It is important to note that lead has been associated with increased risk for hypertension [5], Cd for type 2 diabetes [41] and hypertension [42], and Cd, Pb and As for obesity [43]. Therefore, exposure to these heavy metals might also indirectly influence development or progression of kidney disease.

The aim of the present study was to assess the risk of hospitalization in the population living in the Italian National Priority Contaminated Sites (NPCSs) with a documented contamination of heavy metals for a set of specific well-defined renal diseases (ICD-9-CM codes 580-586) (hereafter referred as "kidney diseases" for simplicity), and, separately, for CKD (ICD-9-CM code 586), by gender and (i) presence or absence of selected type of industrial activities (petrochemical/refinery and steel plants) or other pollution sources (e.g., chemical plants, power plants, waste landfills and dumps, and harbors); (ii) combined heavy metals and solvents contamination or heavy metals only contamination. The study is part of the epidemiological surveillance programme (Italian Epidemiological Study of Residents in National Contaminated Sites (SENTIERI Project)) carried out in NPCSs, which are areas identified to be of national concern for environmental remediation due to heavy soil and water pollution [44].

METHODS
Site's description
The thirty-four NPCSs (Figure 1) included in this study are, or were in the past, characterized by the presence of one or more polluting sources such as refineries, petrochemical and metallurgic plants, thermoelectric power plants, chemical plants, mines, chemical fertilizer and pharmaceutical plants, controlled and illegal waste dumps, and other industrial facilities. All these activities released into the environment the heavy metals considered of interest in the present study due to their well-recognized nephrotoxic action.

The analysis of the environmental surveys carried out to characterise the contamination in each NPCS, in order to define remediation activities, evidenced Cd, Pb, As, and Hg as the metals most responsible for soil and deep-water contamination. They are present in all selected NPCSs. Other metals that are often detected are nickel (Ni), vanadium (V), chromium (Cr) and cobalt (Co). Other chemicals, such as PCDDs, PCDFs, and PCBs, were also detected in some NPCSs studied. The available data are related to the reporting of the presence of the substances that have exceeded the limit value allowed by current Italian regulations in the various environmental matrices (soil, groundwater, and sea).

Case definition and data source
We considered the hospitalization data included in the last update of SENTIERI Project [44]. We included in the study all the subjects who resided in the thirty-four Italian contaminated Sites in the years 2006-2013, and who, in the same period, had first-time hospitalization with newly diagnosed kidney diseases, defined as a primary diagnosis at discharge of acute glomerulonephritis (code 580), nephrotic syndrome (code 581), chronic glomerulonephritis (code 582), nephritis and nephropathy not specified as acute or chronic (code 583), acute kidney failure (code 584), chronic kidney disease (CKD) (code 585), renal failure and renal failure unspecified (code 586), using the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes [45].

Hospitalization data were extracted from the Italian Hospital Discharge Register (HDR) of the Service of Statistics of the Italian National Health Institute (Istituto Superiore di Sanità), for each calendar year for the period 2006-2013. The HDR is a register of all hospitalization episodes that all public and private hospitals are required to report. The HDR has complete national coverage since 2001. It contains administrative, clini-

cal, and treatment data. The primary and five secondary diagnoses, and treatment data on the HDR are coded according to the International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM) [45]. Data contained in the Hospital Discharge Register are provided anonymously. Each Hospital Discharge Record reports a main diagnosis and up to five secondary diagnoses. We chose to take into consideration only the primary diagnosis as Italian and international studies have shown that measures based only on the primary diagnosis are more specific (less false positives), whereas measures that consider all diagnoses are more sensitive (less false negatives) [46, 47].

Design of analysis

Pooled Standardized hospitalization ratios (SHRs), with their relative 95% confidence intervals, were calculated for all the NPCSs together, and separately according to the presence/absence of specific well-defined industrial activities (see below) and the presence/absence of solvents contamination.

The pooled estimates were obtained through the meta-analysis method using the "metan" routine by Stata statistics software (version 15 for Windows Stata Corporation, 2017) [48]. Heterogeneity in meta-analysis refers to the variation in study outcomes between studies. Between-study heterogeneity was assessed by inspecting the forest plots and the chi-squared test for heterogeneity (I^2). The I^2 statistic describes the percentage of variation across studies that is due to heterogeneity rather than chance [49, 50]. When the heterogeneity between studies is low, then I^2 will be low and a "fixed effects" model may be appropriate. The main assumption of the fixed effects model is that all studies examined are conducted under similar conditions – the only difference between the studies is their power to detect the outcome of interest. An alternative approach is that of "random effects", which allows to study heterogeneous studies in a valid way. In our study, I^2 statistic with a value above 50% was interpreted as representing high heterogeneity, and therefore, a random-effects model analysis was performed using the model described by DerSimonian and Laird [51], with the estimate of heterogeneity being taken from the Mantel-Haenszel model. Results of the meta-analysis were reported as pooled SHR with 95% confidence intervals (CIs), p-values <0.05 were considered statistically significant.

Among all the Italian NPCSs, we selected those where the chemical characterization of environmental pollution detected a high concentration of heavy metals in soil and/or water. This constituted the main inclusion criteria for the study and led to the selection of thirty-four distinct NPCSs. These NPCSs were divided in 4 classes, as shown in *Table 1*, for the subsequent statistical analysis. The classes identify sites with different industrial patterns: Class 1, NPCSs where petrochemical/refinery and steel plants are located; Class 2, NPCSs where a steel plant is only present; Class 3, NPCSs where petrochemical/refinery plants are only present; and Class 4, NPCSs with presence of other type of industrial facilities, and with the exclusion of petrochemical, refinery and steel plants. The geographi-
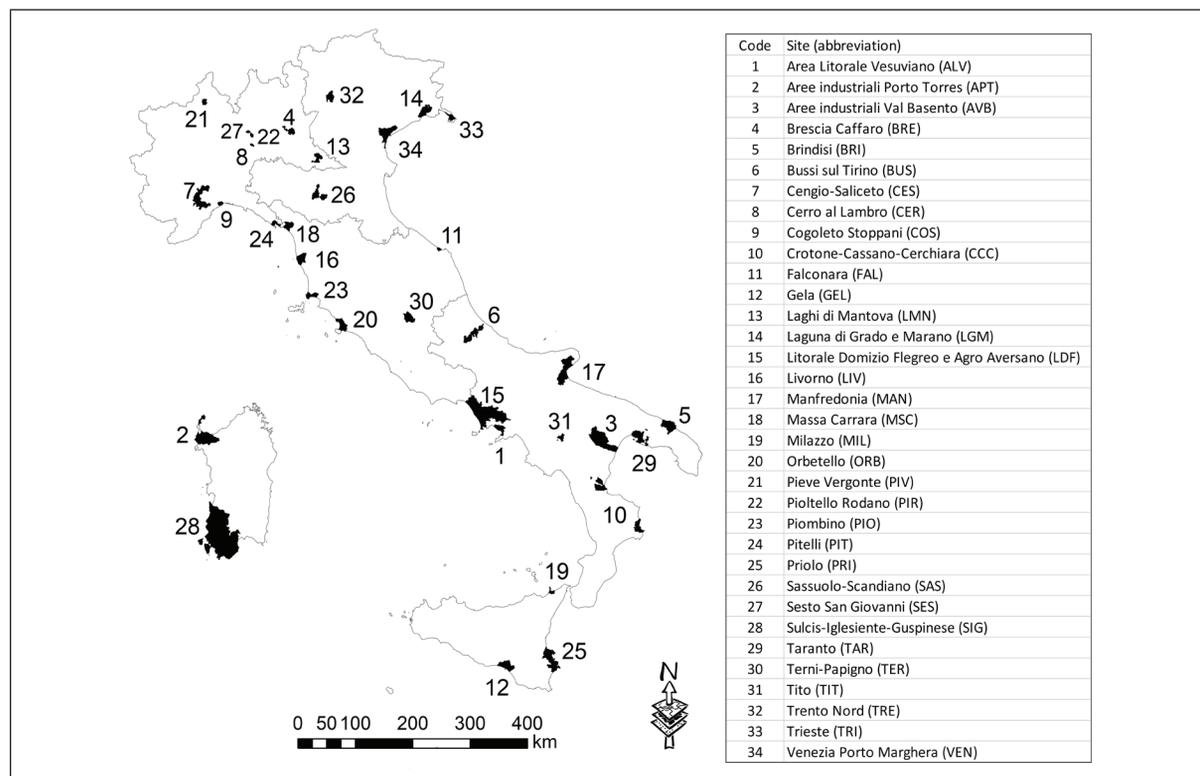

Figure 1
Italian National Priority Contaminated Sites (NPCSs).

Table 1
National Priority Contaminated Sites (NPCSs) information on pollution sources

Class	Presence/absence of selected industrial activities or presence of other pollution sources*	Number of sites	NPCSs abbreviation
1	Presence of petrochemical/refinery and steel plants	4	MSC, MIL, TAR, TRI
2	Presence of only steel plants	4	PIO, SES, TER,TIT
3	Presence of only petrochemical/refinery plants	8	APT, BRI, FAL, GEL, LMN, LIV, PRI, VEN
4	Presence of other pollution sources*, with absence of petrochemical/refinery and steel plants	18	ALV, AVB, BRE, BUS, CES, CER, COS, CCC, LGM, LDF, MAN, ORB, PIV, PIR, PIT, SAS, SIG,TRE
Class	**Presence/absence of solvents contamination**	**Number of sites**	**NPCSs abbreviation**
5	Presence heavy metals and solvents	25	APT, AVB, BRI, BUS, CES, CCC,FAL, GEL,LMN, LDF, LGM, LIV, MAN, MIL, PIV, PIR, PIO, PIT, PRI, SES, SIG, TAR, TIT,TRI, VEN
6	Presence of heavy metals and absence of solvents	9	ALV, BRE, CER, COS, MSC, ORB, SAS, TER, TRE

ALV: Area Litorale Vesuviano; APT:Aree industriali Porto Torres; AVB:Aree industriali Val Basento; BRE: Brescia Caffaro; BRI: Brindisi; BUS: Bussi sul Tirino; CES: Cengio-Saliceto; CER: Cerro al Lambro; COS: Cogoleto Stoppani; CCC: Crotone-Cassano-Cerchiara; FAL: Falconara; GEL: Gela; LMN: Laghi di Mantova; LGM: Laguna di Grado e Marano; LDF: Litorale Domizio Flegreo e Agro Aversano; LIV: Livorno; MAN: Manfredonia; MSC: Massa Carrara; MIL: Milazzo; ORB: Orbetello; PIV: Pieve Vergonte; PIR: Pioltello Rodano; PIO: Piombino; PIT: Pitelli; PRI: Priolo; SAS: Sassuolo-Scandiano; SES: Sesto San Giovanni; SIG: Sulcis-Iglesiente-Guspinese; TAR: Taranto; TER: Terni-Papigno; TIT: Tito; TRE: Trento Nord; TRI: Trieste; VEN: Venezia Porto Marghera.
*This indicates the presence of one or more of the following pollution sources: chemical plants, power plants, waste landfills and dumps, and harbors.

cal distribution shows fourteen plants in the North of Italy, thirteen in the South and seven in Central Italy. Petrochemical and refinery plants are more numerous in the South, while steel plants are equally distributed among geographical regions.

In addition, to investigate the population health effects due to the combined exposure to heavy metals and solvents, the abovementioned thirty-four NPCSs studied were classified and analysed according to the presence or absence of solvents. Twenty-five NPCSs were classified with solvent contamination, while nine NPCSs were classified without (Class 5 and 6 in *Table 1*). The chemical characterization showed that the benzene, toluene and xylene (BTX) mixtures are the most present solvents in fourteen sites. The other solvents occasionally detected are trichloroethylene, tetrachloroethylene, and chlorobenzene. All NPCSs with refineries and/or petrochemical plants are included in Class 5 since the production cycle and the raw materials processed cause the emission of solvents.

Data on the presence of heavy metals and solvents in the NPCSs were derived from the legislative national decrees where the NPCSs are defined, in addition to data from surveys carried out by regional environmental agencies [52, 53].

RESULTS

Thirty-four NPCSs, included in the SENTIERI Project, were considered eligible for the study due to the presence of heavy metals or the combined presence of heavy metals and solvents considered to be risk factors for kidney disease.

The number of observed and expected cases, and of the population residing in the thirty-four NPCSs, by gender and type of kidney disease are shown in *Table 2*. Results of pooled SHRs, with their 95% confidence intervals (95% CIs), by gender, typology of industrial facilities, presence or absence of solvents, and for the thirty-four NPCSs overall are shown in *Table 3*.

The pooled SHRs for all thirty-four NPCSs together

showed a statistically significant excess of hospitalization for CKD (ICD-9-CM code 585) in both genders, while for "kidney diseases" (ICD-9-CM codes 580-585), a statistically significant excess was found in females.

In the analysis by typology of industrial facilities present in the NPCSs, the pooled SHRs for CKD showed in males a non-significant excess risk of 16% in Class 1 (presence of petrochemical, refinery and steel plants), of 12% in Class 3 (presence of only petrochemical and refinery plants), and of 4% in Class 4 (presence of other pollution sources, with absence of petrochemical/refinery and steel plants). In females, the pooled SHRs for CKD showed a non-significant excess risk of 12% in Class 1, of 9% in Class 3, and of 10% in Class 4 (presence of other pollution sources, with absence of petrochemical/refinery and steel plants).

In the analysis by heavy metals contamination and presence/absence of solvents contamination (Class 5 and 6), the pooled SHR for CKD showed a significant excess risk of 9% in males and of 11% in females in Class 5 (presence of heavy metal and solvents), while for "kidney diseases" a significant excess risk of 8% was found in females. In the NPCSs in Class 6 (presence of heavy metals and absence of solvents) non-statistically significant excesses for "kidney diseases" were observed in both genders.

The excesses observed for "kidney diseases" might be due to the inclusion of CKD cases in this group of kidney diseases.

Considering that CKD has been recognized as a leading public health problem worldwide [1, 2], in the present paper the only forest plots of CKD meta-analyses are shown *(Figures 2 and 3)*. In each analysis, the test of heterogeneity showed a high value of I squared, highlighting the heterogeneity of individual estimates. The *Figures* showed a high variability of the estimates for some NPCSs (TIT, PIR, PIV, ORB, CER), due to a low number of cases. In these NPCSs, the observed excesses were not statistically significant. In the analyses

Table 2
Number of observed and expected cases, and of the population residing in the thirty-four NPCSs, by gender and type of kidney disease, 2006-2013

| NPCS (abbreviation) | Kidney diseases (ICD-9-CM codes 580-586) | | | | Chronic kidney disease (CKD) (ICD-9-CM code 585) | | | | Annual resident population* | |
| | Males | | Females | | Males | | Female | | Males | Females |
	Observed cases	Expected cases	Observed cases	Expected cases	Observed cases	Expected cases	Observed cases	Expected cases		
Area Litorale Vesuviano (ALV)	1,738	2,153.46	1,336	1,714.80	1,304	1,568.44	983	1,198.38	213,870	230,534
Aree industriali Porto Torres (APT)	749	625.18	582	551.27	406	391.57	276	331.76	70,026	75,889
Aree industriali Val Basento (AVB)	142	162.60	132	133.38	77	88.86	63	69.12	19,001	19,724
Brescia Caffaro (BRE)	1,126	899.22	921	763.46	588	464.70	338	327.62	96,097	107,696
Brindisi (BRI)	590	470.16	486	420.19	465	319.42	386	275.36	42,354	46,207
Bussi sul Tirino (BUS)	561	431.24	468	345.94	339	270.83	245	192.29	41,227	44,568
Cengio e Saliceto (CES)	218	681.06	137	164.54	132	126.65	85	78.40	18,417	19,309
Cerro al Lambro (CER)	28	29.72	32	19.97	12	15.85	11	9.09	4,461	4,514
Cogoleto Stoppani (COS)	127	155.43	103	121.07	80	90.11	62	60.79	9,864	10,828
Crotone-Cassano-Cerchiara (CCC)	496	365.46	393	275.10	380	259.44	293	184.6	38,459	40,615
Falconara (FAL)	170	140.96	119	109.26	112	81.82	72	57.38	13,012	14,088
Gela (GEL)	371	413.29	314	292.85	292	303.79	238	210.08	36,813	38,204
Laghi di Mantova (LMN)	290	295.03	217	255.87	179	151.82	124	108.89	28,546	32,566
Laguna di Grado e Marano (LGM)	79	128.42	69	86.56	48	70.20	35	39.96	15,485	16,108
Litorale Domizio Flegreo e Agro Aversano (LDF)	5,110	5,880.21	3,952	4,507.07	3,661	4,263.11	2,640	3,141.69	680,318	714,171
Livorno (LIV)	1,017	944.96	911	759.81	471	532.84	311	380.51	82,819	90,634
Manfredonia (MAN)	405	393.48	342	324.78	301	265.89	214	212.06	34,500	35,174
Massa Carrara (MSC)	628	654.06	454	567.86	329	375.74	228	285.58	63,843	69,435
Milazzo (MIL)	399	297.85	279	239.45	334	220.94	222	172.05	22,124	23,470
Orbetello (ORB)	124	79.07	110	64.96	51	45.07	43	32.72	6,933	7,745
Pieve Vergonte (PIV)	26	25.06	15	17.95	16	13.52	10	8.58	2,928	3,049
Pioltello Rodano (PIR)	146	131.98	113	89.17	59	69.75	46	40.49	19,208	19,321
Piombino (PIO)	155	208.36	134	165.94	80	117.73	63	83.03	16,300	17,943
Pitelli (PIT)	931	754.31	930	678.21	554	433.91	468	334.23	48,077	54,677
Priolo (PRI)	1,059	1,101.23	889	868.30	773	815.70	637	624.66	88,576	91,909
Sassuolo – Scandiano (SAS)	484	465.50	389	361.87	220	241.07	141	157.84	56,706	58,091
Sesto San Giovanni (SES)	565	543.97	439	405.58	284	285.37	169	178.91	59,415	64,219
Sulcis-Iglesiente-Guspinese (SIG)	1,356	1,213.97	1,254	989.26	916	756.69	823	592.34	130,414	133,681
Taranto (TAR)	1,245	1,175.13	1,205	1,078.00	980	798.20	909	707.95	102,992	112,349
Terni – Papigno (TER)	505	419.86	368	314.99	340	251.93	229	173.00	51,119	57,466
Tito (TIT)	23	24.46	20	19.38	12	13.49	9	10.03	3,520	3,522
Trento Nord (TRE)	355	402.10	297	340.09	175	160.45	146	131.97	53,760	58,978
Trieste (TRI)	920	893.85	696	694.66	520	484.47	347	312.20	95,250	107,955
Venezia Porto Marghera (VEN)	1,159	1,120.08	922	937.64	676	541.26	474	390.26	123,300	138,667

NPCSs: National Priority Contaminated Sites; ICD-9-CM: International Classification of Diseases, Ninth Revision, Clinical Modification [45].
*Data refer to the annual average of resident population for 2006-2013.

Table 3
Pooled Standardized Hospitalization Ratios (SHRs) for kidney diseases, by gender and NPCS class

NPCS		Kidney diseases (ICD-9-CM codes 580-586)		Chronic kidney disease (CKD) (ICD-9-CM code 585)	
		Males	Females	Males	Females
		SHR (95% CI)	SHR (95% CI)	SHR (95% CI)	SHR (95% CI)
	34 NPCSs overall	1.03 (0.97-1.09)	1.07 (1.00-1.13)	1.07 (1.00-1.15)	1.09 (1.01-1.17)
Classes by presence/absence of specific industrial activities or presence of other pollution sources*					
1	Presence of petrochemical/refinery, and steel plants	1.08 (0.96-1.20)	1.02 (0.85-1.18)	1.16 (0.95-1.38)	1.12 (0.88-1.36)
2	Presence of only steel plants	0.99 (0.77-1.21)	1.03 (0.85-1.20)	0.99 (0.68-1.30)	0.99 (0.72-1.27)
3	Presence of only Petrochemical and refinery plants	1.06 (0.98-1.15)	1.06 (0.99-1.13)	1.12 (0.99-1.26)	1.09 (0.94-1.24)
4	Presence of other pollution sources*, with absence of petrochemical/refinery and steel plants	1.00 (0.90-1.11)	1.10 (0.99-1.2)	1.04 (0.94-1.14)	1.10 (0.99-1.22)
Classes by presence/absence of solvents contamination					
5	Presence of heavy metals and solvents	1.03 (0.96-1.10)	1.08 (1.01-1.16)	1.09 (1.01-1.18)	1.11 (1.01-1.21)
6	Presence of heavy metals and absence of solvents	1.04 (0.89-1.19)	1.04 (0.88-1.20)	1.02 (0.87-1.16)	1.01 (0.89-1.14)

NPCS: National Priority Contaminated Site; CI: Confidence Interval; ICD-9-CM: International Classification of Diseases, Ninth Revision, Clinical Modification [45].
*This indicates the presence of one or more of the following pollution sources: chemical plants, power plants, waste landfills/dumps, and harbors.
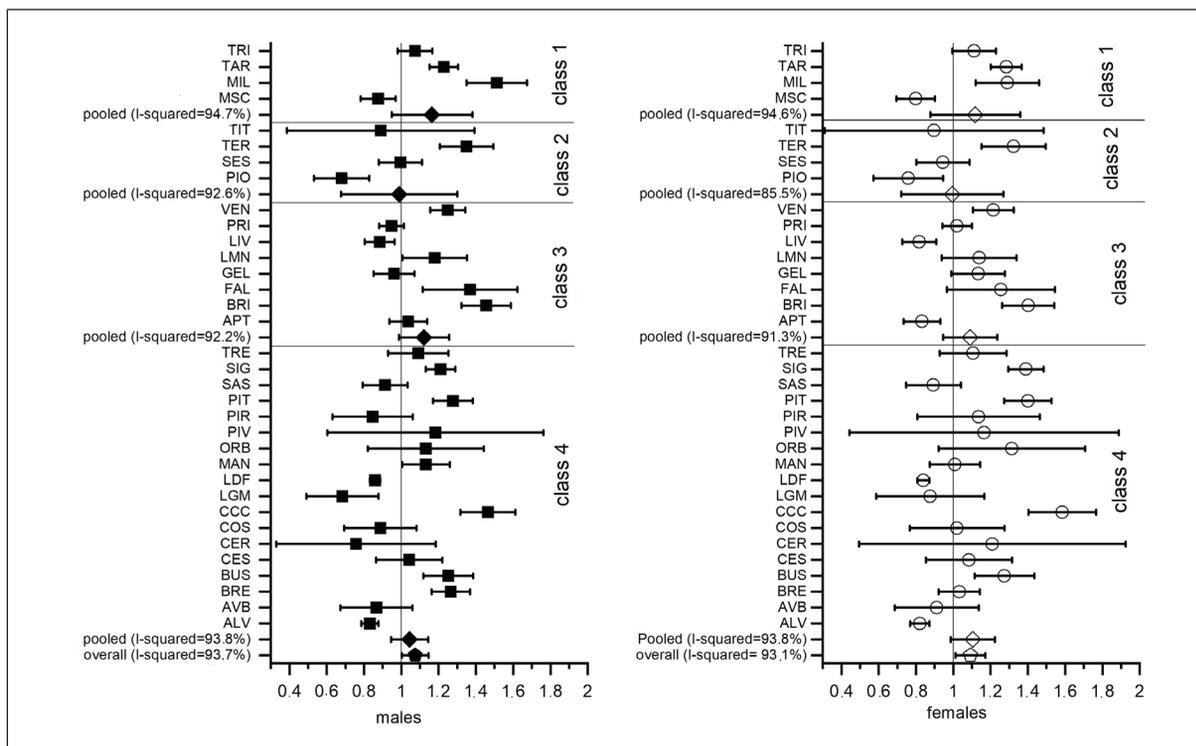
Figure 2
Forest plots of pooled Standardized hospitalization ratios (SHRs) for chronic kidney disease (CKD) in classes 1-4, by gender.
Class 1 = presence of petrochemical/refinery and steel plants; Class 2 =p resence of only steel plants; Class 3 = presence of only petrochemical and refinery plants; Class 4 = presence of other pollution sources (e.g., chemical plants, power plants, waste landfills/dumps, and harbors), and absence of petrochemical/refinery and steel plants.

shown in *Figure 2* and *3*, excesses of hospitalization for CKD were more frequent than deficits.

The pooled excess of CKD, highlighted in females in the analysis of Class 4 (presence of other industrial facilities and absence of petrochemical/refinery and steel plants *(Figure 2))*, seems to be more influenced by the risk excesses of the BUS, PIT, SIG and CCC NPCSs. The pooled excess of CKD, highlighted in both gender

in the analysis of Class 5, seems to be more influenced by the risk excesses of VEN, TAR, SIG, PIT, MIL, FAL, CCC, BUS and BRI sites *(Figure 3)*.

DISCUSSION

In the meta-analysis of ecological studies, the heterogeneity between studies is often high, so the most appropriate method to reduce the bias on the results
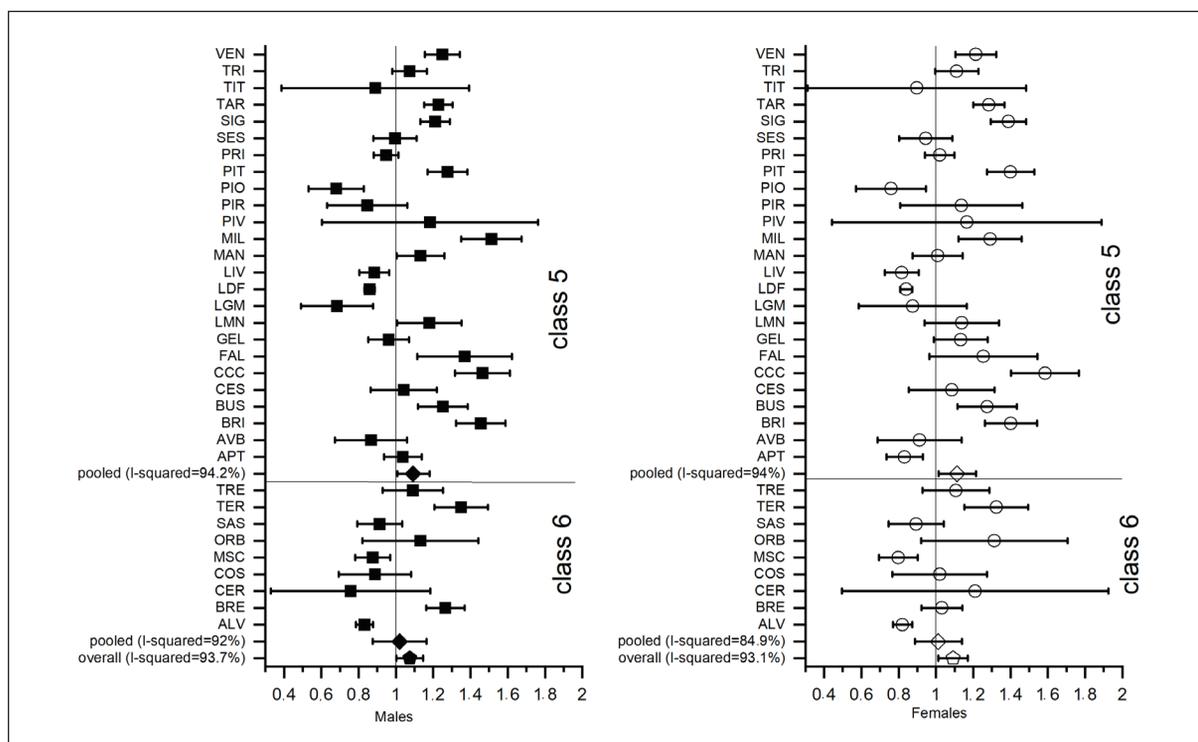
Figure 3
Forest plots of pooled Standardized hospitalization ratios (SHRs) for chronic kidney disease (CDK) in classes 5-6, by gender.
Class 5 = presence of heavy metals and solvents; Class 6 = presence of heavy metals and absence of solvents.

is that of random effects which under such conditions provides more accurate results than those provided by the fixed effects model.

In the present study, the meta-analysis of the combined thirty-four NPCSs, selected on the basis of the presence of heavy metals contamination, showed a statistically significant excess of hospitalization for CKD (ICD-9-CM code 585) in both genders, and for kidney diseases (ICD-9-CM codes 580-586) in females; the excess of kidney diseases among men was non-statistically significant.

In the analyses by classes of NPCSs, statistically significant excesses were found for CKD in males and females, and for kidney diseases in females, residing in the twenty-five NPCSs characterized by the combined presence of heavy metals and solvents (Class 5). The NPCSs that seem to mainly contribute to the excesses observed in Class 5 are characterized by the "presence of other pollution sources, and absence of petrochemical/refinery and steel plants" (SIG, PIT, CCC, BUS), and by "the presence of only petrochemical/refinery plants" (VEN, FAL, BRI).

Several non-significant excesses for CKD and kidney diseases were found in residents (both gender) in the four NPCSs characterized by the presence of petrochemical/refinery and steel plant (Class 1) and in the eight NPCSs with presence of only petrochemical/refinery plants (Class 3). No excesses were observed in resident in NPCSs characterized by the presence of only steel plant (Class 2).

The excesses observed for CKD and kidney diseases in Class 5 (presence of heavy metals and solvents),

mainly in females, are suggestive of a possible adverse effect associated to the combined exposure to heavy metals and solvents. It is worth noting that excesses for CKD and kidney diseases have also been observed in the NPCSs with presence of petrochemical/refinery and steel plants or only petrochemical/refinery plants (Classes 1 and 4), where the exposure to solvents, as well as to heavy metals, is likely to occur. However, the effect of other factors (e.g., random variability, confounding) cannot be excluded, due to the adoption of an ecological study design.

The results obtained for all the thirty-four NPCSs are strongly influenced by the fact that the twenty-five NPCSs in Class 5 (characterized by the combined presence of heavy metals and solvents) represent 74% of all NPCSs, as it is highlighted by the substantial overlapping of the corresponding SHR pooled values.

To the best of our knowledge, this is the first study describing hospitalization risk for selected kidney diseases in residents near contaminated sites according to the presence/absence of some type of industrial facilities releasing heavy metals in the environment, and to the presence/absence of solvents contamination.

There are some limitations to our study, mainly due to the ecological study design. The most important is the lack of a quantitative indicator of residential exposure to heavy metals and solvents. However, this study restricted the analyses only to NPCSs where the environmental contamination by heavy metals, and in some cases solvents, possibly related to renal disease was a priori documented, thus reducing the possibility to observe spurious association only due to chance. Another

limitation is that we could not exclude the possibility of residual confounding factors (e.g., diabetes, hypertension, obesity, age), and of the action or co-exposure to other chemicals (e.g., dioxins, furans, and PCB), recently related to renal dysfunction. Moreover, as most patients with kidney disorders, even chronic kidney disorders are often asymptomatic or may never have been hospitalized, there may have been an underestimation of the cases of the kidney diseases studied.

CONCLUSIONS

According to the results of our study, living in proximity of petrochemical, refinery and steel plants, and particularly, in proximity of contaminated sites with a combined presence of heavy metals and solvents contamination might be considered a potential risk factor for the kidney diseases studied.

This study results support the need for further analytical epidemiological studies to be performed. Future analytical studies should attempt to collect information on occupational and residential history, diabetes, hypertension, obesity, tobacco smoking, and family history of kidney disease. Moreover, the analysis should be performed by specific age-classes as aging is characterized by a progressive decline in renal function [54].

In addition, considering that: (*i*) cadmium and lead nephrotoxic effects may progress even after exposure reduction; (*ii*) CKD can progress into end stage kidney disease, a condition associated with significant mortality; (*iii*) patients with CKD have an increased risk of cardiovascular disease and death; and (*iiii*) the healthcare costs of renal replacement therapy dialysis and/or kidney transplants needed for survival, consume 2-3% of the annual health-care budget in high income countries [55], efforts should be made in development of remediation plans in the NPCSs in order to reduce exposure to heavy metals and solvents.

Due to the findings of several excesses of hospitalization for kidney diseases observed in the present study, an epidemiological surveillance of residents in the NPCSs studied is warranted. Moreover, as kidney disease causes no symptoms until its later stages and the onset and progression of kidney disease is often preventable, for the residents in the NPCSs studied it should be considered to incorporate early detection into current screening protocols, using biomarkers of early effects [56, 57], capable of detecting renal effects at a relatively early stage when they are still reversible, and consequently, preventing the progression to complete renal failure. In future surveillance studies, it is also recommended to use biomonitoring of heavy metals and solvents (particularly of lead and cadmium due to their ability to accumulate in the body and their long half-life) in selected subpopulations residing in the NPCSs studied, in order to validate present and/or past exposure to these chemicals/elements. Besides, biomonitoring can provide a more precise exposure assessment than estimating exposure only based on concentrations in environmental matrices.

Authorship contributions

MB collected and reviewed the scientific literature, MB, FM, MES, LF contributed to study design and wrote the article. FM performed meta-analysis. VM collected cases and computed standardized hospitalization ratios. MB, FM, MES, LF reviewed the manuscript and approved the final version of the manuscript.

Conflict of interest statement

The Authors declare no conflicts of interest.

REFERENCES

1. Tsai HJ, Wu PY, Huang JC, Chen SC. Environmental pollution and chronic kidney disease. Int J Med Sci. 2021;18(5):1121-9. doi:10.7150/ijms.51594

2. Foreman KJ, Marquez N, Dolgert A, et al. Forecasting life expectancy, years of life lost, and all-cause and cause-specific mortality for 250 causes of death: reference and alternative scenarios for 2016-40 for 195 countries and territories. Lancet. 2018;392(10159):2052-90. doi:10.1016/S0140-6736(18)31694-5

3. Genchi G, Sinicropi MS, Lauria G, Carocci A, Catalano A. The effects of cadmium toxicity. Int J Environ Res Public Health. 2020;17(11):3782. doi: 10.3390/ijerph17113782

4. Satarug S, C Gobe G, A Vesey D, Phelps KR. Cadmium and lead exposure, nephrotoxicity, and mortality. Toxics. 2020;8(4):86. doi: 10.3390/toxics8040086

5. Harari F, Sallsten G, Christensson A, et al. Blood lead levels and decreased kidney function in a population-based cohort. Am J Kidney Dis. 2018;72(3):381-9. doi:10.1053/j.ajkd.2018.02.358

6. Chapman E, Haby MM, Illanes E, Sanchez-Viamonte J, Elias V, Reveiz L. Risk factors for chronic kidney disease of non-traditional causes: a systematic review. Rev Panam Salud Publica. 2019;43:e35. doi:10.26633/RPSP.2019.35

7. Kim NH, Hyun YY, Lee KB, et al. Environmental heavy metal exposure and chronic kidney disease in the general population [published correction appears in J Korean Med Sci. 2015;30(4):507. Rhu, Seungho [corrected to Ryu, Seungho]. J Korean Med Sci. 2015;30(3):272-7. doi:10.3346/jkms.2015.30.3.272

8. Sanders AP, Mazzella MJ, Malin AJ, et al. Combined exposure to lead, cadmium, mercury, and arsenic and kidney health in adolescents age 12-19 in NHANES 2009-2014. Environ Int. 2019;131:104993. doi:10.1016/j.envint.2019.104993

9. Satarug S, Gobe GC, Ujjin P, Vesey DA. A comparison of the nephrotoxicity of low doses of cadmium and lead. Toxics. 2020;8(1):18. doi:10.3390/toxics8010018

10. Orr SE, Bridges CC. Chronic kidney disease and exposure

to nephrotoxic metals. Int J Mol Sci. 2017;18(5):1039. doi:10.3390/ijms18051039

11. Arain MB, Kazi TG, Baig JA, et al. Co-exposure of arsenic and cadmium through drinking water and tobacco smoking: risk assessment on kidney dysfunction. Environ Sci Pollut Res Int. 2015;22(1):350-7. doi:10.1007/s11356-014-3339-0

12. Hambach R, Lison D, D'Haese PC, et al. Co-exposure to lead increases the renal response to low levels of cadmium in metallurgy workers. Toxicol Lett. 2013;222(2):233-8. doi:10.1016/j.toxlet.2013.06.218

13. Satarug S. Cadmium Sources and Toxicity. Toxics. 2019;7(2):25. doi: 10.3390/toxics7020025

14. Fatima G, Raza AM, Hadi N, Nigam N, Mahdi AA. Cadmium in human diseases: It's more than just a mere metal. Indian J Clin Biochem. 2019;34(4):371-8. doi:10.1007/s12291-019-00839-8

15. Wu CD, Chern YR, Pan WC, et al. Effects of surrounding environment on incidence of end stage renal disease. Sci Total Environ. 2020;723:137915. doi:10.1016/j.scitotenv.2020.137915

16. Afsar B, Elsurer Afsar R, Kanbay A, Covic A, Ortiz A, Kanbay M. Air pollution and kidney disease: review of current evidence. Clin Kidney J. 2019;12(1):19-32. doi:10.1093/ckj/sfy111

17. Xia J, Wang L, Ma Z, et al. Cigarette smoking and chronic kidney disease in the general population: a systematic review and meta-analysis of prospective cohort studies. Nephrol Dial Transplant. 2017;32(3):475-87. doi:10.1093/ndt/gfw452

18. Sasaki T, Horiguchi H, Arakawa A, et al. Hospital-based screening to detect patients with cadmium nephropathy in cadmium-polluted areas in Japan. Environ Health Prev Med. 2019;24(1):8. doi:10.1186/s12199-019-0762-3

19. Said S, Hernandez GT. Environmental exposures, socioeconomics, disparities, and the kidneys. Adv Chronic Kidney Dis. 2015;22(1):39-45. doi:10.1053/j.ackd.2014.09.003

20. Satarug S. Dietary cadmium intake and its effects on kidneys. Toxics. 2018;6(1):15. doi:10.3390/toxics6010015

21. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological profile for lead. Atlanta, GA: US Department of Health and Human Services, Public Health Service; 2020. doi: 10.15620/cdc:95222

22. Faroon O, Ashizawa A, Wright S, et al. Toxicological profile for cadmium. Atlanta (GA): Agency for Toxic Substances and Disease Registry (US); 2012.

23. Gallagher CM, Chen JJ, Kovach JS. The relationship between body iron stores and blood and urine cadmium concentrations in US never-smoking, non-pregnant women aged 20-49 years. Environ Res. 2011;111(5):702-7. doi:10.1016/j.envres.2011.03.007

24. Satarug S, Gobe GC, Ujjin P, Vesey DA. A comparison of the nephrotoxicity of low doses of cadmium and lead. Toxics. 2020;8(1):18. doi:10.3390/toxics8010018

25. Calvert GM, Ruder AM, Petersen MR. Mortality and end-stage renal disease incidence among dry cleaning workers. Occup Environ Med. 2011;68(10):709-16. doi:10.1136/oem.2010.060665

26. Radican L, Wartenberg D, Rhoads GG, et al. A retrospective occupational cohort study of end-stage renal disease in aircraft workers exposed to trichloroethylene and other hydrocarbons. J Occup Environ Med. 2006;48(1):1-12. doi:10.1097/01.jom.0000190300.51629.e0

27. Lash LH. Environmental and genetic factors influencing kidney toxicity. Semin Nephrol. 2019;39(2):132-40. doi:10.1016/j.semnephrol.2018.12.003

28. Jacob S, Héry M, Protois JC, Rossert J, Stengel B. New insight into solvent-related end-stage renal disease: occupations, products and types of solvents at risk. Occup Environ Med. 2007;64(12):843-8. doi:10.1136/oem.2007.032482

29. Huang CY, Wu CL, Wu JS, et al. Association between blood dioxin level and chronic kidney disease in an endemic area of exposure. PLoS One. 2016;11(3):e0150248. doi:10.1371/journal.pone.0150248

30. Everett CJ, Thompson OM. Association of dioxins, furans and dioxin-like PCBs in human blood with nephropathy among US teens and young adults. Rev Environ Health. 2016;31(2):195-201. doi:10.1515/reveh-2015-0031

31. Hodgson S, Nieuwenhuijsen MJ, Elliott P, Jarup L. Kidney disease mortality and environmental exposure to mercury. Am J Epidemiol. 2007;165(1):72-7. doi:10.1093/aje/kwj345

32. Hodgson S, Nieuwenhuijsen MJ, Hansell A, et al. Excess risk of kidney disease in a population living near industrial plants. Occup Environ Med. 2004;61(8):717-9. doi:10.1136/oem.2003.010629

33. Thomas LD, Hodgson S, Nieuwenhuijsen M, Jarup L. Early kidney damage in a population exposed to cadmium and other heavy metals. Environ Health Perspect. 2009;117(2):181-4. doi:10.1289/ehp.11641

34. Nakagawa H, Nishijo M, Morikawa Y, et al. Urinary cadmium and mortality among inhabitants of a cadmium-polluted area in Japan. Environ Res. 2006;100(3):323-9. doi:10.1016/j.envres.2005.08.014

35. Nishijo M, Morikawa Y, Nakagawa H, et al. Causes of death and renal tubular dysfunction in residents exposed to cadmium in the environment. Occup Environ Med. 2006;63(8):545-50. doi:10.1136/oem.2006.026591

36. Jarup L. Land contamination and renal dysfunction. Occup Environ Med. 2003;60(7):461-2. doi:10.1136/oem.60.7.461

37. Hellström L, Elinder CG, Dahlberg B, et al. Cadmium exposure and end-stage renal disease. Am J Kidney Dis. 2001;38(5):1001-8. doi:10.1053/ajkd.2001.28589

38. Benedetti M, De Santis M, Manno V, et al. Spatial distribution of kidney disease in the contaminated site of Taranto (Italy). Am J Ind Med. 2017;60(12):1088-99. doi:10.1002/ajim.22781

39. Jin Y, Lu Y, Li Y, et al. Correlation between environmental low-dose cadmium exposure and early kidney damage: A comparative study in an industrial zone *vs.* a living quarter in Shanghai, China. Environ Toxicol Pharmacol. 2020;79:103381. doi:10.1016/j.etap.2020.103381

40. Duong TV, Wu PY, Yang E, Lin YF, Chiou HY, Yang SH. Associations of waist circumference, socioeconomic, environmental, and behavioral factors with chronic kidney disease in normal weight, overweight, and obese people. Int J Environ Res Public Health. 2019;16(24):5093.

41. Trouiller-Gerfaux P, Podglajen E, Hulo S, et al. The association between blood cadmium and glycated haemoglobin among never-, former, and current smokers: A cross-sectional study in France. Environ Res. 2019;178:108673. doi:10.1016/j.envres.2019.108673

42. Martins AC, Almeida Lopes ACB, Urbano MR, et al. An updated systematic review on the association between Cd exposure, blood pressure and hypertension. Ecotoxicol Environ Saf. 2021;208:111636. doi:10.1016/j.ecoenv.2020.111636

43. Tinkov AA, Filippini T, Ajsuvakova OP, et al. The role of cadmium in obesity and diabetes. Sci Total Environ. 2017;601-602:741-55. doi:10.1016/j.scitotenv.2017.05.224

44. Zona A, Iavarone I, Buzzoni C, Conti S, Santoro M, Fazzo L, Pasetto R, Pirastu R, Bruno C, Ancona C, Bi-

anchi F, Forastiere F, Manno V, Minelli G, Minerba A, Minichilli F, Stoppa G, Pierini A, Ricci P, Scondotto S, Bisceglia L, Cernigliaro A, Ranzi A, Comba P; Gruppo di Lavoro SENTIERI, Gruppo di Lavoro AIRTUM-SENTIERI, Gruppo di Lavoro Malformazioni Congenite-SENTIERI. SENTIERI – Studio epidemiologico nazionale dei territori e degli insediamenti esposti a rischio da inquinamento: Quinto rapporto. Epidemiol Prev. 2019;43(Suppl. 1):1-208.

45. ICD-9-CM 2007. Classificazione delle malattie, dei traumatismi, degli interventi chirurgici e delle procedure diagnostiche e terapeutiche. Italian version of the "International Classification of Diseases, 9th Revision, Clinical Modification. 2007. Available from: www.salute.gov.it/imgs/C_17_pubblicazioni_2251_allegato.pdf.

46. Del Rio RA, Goldman M, Kapella BK, Sulit L, Murray PK. The accuracy of minimum data set diagnoses in describing recent hospitalization at acute care facilities. J Am Med Dir Assoc. 2006;7(4):212-8. doi:10.1016/j.jamda.2005.12.007

47. Agabiti N, Davoli M, Fusco D, Stafoggia M, Perucci CA; Dipartmento di Epidemiologia Servizio Sanitario Regionale, Lazio. Valutazione comparativa di esito degli interventi sanitari [Comparative evaluation of health services outcomes]. Epidemiol Prev. 2011;35(Suppl. 1):1-80.

48. Fisher D, Harris R, Bradburn M, Deeks J, Harbord R, Altman D, Steichen T, Sterne J, Higgins J, 2006. METAN: Stata module for fixed and random effects meta-analysis. Statistical Software Components S456798, Boston College Department of Economics, revised 15 Feb 2021.

49. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med. 2002;21(11):1539-58.

50. Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003;327:557-60.

51. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials. 1986;7(3):177-88. doi: 10.1016/0197-2456(86)90046-2

52. Musmeci L, Bellino M, Falleni F, Piccardi A. Caratterizzazione ambientale dei Siti di Interesse Nazionale per le bonifiche (SIN) nel progetto SENTIERI [Environmental characterization of the National Contaminated Sites in SENTIERI project]. Epidemiol Prev. 2011;35(Suppl. 4):20-3.

53. Beccaloni E, Cicero MR, Falleni F, et al. Prospettive nella caratterizzazione ambientale e valutazione dell'esposizione [Environmental characterization and exposure evaluation]. Epidemiol Prev. 2014;38(Suppl. 1):137-43.

54. O'Sullivan ED, Hughes J, Ferenbach DA. Renal Aging: Causes and Consequences. J Am Soc Nephrol. 2017;28(2):407-20. doi:10.1681/ASN.2015121308

55. Li PK, Garcia-Garcia G, Lui SF, et al. Kidney Health for Everyone Everywhere - From prevention to detection and equitable access to care. Nefrologia. 2020;40(2):133-41. doi:10.1016/j.nefro.2019.11.001

56. Liu KZ, Tian G, Ko AC, Geissler M, Brassard D, Veres T. Detection of renal biomarkers in chronic kidney disease using microfluidics: progress, challenges and opportunities. Biomed Microdevices. 2020;22(2):29. doi:10.1007/s10544-020-00484-6

57. Gaitonde DY, Cook DL, Rivera IM. Chronic Kidney Disease: detection and evaluation. Am Fam Physician. 2017;96(12):776-83.

ORIGINAL ARTICLES AND REVIEWS

Suicidal ideation among Italian medical students: prevalence and associated factors from a multicenter study

Paolo Leombruni[1], Giuseppina Lo Moro[2], Fabrizio Bert[2] and Roberta Siliquini[2]

[1]*Unità di Psicologia Clinica e Oncologica, Dipartimento di Neuroscienze, Università degli Studi di Torino, Turin, Italy*
[2]*Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università degli Studi di Torino, Turin, Italy*

Abstract
Objectives. To estimate Suicidal Ideation (SI) prevalence among Italian medical students and explore associated factors.
Methods. Multicentre cross-sectional study (2018). Students were enrolled through convenience sampling (sample size=2513). Questionnaires included socio-demographic items and Beck Depression Inventory-II, which has an item on SI. A multivariable regression was performed (p-value<0.05 significant).
Results. SI prevalence was 13.7%. Among students with and without depression SI prevalence was 36.0% and 4.3%. Being female, being in a relationship, good/excellent family cohesion, perceived good economic status were significantly associated with lower SI. Increasing age, bisexual/asexual orientation, psychiatric family history, negative judgment on medical school choice, competitive/hostile climate and unsatisfying friendships among classmates, being worried about not measuring up to the profession significantly increased SI.
Conclusion. There was a high SI prevalence among Italian medical students, consistently with worldwide data. Demographic, relational, and motivational factors seem to influence SI. Determinants should be further investigated to plan university-level interventions.

INTRODUCTION

Worldwide, almost 800,000 people die due to suicide every year and many more attempt suicide [1]. Suicide accounts for 1.5% of all deaths and is the second cause of death among 15-29-year-olds [1]. Despite medical students are recognized as a high-risk population for depressive disorders [2], a gap exists in knowledge of medical students' Suicidal Ideation (SI) [2, 3].

Indeed, in 2016, a meta-analysis reported a pooled prevalence of SI among medical students of 11.1% (data extracted from 24 studies from 15 countries, with a total of 21,002 students) [2], and, in 2018, a systematic review showed a prevalence of SI among medical students that ranged from 1.8% to 53.6% (data extracted from 17 studies from 13 countries, with a total of 13,244 students) [3]. However, such relevant works did not include studies from Southern Europe, except for one study [4]. Currently, data from this area come only from a single-site study from Portugal where a survey on 456 medical students showed a SI prevalence of 3.9% during medical school [4]. Given the above, there

is the need to extend information about SI, especially collecting data from less studied populations. The present paper aimed to estimate the prevalence of SI and to identify factors associated with SI among a large sample of medical students in Italy.

METHODS

The present study is part of the Psychosocial Report in Italian MEdical Students (PRIMES), which was a multicenter cross-sectional survey performed in 12 Italian medical schools in November 2018 [5]. The Ethics Committee of the University of Turin approved the protocol. Participants were recruited by convenience in the 1st, 4th, 6th year of course and informed consents were obtained from all participants. Participation was voluntary and anonymous, and participants received no compensation. Raosoft® was used to determine that the minimum sample size was 383, based on a 5% margin of error, 95% confidence level, 50% response distribution and population of 78,101 (medical students in 2017). Detailed methods are presented in the PRIMES main paper [5].

Address for correspondence: Fabrizio Bert, Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università degli Studi di Torino, Via Santena 5 bis, 10126 Turin, Italy. E-mail: fabrizio.bert@unito.it.

The questionnaire

The self-administered questionnaire included a 30-item section, designed after a research about factors that might influence mental health of medical students [5], and the Beck Depression Inventory-II (BDI-II), an instrument to assess depressive symptoms over the past two weeks [6]. The 30-item section mainly included socio-demographic questions, information about social support, health-related and university-related data.

The BDI-II has an item on suicidal thoughts/wishes (item 9), whose options are: (a) "I don't have any thoughts of killing myself", (b) "I have thoughts of killing myself, but I would not carry them out", (c) "I would like to kill myself", (d) "I would kill myself if I had the chance" [6]. To perform the analyses, the binary outcome on SI was created by grouping the last three options, as proposed by other authors [7, 8]. The presence of depressive symptoms was defined with a BDI-II score≥14, which is a widely used cut-off [2, 5]. Total scores from 0-13 represent no/minimal depression, 14-19 represent mild depression, 20-28 represent moderate depression, and 29-63 represent severe depression [6]. Overall, an increasing BDI-II score corresponds to a great severity of depressive symptoms.

Data analysis

Descriptive analyses were carried out for all variables. The BDI-II score and age reported non-normal distributions (Shapiro-Wilk test) and, therefore, they were expressed as median and interquartile range (IQR). The SI prevalence was computed with a 95% bootstrap CI (simple random sampling method). Concerning categorical variables, chi-squared tests and adjusted residuals were calculated to evaluate differences between participants with/without SI. To assess the difference in BDI-II score and age between participants with/without SI, a non-parametric test, i.e. the Mann-Whitney U test, was used due to the non-normal distribution of these variables.

To explore SI predictors, a multivariable logistic regression model adjusted for age and gender was performed. The independent variables were coded from the items of the socio-demographic section. In particular, the variables entered at the first step were mainly selected because they can be related to medical students' mental health as further explained in the PRIMES main paper [5]. Such variables were: school geographical area, nationality, relationship status, sexual orientation, living condition, family cohesion, working condition, distance from home and economic status, having a hobby, practicing sport, family history of psychiatric disorders and suicides/attempts, personal chronic disease, stimulants consumption, medical school choice judgment, friendships satisfaction, classmates climate, hindrances by medical school, career motivations and worries about future. To achieve the final model (Model 1), a backward elimination method was used (likelihood-ratio statistic greater than 0.10 as removal criterion). Similarly, a model only for 4th-6th year students (Model 2) was executed by adding the variable "grade average and being on time with exams" at the first step.

In addition, we computed another multivariable model (Model 3) to explore if the variables included in Model 1 were associated with SI independently from the severity of the other depressive symptoms. Thus, we added to Model 1 the BDI-II score (subtracting the score of SI, i.e. item 9) as independent variable.

The results of the multivariable models were expressed as adjusted Odds Ratios (adjOR) with 95% Confidence Interval (CI).

Univariable regressions with the final selected variables of Model 1 as independent variables are presented along with the multivariable models to provide an overview of the relationships before adjusting for covariates. The results of the univariable regressions were expressed as Odds Ratios (OR) with 95% CI.

The data were analyzed using IBM SPSS Statistics software version 25.0 (IBM Corp., USA) and a two-tailed p-value <.05 was considered to be significant. Missing values were excluded by pairwise deletion in descriptive analyses and by listwise deletion in regressions.

RESULTS

PRIMES participants were 2,513. In the present paper, we considered 2,457 students (97.8%) who completed the SI item. Females accounted for 61.6% (n = 1,506) and the median age was 22 (IQR = 4). The majority was Italian (n=2416, 98.6%). First-year students were 42.0% (n = 1,033), while fourth- and sixth-year students were 28.4% (n = 699) and 29.5% (n = 725), respectively. About half of the sample was involved in a relationship (n = 1,264, 51.6%). A total of 13.8% had a sexual orientation different from heterosexuality (42 homosexuals, 276 bisexuals, 17 asexuals). Family cohesion was very poor/poor/excessive for 267 students (10.9%), good for 918 (37.5%), and excellent for 1,265 (51.6%). The majority had a good economic status (n = 2,206, 89.9%). A total of 24.1% (n = 589) declared to have a 1st/2nd degree relative with a psychiatric disorder. Participants who declared to see a psychologist/psychiatrist at the time of the survey were 138 (5.6%).

One out of five judged negatively the choice of medical school or had no opinion (n = 496). Few students had unsatisfying friendships with classmates (n = 125, 5.2%) and 390 students felt the climate among classmates was competitive and hostile (16.0%). About half of participants thought that medical school hindered having hobbies (n = 1,286, 52.5%), 46.6% were worried to not measured up to the profession (n = 1,141) and 46.3% were worried about specialty/job limited chances (n = 1,133). Considering only 4th and 6th year students, participants with a high grade average were 62.2% (731 students were on time with exams and 148 not on time) and people with a low/medium average were 37.8% (301 students were on time with exams and 234 not on time. More details about the characteristics of the sample can be found in the PRIMES main paper [5].

A total of 336 students (13.7%, 95% CI 12.5-14.9%) reported SI. Specifically, 286 (11.6%) indicated (b), 43 (1.8%) (c), and 7 (0.3%) (d). In addition, 29.5% of the sample (n = 708) presented depressive symptoms and the median BDI-II score was 9 (IQR = 4-15). SI was

reported by 36.0% of students with depressive symptoms and by 4.3% of students without such symptoms (p <.001). Mild depression was significantly more reported among those who selected (b) (25.7%, adjusted residual = 6.0), moderate depression was significantly more reported among students who chose (b) (32.1%, adjusted residual = 11.9) or (c) (22.0%, adjusted residual = 2.2), and severe depression was significantly more reported among those who marked (b) (17.1%, adjusted residual=10.9), (c) (63.4%, adjusted residual = 18.4) or (d) (57.1%, adjusted residual = 6.8) (p <.001). In particular, students without SI had a median BDI-II score of 8 (IQR = 4-13), while students with SI had a median BDI-II score of 21 (IQR = 14-28) (p <.001). Specifically concerning students with SI, the median BDI-II score was 19 (IQR = 13-26) for participants who selected (b), 32 (IQR = 25-36) for those who selected (c), and 44 (IQR = 25-48) for those who selected (d).

According to the chi-squared tests, SI was significantly differently distributed across all the above-mentioned variables, except across gender (p = 0.780). For instance, the prevalence of SI ideation was higher in the following categories: single students (15.5%), bisexuals (24.6%), asexuals (52.9%), participants with very poor/poor/excessive family cohesion (27.3%), with poor economic status (23.7%), with a 1st/2nd degree relative with a psychiatric disorder (20.0%), 6th year students (15.7%), students negatively judging medical school choice (26.4%), students with unsatisfying friendships (37.6%), students who considered the climate among classmates hostile (25.4%), students thinking that medical schools hinders having hobbies (17.3%), students worried to not measure up to the profession (19.5%) or about the specialty/job limited chances (16.7%). Last, 27.7% of people seeing a psychologist/psychiatrist presented SI (i.e. 12.2% of all students with SI).

The main multivariable model (Model 1) is presented in *Table 1*. Factors associated with a lower probability of reporting SI were being female, being involved in a relation, good and excellent family cohesion, and good economic status. The higher was the age, the more students were prone to show SI. Bisexual and asexual orientation, family history of psychiatric disorders, negative judgment on medical school choice or no opinion, unsatisfying friendships with classmates, competitive and hostile climate among classmates, and being worried about not measuring up to the profession increased the likelihood of declaring SI.

Overall, Model 1 confirmed the results of the univariable analyses *(Table 1)*. However, there were some exceptions. Indeed, year of course, thinking that medical schools hinders having hobbies, and having worries about specialty/job limited chances reported significant relationships with SI in the univariable regressions, while such relationships were no more significant when adjusting for other covariates in Model 1. Conversely, the significant association between gender and SI shown in Model 1 was not found in the univariable analysis.

Moreover, the model with only 4th-6th year students (Model 2) showed also a significance for grade average and timing with exams. Indeed, compared with those with high average and on time with exams, participants with high average and not on time had an OR of 2.44 (95%CI 1.50-3.99, p <.001), students with low/medium average on time of 1.93 (95%CI 1.27-2.94, p = .002) and not on time of 1.82 (95%CI 1.18-2.81, p = .007) (results not shown in the table).

Last, Model 3 showed that some variables kept a significant association with SI even if the model was adjusted for the overall severity of the depressive symptoms *(Table 1)*. Specifically, being female, being involved in a relation, and an excellent family cohesion confirmed to be associated to a lower likelihood of reporting SI. Participants with bisexual and asexual orientation, family history of psychiatric disorders, and unsatisfying friendships with classmates confirmed to be more likely to disclose SI. In addition, the higher was the BDI-II score (excluding item 9), the higher was the probability of declaring SI.

DISCUSSION

The prevalence of SI (13.7%) among medical students of the PRIMES sample resulted consistent with the prevalence reported in the two meta-analyses available in literature: 11.1% (95% CI 9.0%-13.7%) calculated by Rotenstein *et al*. [2] and 11.0% (95% CI 4.0%-19.0%) calculated by Zeng *et al*. [9]. However, there are probably too many differences among the studies considered, both concerning cultural features of the samples and from the methodological point of view. Regarding methods, indeed, tools for measuring SI were different and the time span considered can vary from lifetime to the past 12 months to the last two weeks.

Comparing the SI prevalence of our sample with available data from Europe, our prevalence exceeds estimates by Coentre *et al*. (Portugal, 3.9%) [4] and Wege *et al*. (Germany, 7.4%) [10], is similar to estimates by Chow *et al*. (Germany, 14.7%) [11] and Tyssen *et al*. (Norway, 14%) [12], and is lower than estimates by Miletic *et al*. (Serbia, 23%) [13] and Wallin *et al*. (Sweden, 34-44%) [14]. It should be noted that such European studies were different for included participants and instruments used to evaluate SI. In particular, the studies from Germany considered first year students [10, 11], those from Portugal [4] and Norway [12] enrolled students from the last years, and those from Serbia [13] and Sweden [14] took into account a mixed sample as we did in PRIMES. Interestingly, our study reported a lower prevalence compared with the estimates calculated in samples similar for year of course [13, 14]. About the tools for assessing SI, it is worth highlight two main aspects: the validation and the time span. Most of European studies used one or more items from a validated test (Patient Health Questionnaire-9 [10, 11], Paykel's instrument for measuring suicidal ideation and attempts [12], Suicide Behaviors Questionnaire [13]), while only two works developed *ad hoc* questions [4, 14]. However, there were no clear differences or patterns in prevalence based on this characteristic. Similarly, there are no distinct differences also considering the time span, which was: the last two weeks [10, 11], the last year [12, 14], during medical school [4] and lifetime [13].

Table 1
Univariable logistic regressions and multivariable logistic regression models with Suicidal Ideation as outcome

Outcome: Suicidal ideation	Univariable regression			Multivariable model 1*			Multivariable model 3*		
	OR	95% CI	P value	adjOR	95% CI	P value	adjOR	95% CI	P value
Age	1.09	1.05-1.13	<0.001	1.07	1.01-1.13	0.016	1.04	0.97-1.12	0.261
Gender									
Male	Ref.			Ref.			Ref.		
Female	0.97	0.76-1.23	0.780	0.75	0.56-0.99	0.042	0.54	0.39-0.74	<0.001
Year of course**									
First	Ref.			Ref.			Ref.		
Fourth	2.10	1.58-2.79	<0.001	1.08	0.74-1.58	0.696	1.06	0.69-1.62	0.792
Sixth	1.80	1.35-2.40	<0.001	0.67	0.42-1.08	0.100	0.76	0.44-1.32	0.327
Relationship status									
Single	Ref.			Ref.			Ref.		
Involved	0.74	0.89-0.93	0.010	0.64	0.49-0.83	0.001	0.67	0.50-0.90	0.008
Sexual orientation									
Heterosexual	Ref.			Ref.			Ref.		
Homosexual	2.07	0.98-4.39	0.056	1.34	0.55-3.25	0.519	1.34	0.54-3.33	0.535
Bisexual	2.49	1.83-3.37	<0.001	2.15	1.51-3.05	<0.001	1.93	1.31-2.83	0.001
Asexual	8.55	3.27-22.38	<0.001	7.95	2.71-23.3	<0.001	6.60	1.86-23.4	0.003
Family cohesion									
Very poor/poor/excessive	Ref.			Ref.			Ref.		
Good	0.56	0.41-0.77	<0.001	0.54	0.37-0.78	0.001	0.68	0.45-1.03	0.067
Excellent	0.23	0.17-0.33	<0.001	0.31	0.21-0.45	<0.001	0.40	0.26-0.62	<0.001
Economic status									
Poor	Ref.			Ref.			Ref.		
Good	0.46	0.34-0.63	<0.001	0.63	0.43-0.91	0.014	1.02	0.67-1.55	0.929
1st/2nd degree relatives with psychiatric disorders#	1.91	1.49-2.44	<0.001	1.53	1.15-2.04	0.003	1.40	1.03-1.91	0.032
Judging the choice of medical school									
Positively	Ref.			Ref.			Ref.		
Negatively/No opinion	3.06	2.39-3.91	<0.001	1.79	1.34-2.41	<0.001	1.13	0.81-1.57	0.487
Satisfying friendships with a circle of classmates									
Yes/Not yet	Ref.			Ref.			Ref.		
No	4.29	2.93-6.29	<0.001	2.73	1.74-4.30	<0.001	2.08	1.23-3.52	0.007
Climate among classmates									
Friendly/Competitive but stimulating/No opinion	Ref.			Ref.			Ref.		
Competitive and hostile	2.66	2.04-3.47	<0.001	1.95	1.41-2.69	<0.001	1.19	0.83-1.71	0.333
***Thinking that medical school hinders*: Having hobbies#**	1.94	1.52-2.47	<0.001	1.29	0.97-1.73	0.085	1.01	0.74-1.39	0.941
***Worries about the future°*: Yes, not measured up to the profession#**	2.55	2.00-3.25	<0.001	2.08	1.57-2.77	<0.001	1.19	0.87-1.62	0.281
***Worries about the future°*: Yes, about specialty/job limited chances#**	1.60	1.27-2.02	<0.001	1.27	0.96-1.68	0.089	0.99	0.73-1.34	0.929
BDI-II score (excluding item 9 on suicidal ideation)	1.16	1.14-1.18	<0.001	-	-	-	1.15	1.13-1.17	<0.001

Abbreviations: adjusted Odds Ratios (adjOR); Beck Depression Inventory-II (BDI-II); Confidence Interval (CI); Odds Ratio (OR).
Significant P values in bold.
*The multivariable model 3 is the multivariable model 1 with, in addition, the BDI-II score as independent variable. The results of the multivariable model 2 are described only in the text. Further details about the models are provided in the Methods.
** year of medical school.
# Possible options: "No" and "Yes". "No" considered the reference level.
° Possibility to select more options.

The prevalence of depressive symptoms (29.5%) in our sample is similar to the prevalence of the meta-analysis of Rotenstein *et al.* (27.2%, 95% CI 24.7%-29.9%) [2] and, as expected, depression seems to be an important factor associated with SI in medical students (36.0% of students with depressive symptoms reported SI *vs* 4.3% of students without such symptoms), consistently with literature [3, 4, 7, 13]. Additionally, in the PRIMES sample, being female increased likelihood of showing depression [5] but decreased the probability of reporting SI. Notably, in the literature on MSs, there are mixed evidences about associations between gender and SI [3, 7, 9].

Demographic factors associated with a higher probability of reporting SI were having family history of psychiatric disorders and, differently from literature [2], being older (but no significant relationship between SI and year of course was found by PRIMES, as already seen in literature [2, 3]). Coherently with other studies [3, 4, 1], poor economic status emerged as a factor associated with SI among medical students. In addition, consistently with literature about the high risk for mental disorders in sexual minorities [15], students disclosing bisexual or asexual orientation were more likely to declare SI. Since homosexual orientation was not associated with higher SI risk, our results suggest possible differences in SI among sexual minority subgroups that should be further investigated. Indeed, the systematic review by Plöderl and Tremblay highlighted that, although an elevated risk exist within all sexual minorities, bisexuals have reported higher mental health problems compared with homosexual individuals in several studies [15]. Therefore, it would be worth exploring the determinants of such differences, also taking into account the under-studied asexual orientation, and possibly considering features such as stigma and social acceptance.

As expected by previous findings [3, 7, 12], also some relational factors were associated with a higher likelihood of reporting SI, such as having a poor family cohesion, being single, and feeling classroom climate as hostile and not suitable for friendship.

It should be highlighted that the regression model adjusted for the BDI-II score showed that some of the above-mentioned variables were associated with SI independently from the overall depressive symptoms. Such variables included both demographic (i.e. gender, sexual orientation, family history of psychiatric disorders) and relational (i.e. family cohesion, relationship status, friendship with classmates) factors and, thus, such aspects should be particularly taken into account when studying SI and planning preventive and supportive strategies.

Finally, in line with other works [3, 7], PRIMES revealed some motivational factors that can make the students more prone to SI, such as having a negative judgement about their own career choice, dissatisfaction about their own scholar performance or worries about future profession. Both relational and motivational are probably factors that could be addressed by specific interventions at a university level.

Limitations included the cross-sectional design, the opportunistic sampling, and the use of a single item instead of a multi-item measure for SI. Indeed, a prospective study with structured interview in a random subset of participants would give a more authentic estimate [2].

Nevertheless, PRIMES had the strength to be the first large multicenter study in Italy assessing SI prevalence among medical students. Moreover, it showed that, despite medical students seem a high-risk population for SI, only 12.2% and 14.3% of these students was treated or was followed by a psychologist/psychiatrist, respectively. These findings were similar to the situation Rotenstein and colleagues found in their meta-analysis regarding medical students with depressive symptoms [2], thus underlining the necessity to realize strategies to increase the access to care by accommodating the needs of medical students.

Therefore, given the high SI prevalence, it would be advisable to study more in depth the factors that are involved in SI among medical students in order to implement effective preventive plans and design effective and approachable interventions. Relevant reviews on SI suggest that the primary actions to face SI among medical students should be addressing stigma to reduce barriers to mental health services, identifying depressive symptoms, as they are the most frequent factor associated with SI, and implementing general well-being interventions for all students to lower the overall rates of mental health issues [2, 3]. Based on our findings, we argue that SI interventions should be promoted especially for males and sexual minorities, and the low social support from family and peers that students with SI might experience should be considered when developing such strategies.

gies "GF Ingrassia", University of Catania, Catania, Italy); Laura Brunelli, Silvio Brusaferro, Daniele Celotto (Department of Medicine, University of Udine, Udine, Italy); Maria Chironna, Daniela Loconsole (Department of Biomedical Sciences and Human Oncology, University of Bari - Aldo Moro, Bari, Italy); Vincenzo Baldo, Silvia Cocchio (Department of Cardiac, Thoracic, Vascular Sciences and Public Health, University of Padova, Padova, Italy); Vincenza Cofini, Simona D'Onofrio (Biostatistics and Epidemiology Unit, Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy); Marcello Mario D'Errico (Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy); Martina D'Errico (SAP: psychological counselling for university students, Polytechnic University of Marche, Ancona, Italy); Carolina Marzuillo, Valentina Baccolini (Department of Public Health and Infectious Diseases, Sapienza University of Roma, Rome, Italy); Cesira Pasquarella, Davide Di Fonzo (Department of Medicine and Surgery, University of Parma, Parma, Italy); Maria Pavia (Department of Health Sciences, University "Magna Graecia" of Catanzaro, Catanzaro, Italy); Vincenzo Restivo, Alessandra Casuccio (Department of Science for Health Promotion and Mother to Child Care "G. D'Alessandro", University of Palermo, Palermo, Italy).

ORIGINAL ARTICLES AND REVIEWS

REFERENCES

1. World Health Organization. Depression and other common mental disorders: Global health estimates. WHO; 2017. Available from: https://apps.who.int/iris/handle/10665/254610.

2. Rotenstein LS, Ramos MA, Torre M, et al. Prevalence of depression, depressive symptoms, and suicidal ideation among medical students a systematic review and meta-analysis. JAMA. 2016;316(21):2214-36. doi:10.1001/jama.2016.17324

3. Coentre R, Góis C. Suicidal ideation in medical students: recent insights. Adv Med Educ Pract. 2018;9:873-80. doi:10.2147/amep.s162626

4. Coentre R, Faravelli C, Figueira ML. Assessment of depression and suicidal behaviour among medical students in Portugal. Int J Med Educ. 2016;7:354-63. doi:10.5116/ijme.57f8.c468

5. Bert F, Lo Moro G, Corradi A, et al. Prevalence of depressive symptoms among Italian medical students: The multicentre cross-sectional "PRIMES" study. PLoS One. 2020;15(4):e0231845. doi:10.1371/journal.pone.0231845

6. Beck A, Steer R, Brown G. Beck Depression Inventory-II. San Antonio, TX: The Psychological Corporation Harcourt Brace & Company; 1996.

7. Torres AR, Campos LM, Lima MCP, Ramos-Cerqueira ATA. Suicidal ideation among medical students: Prevalence and predictors. J Nerv Ment Dis. 2018;206(3):160-8. doi:10.1097/NMD.0000000000000734

8. Farabaugh A, Nyer M, Holt D, et al. Screening for suicide risk in the college population. J Ration Cogn-Behav Ther. 2015;33:78-94.

9. Zeng W, Chen R, Wang X, Zhang Q, Deng W. Prevalence of mental health problems among medical students in China: A meta-analysis. Med (United States). 2019;98(18). doi:10.1097/MD.0000000000015337

10. Wege N, Muth T, Li J, Angerer P. Mental health among currently enrolled medical students in Germany. Public Health. 2016;132:92-100. doi:10.1016/j.puhe.2015.12.014

11. Chow WS, Schmidtke J, Loerbroks A, Muth T, Angerer P. The relationship between personality traits with depressive symptoms and suicidal ideation among medical students: A cross-sectional study at one medical school in Germany. Int J Environ Res Public Health. 2018;15(7). doi:10.3390/ijerph15071462

12. Tyssen R, Vaglum P, Grønvold NT, Ekeberg. Suicidal ideation among medical students and young physicians: A nationwide and prospective study of prevalence and predictors. J Affect Disord. 2001;64(1):69-79. doi:10.1016/S0165-0327(00)00205-6

13. Miletic V, Lukovic JA, Ratkovic N, Aleksic D, Grgurevic A. Demographic risk factors for suicide and depression among Serbian medical school students. Soc Psychiatry Psychiatr Epidemiol. 2015;50(4):633-8. doi:10.1007/s00127-014-0950-9

14. Wallin U, Runeson B. Attitudes towards suicide and suicidal patients among medical students. Eur Psychiatry. 2003;18(7):329-33. doi:10.1016/j.eurpsy.2003.03.006

15. Plöderl M, Tremblay P. Mental health of sexual minorities. A systematic review. Int Rev Psychiatry. 2015;27(5):367-85. doi:10.3109/09540261.2015.108394

Network as a language for precision medicine

Lorenzo Farina

Dipartimento di Ingegneria Informatica, Automatica e Gestionale "A. Ruberti", Sapienza Università di Roma, Rome, Italy

Abstract
The article takes as a starting point the observation of a deep and long-standing gap between the views of biologists/physicians and that of physicists/data scientists when dealing with life sciences. This gap has been exacerbated by the advent of large-scale -omics technologies. Here, we focus on the impact of this gap in the field of precision medicine that impedes dialogue between omics data analysts and precision medicine physicians. To try to overcome this cultural divide, here we suggest a new possibility through the use of network science as a shared language composed of a vocabulary of words that have different meanings in each discipline but refer to the same biological entity. By doing so, one can move from biological concepts to network patterns and algorithms and backwards, thus generating a dialogue between "life scientists" and "number scientists". The article presents several simple network concepts with a straightforward biological interpretation as a starting point for such interdisciplinary dialogue.

INTRODUCTION

There is a growing awareness of the large and increasing gap between two visions of life: the vision of research scientists like biologists, physicians, molecular biologists, and so on, and that of research scientists like physicists, engineers, mathematicians, and so on [1]. For the sake of simplicity, we can call the first "life scientists" and the second "number scientists". The two groups are just an idealized and simplified classification, and a wide variety of different approaches are included in the same group, as well as a continuum between the two extremes is conceivable. Nevertheless, life scientists share a common vision that is well defined in the words of Ernst Mayr: "*Owing to their complexity, biological systems are richly endowed with capacities such as reproduction, metabolism, replication, regulation, adaptedness, growth, and hierarchical organization. Nothing of the sort exists in the inanimate world*" [2]. The fundamental separation between the two visions is just in front of us every day. It suffices to quote the philosopher of science Evelyn Fox Keller who wrote: "*I have had ample opportunity to observe failures of communication virtually whenever experimental and mathematical biologists happened to be in the same room*" [3]. Life scientists vision is centered on "concepts" like for example evolution, adaptation, development, speciation, purpose, which are not amenable of a rigorous and immediate mathematical formalization, while number scientists' vision is centered on fitting "patterns" to data [4]. Furthermore, it is a common belief that biology is subject to "universal laws" (yet to be discovered),

and that from these laws one can derive mathematical models, and consequently computations on data, i.e., algorithms. In other words, the underlying idea is that, to make sense of biological data we need to find universal patterns and "general mathematical theories" of biology, just like in physics. Number scientists believe that biological information can be obtained from data only by establishing an all-encompassing mathematical framework from which derive equations that naturally lead to some calculation on data. Simply put, from this perspective, the path from biological properties to algorithms on data is necessarily mediated by a "universal mathematical theory" provided by the coming of the "Newton of biology" and guided by the "law of parsimony" (Ockham's razor) [5]. By contrast, life scientists have an opposing view of the problem of making sense of biological data. The only "law" of biology, although not written in the language of mathematics, is evolution, and their vision is centered on the uniqueness of life as a scientific discipline, with its own language and concepts [2], often (if not always) not amenable of precise mathematical formulation. Apart from the biochemical elements that make life possible on earth (e.g., DNA structure or protein structure), the life scientists usually strongly oppose universality, as clearly explained by Steven Jay Gould "*If nature teaches us any lesson, it loudly proclaims life's diversity. […] In any case, bursting diversity is nature watchword; it should never be submerged by careless abstraction*" [6]. Our aim is not to reconcile such visions or to take the side of one or the other, but to focus

Address for correspondence: Lorenzo Farina, Dipartimento di Ingegneria Informatica, Automatica e Gestionale "A. Ruberti", Sapienza Università di Roma, Via Ariosto 25, 00185 Rome, Italy. E-mail: lorenzo.farina@uniroma1.it.

on the almost complete absence of dialogue between life scientists and number scientists caused by an objective gap between the two cultures. Certainly, we do not support both the idea that biology's dignity as a science depends on its degree of mathematization and the opposite vision that considers mathematics as a simple tool-generator, so that the life scientist must choose the "best" one for his/her purposes, without any interactive dialogue with the number scientist.

Here, we focus on the impact of this gap in the field of precision medicine by presenting an alternative view and propose a contribution to reduce the gap (that we call the "complex data divide") and allow a dialogue, by looking at network science as a vocabulary generator (not tools). It is *not* enough to fit data to patterns if the patterns do not match a biological concept, and vice-versa. Mathematical patterns may be elegant but lacking biological plausibility (like, for example, the Turing mathematical model of morphogenesis [3] just to cite the most popular), and biological concepts may not result in a significant mathematical pattern on available data. Precisely, here we propose networks as a way to represent omics (large scale) data by focusing on relationships among elements and discuss some network pattern (the words of the language) as suggestive of biological features so that, the life scientist and the number scientist, can speak the same language even though they give a different meaning to the same words. It is worth noting that the recently introduced concept of "networks of networks" [7] can be fruitfully used to generate new "words" of the network vocabulary at different scales.

Here, we review some of the most promising examples of "words" of the network language and their precision medicine counterpart and show how they can be used to link biological concepts to algorithms on networks and vice versa, without the need for any "general theory" and, most importantly, to stimulate a truly interdisciplinary dialogue between life scientists and number scientists. As a final comment, we note that the network vocabulary is still in its infancy, but there is growing evidence that such network-based dialogue can effectively reduce the complex data divide.

"OMEXITY": OMICS DATA EXPLOSION AND DISEASE COMPLEXITY

Complexity as emergence. A complex disease is the result of many intertwined factors that include polygenic risk variants, physiological and environmental stresses, lifestyle habits, and many others. Moreover, even Mendelian disorders do not adhere to the one gene – one phenotype model, so that the awareness that virtually all diseases share these properties is increasing [8]. For these reasons, complex diseases have increasingly become the focus of modern medicine, especially in western countries, where age-related pathologies (cardiovascular and respiratory diseases, cancer, or type 2 diabetes) are the leading causes of death globally. According to the World Health Organization (WHO), 7 of the 10 leading causes of death are the so-called "non-communicable diseases". These seven causes accounted for 44% of all deaths or 80% of the top 10 and, together, accounted for 74% of deaths globally in 2019 [9]. Non-communicable diseases (NCDs) are chronic pathologies, that is they tend to develop and persist over a long period. Most importantly, they are all complex diseases, in that their onset and development are inherently multi-factorial.

Complexity in disease onset and development can be fully considered the "rule" rather than "the "exception". Indeed, the definition itself of a "complex" disease as "caused by the interaction of multiple genes and environmental factors" (taken from NIH glossary of genetics term), calls to mind the concept of *interconnection* among factors. As such, complexity arises from the crosstalk among a variety of molecular factors and pathways that prevents the understanding of pathogenesis as a linear causal route from genotype to phenotype. Complex diseases emerge from the interplay of many actors working together [10]. Such multi-factorial character implies that most causal players have just "weak" effects on the disease, whereas mainstream research studies assume the presence of risk elements with a "strong" effect [11].

The emergence of disease as a complex phenomenon makes it intractable from a reductionist perspective, which is the idea that systems can be understood by looking at every single component and disease as a linear chain of molecular interactions [12]. For example, using the terminology borrowed from complexity theory, cancer onset and progression is characterized by many "emergent" properties that reveal its behavior as an adaptive, self-organized system. The hallmarks of cancers include triggering proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [13]. These biological properties cannot be traced back to a single cell or component, but to the web of interconnections of many factors that lead to the "emergence" of both normal and pathological cell behavior.

Omics networks. As already mentioned, a complex disease is also a complex system, in that its properties "emerge" from the interactions between components and the environment. From this perspective, it is quite natural to represent complex systems as *networks* where nodes represent parts (components), and links represent interactions or, more generally, associations among nodes [14], as illustrated in *Figure 1*.

The explosion of omics data has revolutionized the study of complex diseases. The big data produced by omics technologies are pervasive, and their variety and availability increase every day. They include genomics, transcriptomics, proteomics, epigenomics, microbiomics and many others. For example, Next Generation Sequencing (NGS) devices allow the study of inherited genetic factors using exome or targeted panels, or the effects of specific lifestyles by detecting changes that impact the global expression pattern, assessing epigenetic mechanisms (methylation, non-coding RNAs). It also allows to study the impact of environmental factors, such as the microbiome composition and its interactions with the immune system, to identify biomarkers and personalized drug response. The current situation is often referred to as era of "big" data,

and emphasis is given on the quantitative aspect, rather it should be clear that the real challenge is that such large amounts of data are linked to one another in very complex ways, so they should be called "complex data" to better highlight the key question at stake which is *not* merely quantitative, as discussed in what follows.

Types of omics networks. It would be unreasonable to give an exhaustive list of all possible networks that can be constructed using omics data. Indeed, besides *interaction networks*, that is networks in which a link is present due to physical interactions between the two biological entities represented by nodes, *e.g.*, map of protein-protein interaction (PPI), it is very common to build *association networks* where links represent any kind of association rule, thus making the universe of all possible association networks virtually infinite. Associations between two elements (the nodes of the network) can be derived in many ways, by looking at any commonality between them. For example, interesting association networks are the so-called "human disease network" or "diseasome" [15], which is a disease-disease network where diseases are connected if they share a common genetic component (a mutation) or the "patient similarity networks" [16] in which patients are linked based on their similarities in various clinical features, including genomic profiles. A comprehensive list of molecular networks of both types can be found in a recent review by Silverman *et al.* [17].

More is different. Omics data are certainly important because they provide an unprecedented view of a cell's life at the molecular level, but equally important is the role of the availability of large quantities of information, i.e., the large-scale feature of omics data. Indeed, as anticipated by Philip Anderson in 1972, "*more is different*" [18], which means that when we deal with a large number of highly interconnected entities, the properties of the single part fade into the background

and new emergent properties arise, i.e. quantitative difference may become qualitative. This point is illustrated by *Figure 2*.

Omics technology explosion developed independently of awareness of the complexity of diseases. Indeed, complexity theory originated from the studies of the early fifties of the last century initiated by von Bertalanffy [19] and Boulding [20] in the field of holistic general dynamic systems theory [21], while the omics revolution started in the seventies of the last century fostered by discoveries in the field of biotechnology like recombinant DNA technology and Sanger sequencing [21, 22]. Interestingly, those two independent lines of research have now met in a single pathway leading to precision medicine. In other words, the omics/complexity (that we might call "omexity") era has just begun.

Omexity and precision medicine. The rise of "precision medicine" is crucially related both to widespread awareness of the complexity of virtually all pathological conditions and to the availability of increasingly large amounts of molecular omics data which call for improved information processing capabilities to extract relevant information [23]. Medical research in the era of precision medicine cannot but include data analysis and integration of large and heterogeneous sources like DNA/RNA sequencing, proteomics, imaging, digital pathology, laboratory medicine, vital signs, medical records, and so on. Simply put, complexity and omics together, naturally lead to the development of an integrative medical mindset that merges clinical observation and data pattern recognition. The real challenge of omexity and the associated precision medicine approach, is the buildup of abilities from somewhat separate worlds, i.e., the computational sciences and the life sciences. This amounts to saying that a new kind of interdisciplinary mindset is needed to tackle omexity, that is the ability to establish a dialogue between the
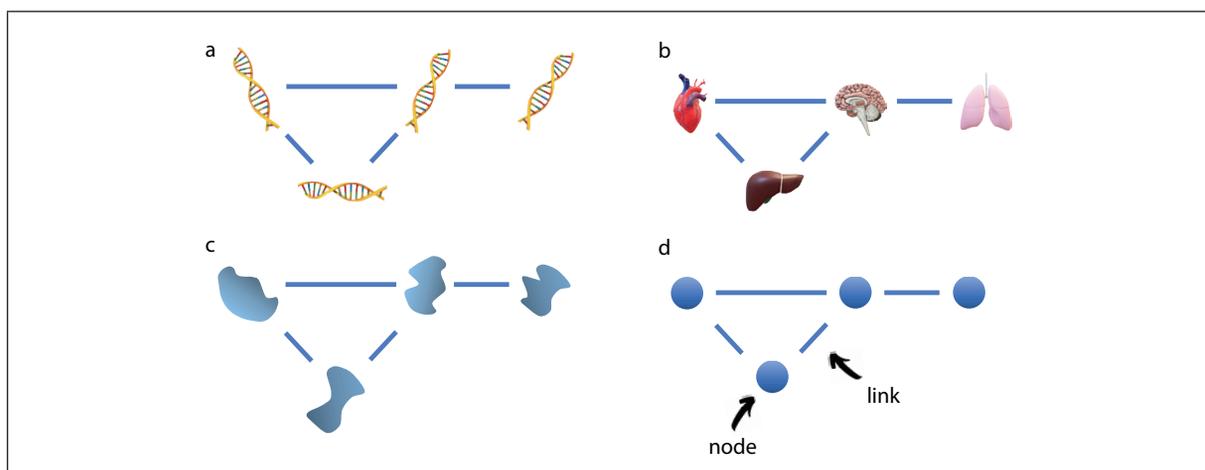

Figure 1

Networks. A network is a collection of nodes and links that connect them. The network representation simplifies reality in that it focuses on relationships, rather than on element properties. In fact, a) DNA-DNA associations may be obtained, for example, if mutations on a pair of genes are related to the same disease or if they are both targets of some transcription factors or miRNA or epigenetic modifier. b) A link between two organs may be established, for example, by considering comorbidities or for sharing some molecular driver. c) Two proteins could be linked if they interact, i.e., if they form a complex. d) the resulting abstract network is the same for all cases considered since the link pattern is the same, even though the elements are completely different.
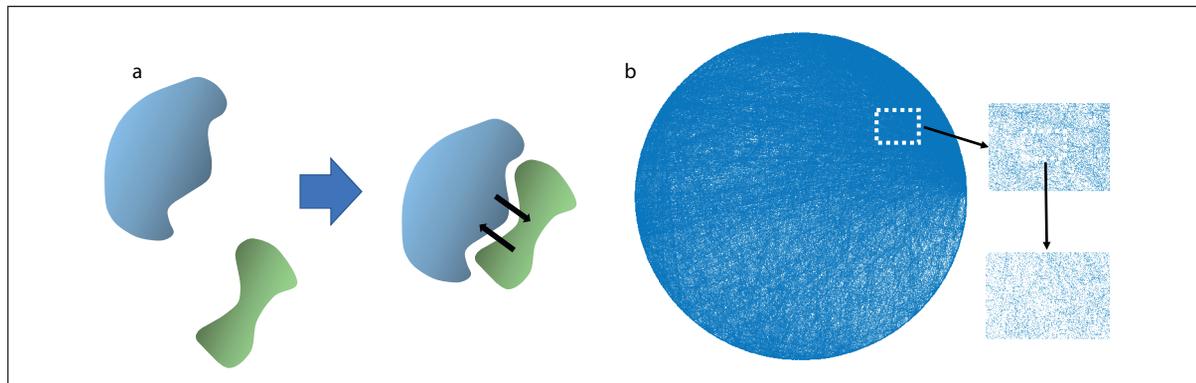
Figure 2
More is different. a) The binding of two proteins can be a very complicated process involving many spatial and biochemical properties of the subunits and the environment. b) When millions of binding events occur, new properties of the ensemble arise, and the properties of the whole cannot be attributable to single parts. Simply put, "more is different".

"calculative" and the "meditative" mind, using a terminology due to Martin Heidegger [24], that in our case corresponds to, on the one hand, the computational aspect of pattern recognition in omics data interpretation, and, on the other hand, to a more holistic view of disease and specificity of the single patient. It is perhaps the greatest challenge of the healthcare sector over the coming decade to integrate all these resources and translate them into clinical practice [23]. Such a deep gap between the medical and the data analyst mindsets can be termed as the "complex data divide".

In the next section, the cultural origins of such a divide will be discussed and possible roads will be suggested to reduce this gap that stalls knowledge advancement and interdisciplinary dialogue. As discussed in what follows, the network language could have a very special role in this dialogue.

THE "COMPLEX DATA DIVIDE" AND INTERDISCIPLINARY DIALOGUE

A document published in 1999 entitled *Medical and societal consequences of the Human Genome Project* [25] predicted that within 10 or 15 years, the impact of the human DNA sequencing would be a radical transformation of medicine. There is a general agreement that the genome project radically changed the rules of medical research, the way of practicing biological discovery, and the ubiquitous digitation of biological sciences. However, there is still a debate on whether there is a real impact on population's life expectancy or any other public health measures [26]. The omics data explosion has increased public expectations on the utility of molecular big data and on artificial intelligence (AI) methodologies for the discovery of new therapies for diseases which still lack effective treatments, like the vast majority of complex diseases, e.g., cancer or diabetes. Moreover, the recent pandemic made it clear to the general public, the urgent need for a personalized treatment based on multi-level profiling able to take into account the large variety of responses observed in the CoViD-19 manifestations, ranging from the absence of symptoms to pneumonia and death. This is a great challenge and an opportunity for omexity research. However, to meet

such high expectations a radical perspective change is needed.

A key issue is the huge gap between the historical and cultural milieu of researchers trained in computational sciences (computer science, statistics, engineering, etc.) and those trained in the life sciences (biologists, physicians, biochemists, etc.). To tackle omexity, which lies at the interface between big heterogeneous omics data analysis (computation) and the extraction of relevant information (biological interpretation), a new interdisciplinary or intercultural dialogue must be established. Although the strict separation of the two domains is well known and often referred to as the difference from theoretical and applied sciences, the situation evidenced by omexity has peculiarities that make it something very different from the past. The omexity challenge cannot proceed directly from theory to applications, simply because we do not have theories like in physics or general quantitative laws that can provide an unambiguous framework for the development of the "best" algorithm for a given biological or medical question. A good illustrative example is the evidence that molecular systems of complex cells are inherently different from simple electronic circuits [27]. In other words, the role of mathematics and computation (algorithms) in the life sciences is extremely different from that of physics or engineering. As regards omexity, the point at stake is not to find universal laws but to establish a dialogue between omics data analysis and its biological interpretations. In other words, what is needed is a sort of "mapping" or "bidirectional flow" from emergent properties of data structures and emergent properties of biological systems. This key point is illustrated in *Figure 3*.

This mapping can be obtained, for example, by using "metaphorical projections", which is a metaphorical correspondence that can be established between two separate worlds, so that the finding in one domain can be translated into the other domain and *vice versa*. It is worth noting that such mapping cannot be devoid of any "theory" since a mechanistic explanation of the mapping is required to provide a solid background of such mapping. In other words, this is *not* the end of theory [28], but (hopefully) the beginning of a peer-to-peer
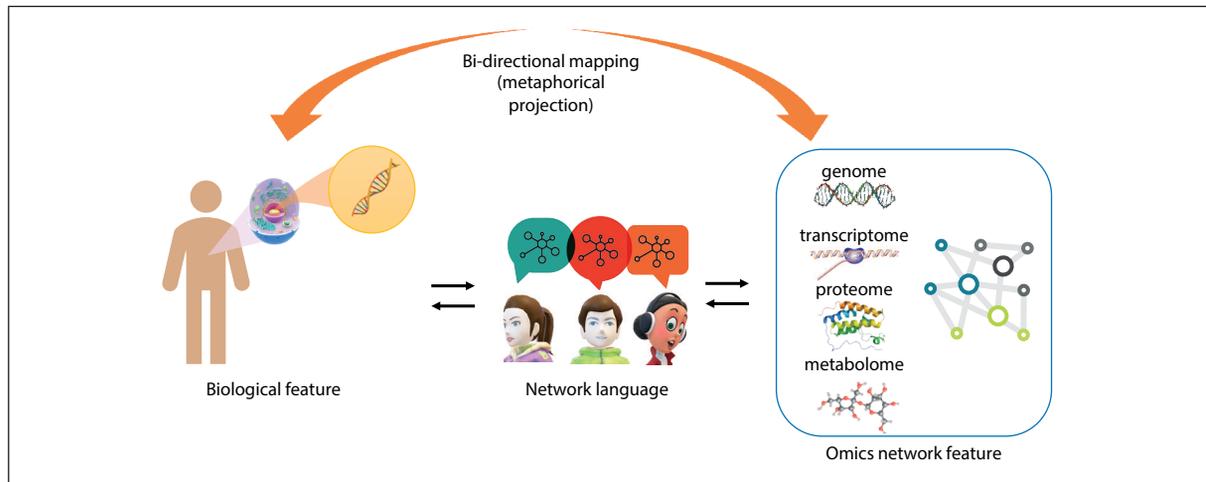
Figure 3
The complex data divide: networks as mediators from biology to omics and back. The bi-directional mapping (or metaphorical projection) from biological to network features is mediated by the network language. The availability of a set of words (language) that have a precise meaning, although different, both for the life scientist and the number scientist, makes the interdisciplinary dialogue possible.

dialogue between separate domains, each with its own rules and procedures that may be theories (like in statistics or physics) or interpretations in the light of biological concepts, like evolution, natural selection, reproduction, metabolism, replication, regulation, adaptedness, growth, hierarchical organization and so on, none of which have counterparts in the inanimate world. As pointed out by Ernst Mayr, the most fundamental difference between biology and the hard sciences is that biology theories are based on "concepts", while in the physical sciences they are based on "natural laws" [2]. Indeed, data do not speak for themselves since science is not about finding patterns but (biological) explanations for those patterns [29]. Most importantly, the goal is not the discovery of a unifying general theory but the finding of a link (a mapping of some sort) between the two domains, each with peculiar theories no matter how mathematical or conceptual they are.

Here we support the idea that network language, as defined and explained in the following sections, can effectively function as a bridge between the computational and the living domains, i.e., able to define single entities that can have a valuable, although different, meaning in both fields of research. Precisely, the large amounts of complex omics data and the awareness of disease complexity, lead straightforwardly to a true inter-disciplinarity dialogue which is possible only in the presence of a common language, like that of networks, and that results in a calculation or algorithm on data to implement precision medicine. This process is pictorially described in *Figure 4*.

THE NETWORK LANGUAGE
A metaphorical projection

A "network" is not necessarily a "real" thing, rather it must be considered a "cognitive schema" [30], which is an abstract collection of concepts used to make sense of the unknown world of life. Precisely, a general characterization of a network can be defined by a bunch

of "nuclei" (where matter, as well as other activities are far more concentrated) linked to each other by edges (streets, power cables, etc.) passing in a much less dense environment [31], just like a lumped-element model of a spatially distributed physical system. From this perspective, in a more abstract sense, nodes are non-dimensional points and edges are one-dimensional lines. But, protein-protein interactions are not "lines" between "points" but very complex phenomena where many spatial and energetic factors are at work. For example, by substituting proteins with nodes and bindings with links, one is "projecting" the network schema onto the protein-protein interaction network and thus performing a "metaphorical projection" [30, 32]. The network is a functional abstraction, a way of rendering complex systems comprehensible using an over-simplified representation of data. And yet, at the same time, only through this distortion of reality operated by a metaphorical projection, the protein-protein interaction network becomes amenable to computations on data. The key point here is that "distortion" is by no means a re-creation of reality but, rather the inevitable re-organization of available knowledge into meaningful forms able to indicate solutions or useful directions of research for a specific purpose or problem of interest. An example of a successful metaphorical projection is the London underground map as designed by Harry Beck in 1933. "The map is not the territory" or "the menu is not the meal" are popular expressions to remember that we cannot confuse models of reality with reality itself. The construction of a map is not an easy task, since the goal is to represent only "relevant" information for the end-user. From the more general perspective of the scientific enterprise, it is worth quoting Gaston Bachelard's statement: "*Contemporary science maintains that quantities which are negligible must be neglected. It is not enough to say they can be neglected.*" [33, p. 220]. In the early 1930s, the map of the London underground was purely geographic and metro stations were represented
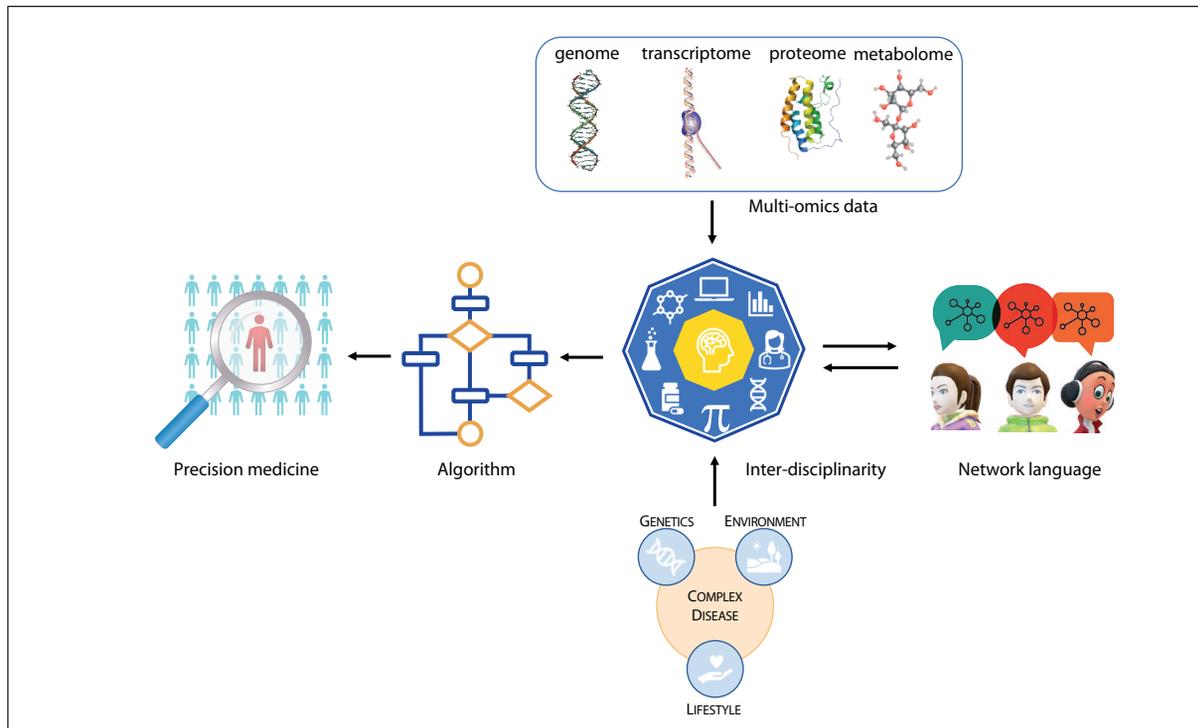
Figure 4
Network language for precision medicine. The network language enables a true interdisciplinary dialogue among the many skills required by the availability of complex data, like omics data. Questions on complex diseases can be answered using omics data through interdisciplinarity that, using the network language, may directly provide algorithms on data to provide answers without resorting to abstract theories of any kind.

on a true scale together with the city's street network, attractions, public institutions, parks, and waterways. The information content was overwhelming, and passengers found it difficult to use it. When the first abstract map developed by Beck was presented to Londoners in 1933, they found it useful and easy to understand [34]. The basic idea was to place stations without any direct correspondence with "true" geographic positions and to use only straight lines and orientations of 0°, 90°, or 45° degrees. An example of the resulting map is reported in *Figure 5*.

Beck's map is considered by many the most celebrated graphic design of the 20th century [34] and it is a perfect example of a metaphorical projection of "real data" (i.e., the geographic map) on an abstract and oversimplified representation which must serve the primary function of helping users to extract the information they need, without too many details that might lead the end-user astray. And this is not a specific problem with maps, but a general paradigm of how scientific enterprise proceeds by neglecting what *must* be neglected. Indeed, network representation of relationships among omics data is certainly an oversimplified vision of the tremendous complexity of life, but it is a necessary step to *make sense* of huge amounts of complex data. Once abstraction of concrete is performed to form representational elements (nodes and links), integration of these elements can be used to identify structures or patterns which, in turn, represent the words that constitute the "network language" able to express a global configuration, i.e. the

act of arranging all the informational elements and their structures to create a whole: this "pattern language" approach is known as "theory of centers" and it has been developed for the design of visual artifacts [34].

In sum, a network is a visual representation of data focused on relationships (links) among elements (nodes) and link configuration is what really matters, whereas peculiarities of the nodes are neglected. The goal is therefore to identify network "patterns" to be metaphorically projected onto biological concepts, thus providing a language that may be able to overcome the complex data divide and allow computational and the biomedical researchers to talk to each other about the same reality from different perspectives. The next section is devoted to the illustration of three popular examples of network patterns that have been used to make sense of omics data in the recent literature.

The three C's of network language

When studying a network, many properties can be of interest, depending on the particular problem at hand. For example, in 1736, when Leonhard Euler studied the bridges of Konigsberg, he was interested in finding a path through the city that would cross each of its seven bridges over the river Pregel only once. However, special focus has been given by researchers in social and biological networks on three properties that are key if we consider the metaphorical projection on a situation in which "messages" or "information" flows from node to node through the links. Not surprisingly, a cell
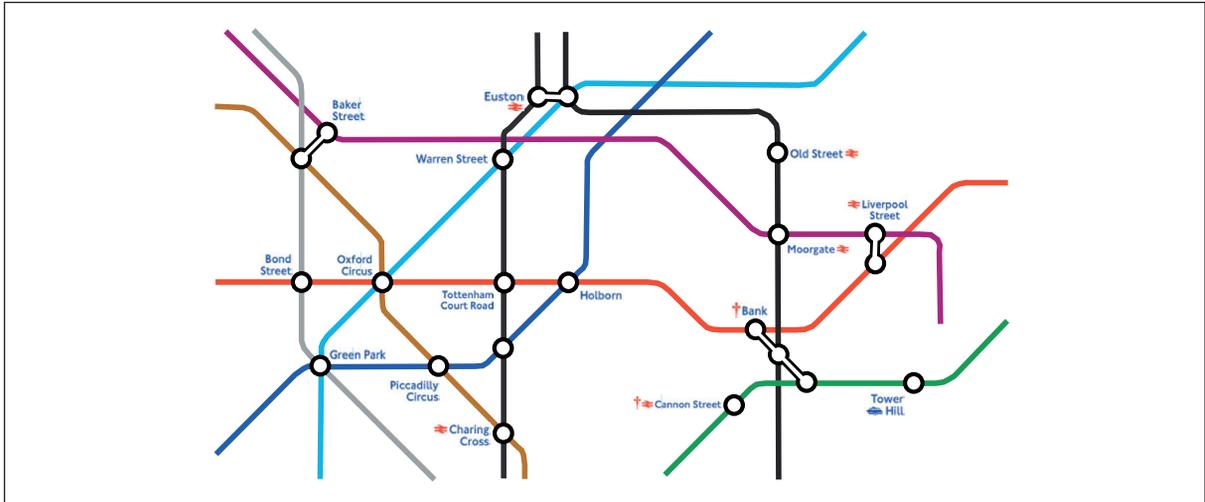
Figure 5

Metro map as metaphorical projection. The London Metro map is a paradigmatic example of a "metaphorical projection". Real geographic coordinates have been eliminated in favor of greater readability. The purpose of the map is not to faithfully reproduce reality but to represent relevant information suitable for finding the best route between stops.

is considered an "information processing unit" by many influential molecular biologists [35, 36]. From this perspective, it is customary to consider three features of a network: the modular organization in communities, the presence of "influential" (or central) nodes in a network or a community, and the presence of nodes connecting different communities. Therefore, the three C's of network analysis are communities, centralities, and connectors (see *Figure 6*).

Besides centrality measures, there are many other important topological properties tightly linked to biological concepts of special interest for precision medicine. The most promising certainly is the "interactome disease module" perturbation model of disease onset and development proposed by Barabasi, Gulbahce and Loscalzo [37] which has been validated on several real cases [38]. Here we focus on the 3C's for the sake of brevity, but the same arguments apply to any pattern that can enrich the vocabulary of the network language for precision medicine.

Communities. Using network science terminology, modularity is often referred to as having a "community structure", *i.e.*, their vertices are organized into groups, called *communities, clusters*, or *modules* [39] as shown in *Figure 7a*.

Modularity is a key feature of living systems. Every cellular event, such as signaling or DNA replication, is the result of the presence of "modules" composed of several molecular machineries or regulatory structures, coordinately interacting directly or indirectly [40]. Indeed, at the molecular scale, the presence of modules is often described as an ensemble of gene products highly coordinated at the functional level, interacting physically and subject to co-regulation [41, 42]. Moreover, modularity may support evolutionary forces and sustain change. The organization of functions in discrete modules (possibly partially overlapped) provide robustness to change but permit changes by modifications of the interconnections among modules. This is key to allow

evolvability in uncertain and noisy environments and, at the same time, maintain adaptability [40, 43]. Modularity is an omnipresent property of genomic data of all living systems which can be found in many kinds of experimental datasets, such as protein-protein or protein-DNA interactions, gene expression measurements, and many others [44]. The modularity structure of a network and identification of communities can be formally characterized in many ways. The most widely used one is the "modularity measure" defined by Newman as the fraction of edges that belong to the given communities minus the expected fraction whether links were randomly distributed [45]. Community finding algorithms using the modularity measure are based, for example, on maximum likelihood [46] or local greedy approach [47].

The identification of modules in a network may provide useful information on how it is organized by emphasizing regions with a sort of "degree of autonomy"
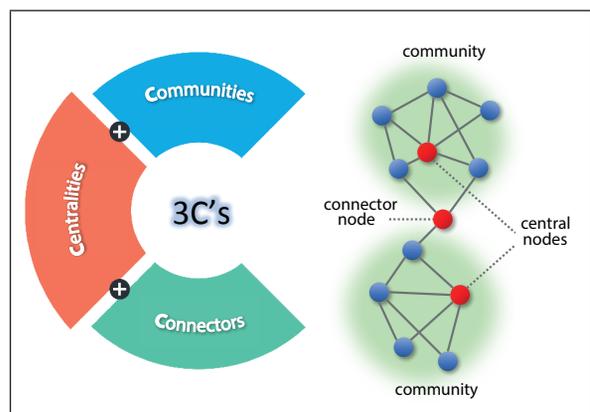
Figure 6

The three C's of network analysis. The most used "words" in the network language correspond both to mathematical patterns (community, centrality, connector) and biological concepts (co-operation, influence, mediator).

or "self-organization" within the network. It allows the classification of nodes, based on their "importance" with respect to their module. For example, we can highlight nodes that are totally embedded within their module from those which lies on the frontier between modules, which may act as "connectors" between them and, as such, play a key role both in holding them together and in the dynamics of spreading "information" throughout the network. Indeed, many tools to identify and study the biological/medical significance of modules are available in the literature, the most widely used are the weighted gene co-expression network analysis (WGC-NA) [48] and the switch miner algorithm (SWIM) [49]. There is a long list of biological and clinical applications of the "module" identification, for example, WGCNA has been applied to hepatocellular carcinoma [50], calcific aortic valve disease [51], cervical cancer [52], and pulmonary artery hypertension [53], just to cite a few. SWIM algorithm has shown key modules (the so-called "switch genes") in drug response [54], miRNA cancer networks [55], glioblastoma stem cells [56], chronic obstructive pulmonary disease [57], breast cancer [58], cancer-miRNA networks [55] and disease/genes associations [59].

Centralities. A fundamental question when studying networks (both at the whole network-level or community-level) is to find candidate nodes for being the "most influential" of the whole network or of the community it belongs to. In the network science language, they are referred to as "central nodes", whilst the "mapped" biological concept can be that of a "key driver", "critical", "switch" gene or mutation, a drug "target" or a "lethal" protein, depending on the context. Therefore, measures of "centrality" summarize a node's involvement in or

contribution to the cohesiveness of the network [60]. Most importantly, centrality values depend solely on the network topology, i.e., on its structure defined by how links and nodes are set up to relate to each other. A commonly used description of a node's centrality is based on three main properties: its connectedness, its role as a mediator, and its closeness to other nodes [61]. The first property may be metaphorically projected onto an interconnected social group exchanging messages (information), thus corresponding to its degree of potential communication activity, the second may be viewed as the potential to control such activity, and the third its efficiency in passing messages to all other nodes [62].

Degree centrality. One of the most popular ways to characterize node importance in terms of its connectedness is to compute its "degree centrality", i.e., the number of connections it has to other nodes (see *Figure 7c*). The underlying idea is clear and simple: degree centrality is a measure of importance based on the number of connections, the more the better. A typical metaphorical interpretation is that of an individual with many friends in a social network or that of an airport with many flights. A mapping between network properties and biological concepts is the well-known "lethality-centrality" correspondence in protein networks [63-65]. The underlying idea is that a single protein, although working as a catalyst or signaling molecule, or building block in a cell, also have a role defined by the network of interactions with other proteins (or DNA/RNA) in which it has a cellular function within functional modules [40]. By studying the *Saccharomyces cerevisiae* PPI, Jeong *et al.* [63] found that the phenotypic consequences of single gene deletion are affected by the number of interactions of its protein product, i.e., by its degree centrality in the PPI
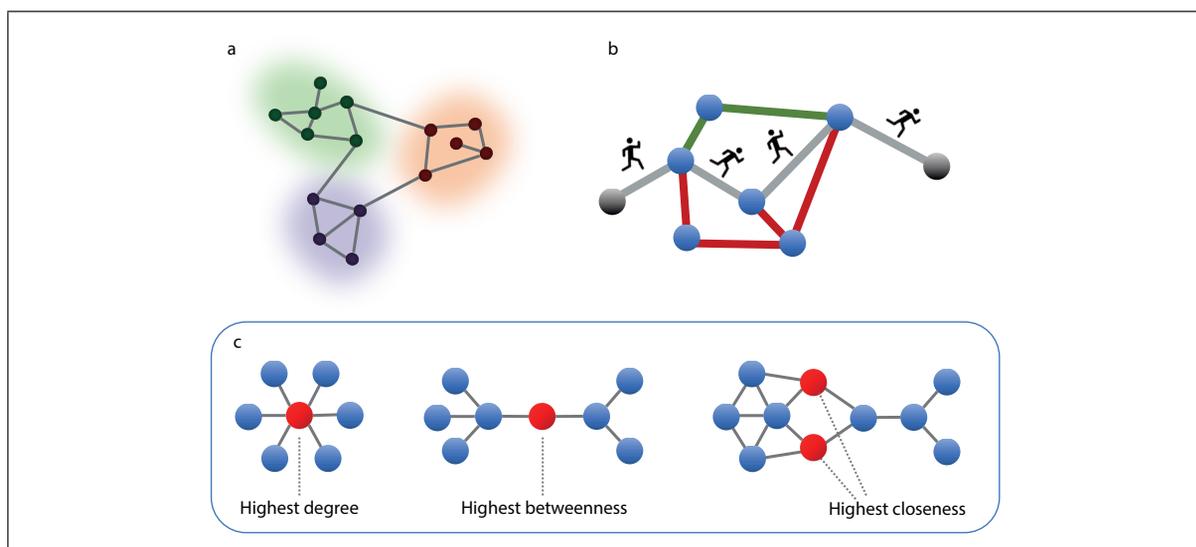
Figure 7
Network patterns. a) Network communities. Apart from computational aspects, modular networks reflect the inherent modularity of living systems and the network language provides a straightforward mapping from a biological concept (cooperating sub-units for a specific purpose or function) and topological properties and, the latter can be translated in an algorithm on data. b) Distance and shortest path(s) between two nodes in a network. The distance between source and target nodes is 4 (minimum number of hops) but there are two shortest paths with the same number of "hops" (green detour). c) Network centrality measures. Three ways to characterize "importance" from a network perspective (degree, betweenness, closeness) and from a biological perspective (e.g., lethality, integrity of functional cordination, effective information flow).

network. They showed that nodes degree is very inhomogeneously distributed with most proteins having few connections and a few of them being highly connected. The latter, it is argued, play a central role in mediating interactions within the global network. Highly connected proteins (called "hubs") are three times more likely to be essential than proteins with only a small number of links to other proteins [63]. The biological interpretation of hubs in the PPI network is still debated [66], but to our purposes, it is important to realize how the network language can function as a mapmaker between "computation on data" (degree of the PPI network) and "biological concept" (essentiality of a protein). The biological significance of degree centrality is witnessed by several publications identifying hubs as a critical feature of the interactome for disease as in diabetes mellitus [67], nephropathies [68], allergic response [69] or cancers [70-73]. Also, other types of networks have shown the relevance of degree centrality as in the gene-interaction network [74-76], miRNA-target gene networks [77], RNA-RNA networks [78], developmental regulatory networks [79], co-expression networks [49, 80, 81] or in the human brain network [82, 83].

Distance and shortest path. Other popular measures of importance are the so-called "betweenness" and "closeness" centralities. Before we discuss the mapping provided by these network characterizations, we preliminary need to define the concept of "shortest path" and "distance" between two nodes in a network (see *Figure 7b*). Given a set of nodes and links connecting them (i.e., a network) the "distance" between two nodes is usually defined as the minimum number of "hops" (links) needed to move from the source node to the target node and the corresponding path(s) are termed "shortest paths". Even if multiple shortest paths can be identified on the network from source to target nodes, the minimum number of links is a single value. An example of the mapping of the concept of "shortest path" on a biological problem, is that of finding repurposable drugs. The best candidate drug for a given set of proteins associated with a given disease, is that "close", i.e., having the smallest shortest paths, to its targets [84]. Now we can define the concept of "betweenness and closeness centrality" of a node in a network.

Betweenness centrality. A popular measure of "importance" of a node in terms of its ability as a "mediator", is called "betweenness centrality". It depends on its ability to allow nodes to reach other nodes, i.e., the extent to which a node lies between other nodes which depend on it [62]. Its formal definition coincides with the sum of the fractions of shortest paths passing through it (they may be more than one as previously shown) for all pairs of nodes [62]. A metaphorical projection on the concept of "information flow" is that a node with a high betweenness is potentially able to control such flow, that is it can facilitate, impede, or bias the "transmission" of "messages" [85] or, more generally, "information". Interestingly, using PPI data, Samokhin *et al.* [86] identified NEDD9 as a critical node in the phenotype transition from adaptive to pathogenic fibrosis using betweenness centrality, Joy *et al.* [87] found that proteins with high betweenness are more likely to be

essential and that evolutionary age of proteins is positively correlated with betweenness and Duron *et al.* [88] showed results indicating robustness of betweenness centrality in the identification of target genes for drug development. Using mammalian transcription networks, Potapov *et al.* [89] showed that the top list of genes displaying high degree and high betweenness, such as P53, C-FOS, C-JUN, and C-MYC, is enriched with genes that are known as having tumor-suppressor or proto-oncogene properties.

Closeness centrality. The last example to show the use of the most popular measures of importance of a node, we consider now the so-called "closeness centrality". The terminology makes it clear that it provides information about the property of a node to be "close" to all other nodes, i.e. to be at the center of a network. The formal definition consists of two steps: first, the sum of its distances to other nodes is computed and, second, its value is defined by the inverse of such a value. In this way, high closeness values correspond to nodes that are close to all others, and the smaller the total distance of a node to other nodes, the higher its closeness is. Using the already mentioned metaphorical projection of a network as a web of "information flow", a node with a high closeness may be considered important since information can rapidly spread to all other nodes very quickly. In biological terms, one may think, for example, of a protein-protein interaction network where a misfolded protein may produce a perturbation that can produce some effect (e.g., by decreasing or increasing the binding strength) to its interaction partners and so on, thus resembling the situation in which a message rapidly spreads over a web of people starting from its "center". For example, Ozgur *et al.* [75], using closeness centrality in a gene interaction prostate cancer network, inferred the presence of 18 new potential disease genes and Amitai *et al.* [90], using a residue interaction network where amino acid residues are the nodes and their interactions with each other are the links, found that active site, ligand-binding and evolutionarily conserved residues, typically have high closeness values. Ma and Zeng [91] showed that nodes with a high closeness in a metabolic network belong to the central metabolism, namely the glycolysis and citric acid cycle pathway.

Clearly, many other centrality measures can be defined to characterize some topological property of a network. For example, we can mention barycenter, cluster rank, decay, diffusion degree, geodesic k-path, leverage, lobby, radiality, eccentricity, Kleinberg's authority scores, and Harary graph, just to cite a few [92]. The proliferation of centrality measures is not a problem, on the contrary, this is the normal and positive development of a language, where new words are "invented" every day thus increasing the vocabulary of the network language. We envisage the birth of a large dictionary of thousands of words that can be used as the building blocks for expressing biomedical properties in this new language. The situation resembles that of sign language for hearing and speech impaired people, where gestures are used to express concepts, feelings, and ideas. The key advantage of the network language is that, once the biologist/physician has found the way of expressing his/

her ideas on the medical problem of interest in terms of networks, then an algorithm on data can be readily obtained, thus avoiding the intermediate step of a unifying general theory of disease and network science.

The key issue here discussed is that the "network language" may be able to provide biological interpretations of such properties therefore drawing the attention of the experimentalist to specific nodes for further analysis.

Connectors. Connectors are nodes in the network that connect modules. This broad definition that needs to be precisely quantified – as discussed in the following – to set up an algorithm, is very interesting from a biological perspective. Indeed, communities of nodes (genes, proteins, etc.) are there because they cooperate for some purpose or function in a cell (a functional module). However, groups of cooperating entities cannot work in isolation, but they must be coordinated for proper global function. In other words, self-organization is required for an appropriate response to internal or external stimuli. The simplest way to map this biological feature on a network is to consider nodes through which different communities can "communicate", thus using the usual "information flow" metaphor. Such nodes are usually referred as "connectors", as shown in *Figure 6*. A very interesting application of this property is reported by Niss *et al.* [93] where the protein-protein interactome of dendritic cells has been studied. They found an intriguing group of 294 proteins each forming a "bow-tie" structure, that is a single protein connecting the majority of protein complexes. The latter are "communities" on the network, and such "knot" proteins at the center of the bow tie, act as connectors. Such proteins resulted to have fundamental biological properties, like multifunctional capabilities, enrichment in essential proteins, and wide expression in other cells and tissues [93].

Since connector nodes, at the community level, may not be connectors on a global scale, they do not have necessarily a high betweenness centrality value. Therefore, new formal definitions are needed and the most popular measures to characterize "connector nodes" have been provided by Guimerà and Amaral [94] and by Paci *et al.* [49]. To characterize connectors nodes in a modular network, Guimerà and Amaral [94] suggest considering two parameters: a measure of internal connectivity called "within-module degree" defined as the degree of a node by counting its links to members of the same community, and a measure of external connectivity called the "participation coefficient", which is defined in such a way that its values are close to one if its links are uniformly distributed among all the modules (or communities) and zero if all its links are within its own module. It is therefore clear, that connector nodes can be computationally identified as those having a low within-module degree and a high participation coefficient, as shown in *Figure 8*. Moreover, the figure makes it clear that four regions can be identified and the other three node's roles identified: local hubs, global hubs, and peripheral nodes.

Using the "clusterphobic coefficient" to measure external connectivity as in [49], instead of the participation coefficient, we can also identify on the co-expression network, a specific class of connectors called "switches" which has been shown to be associated with transitions of cell's state [54-57, 95].

CONCLUSIONS

There is a growing awareness of the large and increasing gap between two visions of life: that of the "life scientists", like biologists, physicians, molecular biologists, and so on, and that of the "number scientist" like physicists, engineers, mathematicians, and so on. Biologists' vision of life is centered on "concepts" like for example evolution, adaptation, development, speciation, purpose, which are not amenable of a rigorous and immediate mathematical formalization, whilst physicists/
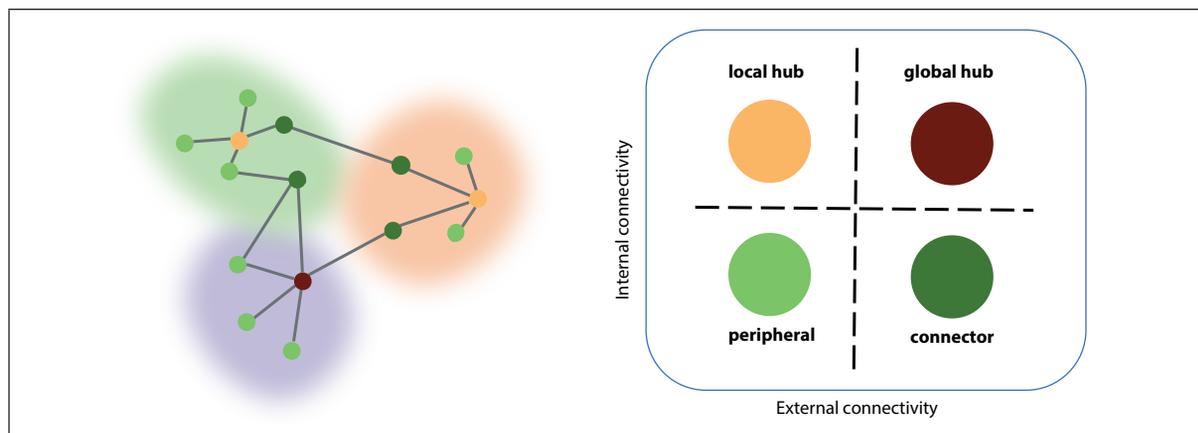

Figure 8
Connectors and other roles in modular networks. A "cartography" of a modular network can be constructed using measures of internal and external connectivity (degree). Given the modules, each node of the network corresponds to a point on the map and each quadrant corresponds to different roles. High external and internal connectivity (first quadrant) correspond to global hubs, low external and high internal connectivity (second quadrant) correspond to local hubs, low external and internal connectivity (third quadrant) correspond to peripheral nodes and high external and low internal connectivity (fourth quadrant) correspond to connectors.

data scientists' vision is centered on fitting "patterns" to data using "universal laws" or "universal organizing principles". The aim of the article is not to reconcile such visions or to take the side of one or the other, but to focus on the almost complete absence of dialogue and on how to stimulate effective interaction between the two in the field of precision medicine. An alternative view is suggested and a contribution to its solution is presented aiming to reduce the gap (that we called the "complex data divide") and allow a dialogue, by looking at network science as a vocabulary-generator filled with "words" that have different meanings in each discipline but refer to the same "thing" (cell behavior, health, disease, etc.). In this way, each researcher can continue to study his/her own discipline independently and, at the same time, engage in a true inter-disciplinary dialogue to implement precision medicine in a clinical setting.

Conflict of interest statement

The Author declares that there is no conflict of interest.

REFERENCES

1. Keller EF. A clash of two cultures. Nature. 2007;445(7128):603. doi: 10.1038/445603a
2. Mayr E. What makes biology unique? Considerations on the autonomy of a scientific discipline. Cambridge: Cambridge University Press; 2004. doi: 10.1017/CBO9780511617188
3. Keller EF. Making sense of life: explaining biological development with models, metaphors, and machines. Cambridge, Massachusetts; London, England: Harvard University Press; 2002. doi: 10.2307/j.ctvjz82ht
4. Steen LA. The science of patterns. Science. 1988;240(4852):611-6. doi: 10.1126/science.240.4852.611
5. Shmulevich I. Model selection in genomics. Environ Health Perspect. 2003;111(6):A328-9. doi: 10.1289/ehp.111-a328b
6. Gould SJ. Hen's teeth and horse's toes: Further reflections in natural history. New York: WW Norton & Company; 1984.
7. Uversky VN, Giuliani A. Networks of networks: An essay on multi-level biological organization. Front Genet. 2021;12:706260. doi: 10.3389/fgene.2021.706260
8. Hunter, D. J. Gene–environment interactions in human diseases. Nature Rev Genet. 6;287-98;2005. doi: 10.1038/nrg1578
9. The top 10 causes of death. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death.
10. Craig J. Complex diseases: Research and applications. Nature Educ. 1(1):184;2008.
11. Buchanan AV, Weiss KM, Fullerton SM. Dissecting complex disease: the quest for the Philosopher's Stone? Int J Epidemiol. 2006;35(3):562-71. doi: 10.1093/ije/dyl001
12. Greene JA, Loscalzo J. Putting the patient back together – social medicine, network medicine, and the limits of reductionism. N Engl J Med. 2017;377(25):2493-9. doi: 10.1056/NEJMms1706744
13. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74. doi: 10.1016/j.cell.2011.02.013
14. Tieri P, Farina L, Petti M, Astolfi L, Paci P, Castiglione F. Network inference and reconstruction in bioinformatics. Encyclopedia of bioinformatics and computational biology. Academic Press; 2019. p. 805-13, doi: 10.1016/B978-0-12-809633-8.20290-2
15. Goh KI, Cusick ME, Valle D, Childs B, Vidal M, Barabási AL. The human disease network. Proc Natl Acad Sci USA. 2007;104(21):8685-90. doi: 10.1073/pnas.0701361104
16. Pai S, Bader GD. Patient Similarity Networks for Precision Medicine. J Mol Biol. 2018;430(18 Pt A):2924-38. doi: 10.1016/j.jmb.2018.05.037
17. Silverman EK, Schmidt HHHW, Anastasiadou E, Altucci L, Angelini M, Badimon L, Balligand JL, Benincasa G, Capasso G, Conte F, Di Costanzo A, Farina L, Fiscon G, Gatto L, Gentili M, Loscalzo J, Marchese C, Napoli C, Paci P, Petti M, Quackenbush J, Tieri P, Viggiano D, Vilahur G, Glass K, Baumbach J. Molecular networks in network medicine: Development and applications. Wiley Interdiscip Rev Syst Biol Med. 2020;12(6):e1489. doi: 10.1002/wsbm.1489
18. Anderson PW. More is different. Science 1972;177(4047):393-6.
19. von Bertalanffy L. The theory of open systems in physics and biology. Science. 1950;111(2872):23-9. doi: 10.1126/science.111.2872.23
20. Boulding KE. General Systems Theory – The Skeleton of Science. Manag Sci. 1956;2(3):197-208. doi: 10.1287/mnsc.2.3.197
21. Manzoni C, Kia DA, Vandrovcova J, Hardy J, Wood NW, Lewis PA, Ferrari R. Genome, transcriptome and proteome: the rise of omics data and their integration in biomedical sciences. Brief Bioinform. 2018;19(2):286-302. doi: 10.1093/bib/bbw114
22. Kandpal R, Saviola B, Felton J. The era of omics unlimited. Biotechniques. 2009;46(5):351-2, 354-5. doi: 10.2144/000113137
23. D'Adamo GL, Widdop JT, Giles EM. The future is now? Clinical and translational aspects of "Omics" technologies. Immunol Cell Biol. 2021 Feb;99(2):168-76. doi: 10.1111/imcb.12404
24. Malloy DC, Martin R, Hadjistavropoulos T, Liu P, McCarthy EF, Park I, Shalani N, Murakami M, Paholpak S. Discourse on medicine: meditative and calculative approaches to ethics from an international perspective. Philos Ethics Humanit Med. 2014;9:18. doi: 10.1186/1747-5341-9-18
25. Collins FS. Shattuck lecture – medical and societal consequences of the Human Genome Project. N Engl J Med. 1999;341(1):28-37.

26. Joyner MJ, Paneth N. Promises, promises, and precision medicine. J Clin Invest. 2019;129(3):946-8. doi: 10.1172/JCI126119

27. Antebi YE, Linton JM, Klumpe H, Bintu B, Gong M, Su C, McCardell R, Elowitz MB. Combinatorial signal perception in the BMP Pathway. Cell. 2017;170(6):1184-1196.e24. doi: 10.1016/j.cell.2017.08.015

28. Anderson C. The end of theory: The data deluge makes the scientific method obsolete. Wired. Jun 23; 2008.

29. Pigliucci M. The end of theory in science? EMBO Rep. 2009;10(6):534. doi: 10.1038/embor.2009.111

30. Kilgore C. Rhetoric of the network: toward a new metaphor. Mosaic: a journal for the interdisciplinary study of literature. 2013;46:37-58. doi: 10.1353/mos.2013.0044

31. Palumbo MC, et al. Networks everywhere? Some general implications of an emergent metaphor. Curr Bioinformatics 1 (2006): 219-234.

32. Fauconnier G, Turner M. The way we think: Conceptual blending and the mind's hidden complexities. Basic Books; 2002.

33. Bachelard G, The formation of the scientific mind,: a contribution to a psychoanalysis of objective knowledge. Clinamen Press; 2002.

34. Degani A. A tale of two maps: Analysis of the London underground. Diagram. Ergonomics in Design. 2013;21(3):7-16. doi: 10.1177/1064804613489125

35. Nurse P. Systems biology: understanding cells. Nature. 2003;424(6951):883. doi: 10.1038/424883a

36. Brenner S. Sequences and consequences. Philos Trans R Soc Lond B Biol Sci. 2010;365(1537):207-12. doi: 10.1098/rstb.2009.0221

37. Barabási AL, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. Nat Rev Genet. 2011;12(1):56-68. doi:10.1038/nrg2918

38. Lee LY, Loscalzo J. Network medicine in pathobiology. Am J Pathol. 2019;189(7):1311-26. doi: 10.1016/j.ajpath.2019.03.009

39. Santo Fortunato, Darko Hric, Community detection in networks: A user guide. Phys Rep. 2016;659:1-44.

40. Hartwell LH, Hopfield JJ, Leibler S, Murray AW. From molecular to modular cell biology. Nature. 1999;402(6761 Suppl):C47-52. doi: 10.1038/35011540

41. Mitra K, Carvunis AR, Ramesh SK, Ideker T. Integrative approaches for finding modular structure in biological networks. Nat Rev Genet. 2013;14(10):719-32. doi: 10.1038/nrg3552

42. Wagner GP, Pavlicev M, Cheverud JM. The road to modularity. Nat Rev Genet. 2007;8(12):921-31. doi: 10.1038/nrg2267

43. Hintze A, Adami C. Evolution of complex modular biological networks. PLoS Comput Biol. 2008;4(2):e23. doi: 10.1371/journal.pcbi.0040023

44. Fraser HB. Coevolution, modularity and human disease. Curr Opin Genet Dev. 2006;16(6):637-44. doi: 10.1016/j.gde.2006.09.001

45. Newman ME. Modularity and community structure in networks. Proc Natl Acad Sci USA. 2006;103(23):8577-82. doi: 10.1073/pnas.0601602103

46. Newman, M. (2011). Communities, modules and large-scale structure in networks. Nature Physics. 2011; 8:25-31. doi: 10.1038/nphys2162

47. Blondel VD, Guillaume J-L, Lambiotte R, Lefebvre, E. Fast unfolding of communities in large networks. Journal of Statistical Mechanics: Theory and Experiment. 2008, P10008.

48. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008;9:559. doi: 10.1186/1471-2105-9-559

49. Paci P, Colombo T, Fiscon G, Gurtner A, Pavesi G, Farina L. SWIM: a computational tool to unveiling crucial nodes in complex biological networks. Sci Rep. 2017;7:44797. doi: 10.1038/srep44797. Erratum in: Sci Rep. 2017;7:46843.

50. Li L, Xia S, Shi X, Chen X, Shang D. The novel immune-related genes predict the prognosis of patients with hepatocellular carcinoma. Sci Rep. 2021;11(1):10728. doi: 10.1038/s41598-021-89747-7

51. Sun JY, Hua Y, Shen H, Qu Q, Kan JY, Kong XQ, Sun W, Shen YY. Identification of key genes in calcific aortic valve disease via weighted gene co-expression network analysis. BMC Med Genom. 2021;14(1):135. doi: 10.1186/s12920-021-00989-w

52. Deng ZM, Dai FF, Zhou Q, Cheng YX. Hsa_circ_0000301 facilitates the progression of cervical cancer by targeting miR-1228-3p/IRF4 Axis. BMC Cancer. 2021;21(1):583. doi: 10.1186/s12885-021-08331-4.

53. Bai Z, Xu L, Dai Y, Yuan Q, Zhou Z. ECM2 and GLT8D2 in human pulmonary artery hypertension: fruits from weighted gene co-expression network analysis. J Thorac Dis. 2021;13(4):2242-54. doi: 10.21037/jtd-20-3069

54. Falcone R, Conte F, Fiscon G, Pecce V, Sponziello M, Durante C, Farina L, Filetti S, Paci P, Verrienti A. BRAFV600E-mutant cancers display a variety of networks by SWIM analysis: prediction of vemurafenib clinical response. Endocrine. 2019;64(2):406-413. doi: 10.1007/s12020-019-01890-4

55. Fiscon G, Conte F, Farina L, Pellegrini M, Russo F, Paci P. Identification of disease-miRNA networks across different cancer types using SWIM. Methods Mol Biol. 2019;1970:169-81. doi: 10.1007/978-1-4939-9207-2_10

56. Fiscon G, Conte F, Paci P. SWIM tool application to expression data of glioblastoma stem-like cell lines, corresponding primary tumors and conventional glioma cell lines. BMC Bioinformatics. 2018;19(Suppl. 15):436. doi: 10.1186/s12859-018-2421-x

57. Paci P, Fiscon G, Conte F, Licursi V, Morrow J, Hersh C, Cho M, Castaldi P, Glass K, Silverman EK, Farina L. Integrated transcriptomic correlation network analysis identifies COPD molecular determinants. Sci Rep. 2020;10(1):3361. doi: 10.1038/s41598-020-60228-7

58. Fiscon G, Pegoraro S, Conte F, Manfioletti G, Paci P. Gene network analysis using SWIM reveals interplay between the transcription factor-encoding genes HMGA1, FOXM1, and MYBL2 in triple-negative breast cancer. FEBS Lett. 2021; Apr 9. doi: 10.1002/1873-3468.14085. Epub ahead of print.

59. Paci P, Fiscon G, Conte F, Wang RS, Farina L, Loscalzo J. Gene co-expression in the interactome: moving from correlation toward causation via an integrated approach to disease module discovery. NPJ Syst Biol Appl. 2021;7(1):3. doi: 10.1038/s41540-020-00168-0

60. Borgatti SP, Everett MG. A graph-theoretic perspective on centrality. Soc Netw. 2006;8:466-84.

61. Freeman LC. Centrality in networks: I. Conceptual clarifications. Soc Netw. 1979;1:215-39.

62. Zweig KA. Network analysis literacy. Lecture Notes in Social Networks, springer, Vienna, 2016

63. Jeong H, Mason SP, Barabási AL, Oltvai ZN. Lethality and centrality in protein networks. Nature. 2001;411(6833):41-2. doi: 10.1038/35075138

64. Hahn MW, Kern AD. Comparative genomics of centrality and essentiality in three eukaryotic protein-interaction networks. Mol Biol Evol. 2005;22(4):803-6. doi: 10.1093/molbev/msi072

65. Joyce AR, Palsson BØ. Predicting gene essentiality using genome-scale in silico models. Meth Mol Biol.

2008;416:433-57. doi: 10.1007/978-1-59745-321-9_30

66. Goymer P. Network biology: why do we need hubs? Nat Rev Genet. 2008;9(9):650. doi: 10.1038/nrg2450.

67. Ambedkar C, Reddi KK, Muppalaneni NB, Kalyani D. Application of centrality measures in the identification of critical genes in diabetes mellitus. Bioinformation. 2015;11(2):90-5. doi: 10.6026/97320630011090

68. Abedi M, Gheisari Y. Nodes with high centrality in protein interaction networks are responsible for driving signaling pathways in diabetic nephropathy. Peer J. 2015;3:e1284. doi: 10.7717/peerj.1284

69. Lu X, Jain VV, Finn PW, Perkins DL. Hubs in biological interaction networks exhibit low changes in expression in experimental asthma. Mol Syst Biol. 2007;3:98. doi: 10.1038/msb4100138

70. Jonsson PF, Bates PA. Global topological features of cancer proteins in the human interactome. Bioinformatics. 2006;22(18):2291-7. doi: 10.1093/bioinformatics/btl390

71. Li Z, Ivanov AA, Su R, Gonzalez-Pecchi V, Qi Q, Liu S, Webber P, McMillan E, Rusnak L, Pham C, Chen X, Mo X, Revennaugh B, Zhou W, Marcus A, Harati S, Chen X, Johns MA, White MA, Moreno C, Cooper LA, Du Y, Khuri FR, Fu H. The OncoPPi network of cancer-focused protein-protein interactions to inform biological insights and therapeutic strategies. Nat Commun. 2017;8:14356. doi: 10.1038/ncomms14356. Erratum in: Nat Commun. 2017;8:15350.

72. Sun J, Zhao Z. A comparative study of cancer proteins in the human protein-protein interaction network. BMC Genomics. 2010;11(Suppl 3):S5. doi: 10.1186/1471-2164-11-S3-S5

73. [82. van den Heuvel MP, Sporns O. Network hubs in the human brain. Trends Cogn Sci. 2013;17(12):683-96. doi: 10.1016/j.tics.2013.09.012

74. Lehner B, Crombie C, Tischler J, Fortunato A, Fraser AG. Systematic mapping of genetic interactions in *Caenorhabditis elegans* identifies common modifiers of diverse signaling pathways. Nat Genet. 2006;38(8):896-903. doi: 10.1038/ng1844

75. Ozgür A, Vu T, Erkan G, Radev DR. Identifying gene-disease associations using centrality on a literature mined gene-interaction network. Bioinformatics. 2008;24(13):i277-85. doi: 10.1093/bioinformatics/btn182

76. Yu D, Lim J, Wang X, Liang F, Xiao G. Enhanced construction of gene regulatory networks using hub gene information. BMC Bioinformatics. 2017;18(1):186. doi: 10.1186/s12859-017-1576-1

77. Mukherjee S, Akbar I, Bhagat R, Hazra B, Bhattacharyya A, Seth P, Roy D, Basu A. Identification and classification of hubs in microRNA target gene networks in human neural stem/progenitor cells following Japanese Encephalitis Virus Infection. mSphere. 2019;4(5):e00588-19. doi: 10.1128/mSphere.00588-19

78. Banerjee S, Kalyani Yabalooru SR, Karunagaran D. Identification of mRNA and non-coding RNA hubs using network analysis in organ tropism regulated triple negative breast cancer metastasis. Comput Biol Med. 2020;127:104076. doi: 10.1016/j.compbiomed.2020.104076

79. Seo CH, Kim JR, Kim MS, Cho KH. Hub genes with positive feedbacks function as master switches in developmental gene regulatory networks. Bioinformatics. 2009;25(15):1898-904. doi: 10.1093/bioinformatics/btp316

80. Liu Y, Gu HY, Zhu J, Niu YM, Zhang C, Guo GL. Identification of hub genes and key pathways associated with bipolar disorder based on weighted gene co-expression network analysis. Front Physiol. 2019;10:1081. doi: 10.3389/fphys.2019.01081

81. Zhu Z, Jin Z, Deng Y, Wei L, Yuan X, Zhang M, Sun D. Co-expression network analysis identifies four hub genes associated with prognosis in soft tissue sarcoma. Front Genet. 2019;10:37. doi: 10.3389/fgene.2019.00037

82. van den Heuvel MP, Sporns O. Network hubs in the human brain. Trends Cogn Sci. 2013;17(12):683-96. doi: 10.1016/j.tics.2013.09.012

83. Palva S. The importance of hubs in large-scale networks. Nat Hum Behav. 2018;2(10):724-5. doi: 10.1038/s41562-018-0438-9

84. Yildirim MA, Goh KI, Cusick ME, Barabási AL, Vidal M. Drug-target network. Nat Biotechnol. 2007;25(10):1119-26. doi: 10.1038/nbt1338

85. Freeman LC. A set of measures of centrality based upon betweenness. Sociometry. 1977;40:35-41

86. Samokhin AO, Stephens T, Wertheim BM, Wang RS, Vargas SO, Yung LM, Cao M, Brown M, Arons E, Dieffenbach PB, Fewell JG, Matar M, Bowman FP, Haley KJ, Alba GA, Marino SM, Kumar R, Rosas IO, Waxman AB, Oldham WM, Khanna D, Graham BB, Seo S, Gladyshev VN, Yu PB, Fredenburgh LE, Loscalzo J, Leopold JA, Maron BA. NEDD9 targets COL3A1 to promote endothelial fibrosis and pulmonary arterial hypertension. Sci Transl Med. 2018;10(445):eaap7294. doi: 10.1126/scitranslmed.aap7294

87. Joy MP, Brock A, Ingber DE, Huang S. High-betweenness proteins in the yeast protein interaction network. J Biomed Biotechnol. 2005;2005(2):96-103. doi: 10.1155/JBB.2005.96

88. Durón C, Pan Y, Gutmann DH, Hardin J, Radunskaya A. Variability of Betweenness Centrality and Its Effect on Identifying Essential Genes. Bull Math Biol. 2019;81(9):3655-73. doi: 10.1007/s11538-018-0526-z

89. Potapov AP, Voss N, Sasse N, Wingender E. Topology of mammalian transcription networks. Genome Inform. 2005;16(2):270-8.

90. Amitai G, Shemesh A, Sitbon E, Shklar M, Netanely D, Venger I, Pietrokovski S. Network analysis of protein structures identifies functional residues. J Mol Biol. 2004;344(4):1135-46. doi: 10.1016/j.jmb.2004.10.055

91. Ma HW, Zeng AP. The connectivity structure, giant strong component and centrality of metabolic networks. Bioinformatics. 2003;19(11):1423-30. doi: 10.1093/bioinformatics/btg177

92. Ashtiani M, Salehzadeh-Yazdi A, Razaghi-Moghadam Z, Hennig H, Wolkenhauer O, Mirzaie M, Jafari M. A systematic survey of centrality measures for protein-protein interaction networks. BMC Syst Biol. 2018;12(1):80. doi: 10.1186/s12918-018-0598-2

93. Niss K, Gomez-Casado C, Hjaltelin JX, Joeris T, Agace WW, Belling KG, Brunak S. Complete topological mapping of a cellular protein interactome reveals bow-tie motifs as ubiquitous connectors of protein complexes. Cell Rep. 2020;31(11):107763. doi: 10.1016/j.celrep.2020.107763

94. Guimerà R, Amaral LA. Cartography of complex networks: modules and universal roles. J Stat Mech. 2005;2005(P02001):nihpa35573. doi: 10.1088/1742-5468/2005/02/P02001

95. Fiscon G, Conte F, Farina L, Paci P. SAveRUNNER: A network-based algorithm for drug repurposing and its application to COVID-19. PLoS Comput Biol. 2021;17(2):e1008686. doi: 10.1371/journal.pcbi.1008686

Clinical practice guideline for the integrated management of major trauma by the Italian National Institute of Health: process and methods

Alice Josephine Fauci[1*], **Daniela Coclite**[1*], **Antonello Napoletano**[1*], **Daniela D'Angelo**[1], **Annalisa Biffi**[2], **Greta Castellini**[3], **Silvia Gianola**[3], **Laura Iacorossi**[1], **Roberto Latina**[1], **Gloria Porcu**[2], **Katia Salomone**[1], **Osvaldo Chiara**[4**], **Primiano Iannone**[1**] and the Italian National Institute of Health Guideline Working Group on Major Trauma[***]

[1]*Centro per l'Eccellenza Clinica, la Qualità e la Sicurezza delle Cure, Istituto Superiore di Sanità, Rome, Italy*
[2]*Dipartimento di Statistica e Metodi Quantitativi, Università degli Studi di Milano-Bicocca, Milan, Italy*
[3]*Unità di Epidemiologia, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy*
[4]*Chirurgia Generale e Traumatologia, ASST Grande Ospedale Metropolitano Niguarda, Università degli Studi di Milano, Milan, Italy*

*These Authors contributed equally as co-first authors
**These Authors contributed equally as co-last authors
***The members of the Italian National Institute of Health Guideline Working Group on Major Trauma are listed before the References

Abstract

Background. Major trauma describes serious injuries requiring lifesaving interventions or resulting in long-term disability; it represents about 8% of all deaths worldwide. Specific guidelines can help reduce deaths and disabilities, provided they adhere to high quality and trustworthiness standards. This article aims at introducing the development process of the Istituto Superiore di Sanità, ISS (Italian National Institute of Health) guideline for major trauma integrated management.
Methods. We applied the ISS methodological standards including the GRADE-ADOLOPMENT approach for adoption, adaptation, and *de novo* development of trustworthy guidelines.
Results. The scope was formulated by the multidisciplinary panel with stakeholders' involvement; two guidelines were identified as appropriate sources for adolopment. Forty questions from the two source guidelines were prioritised and five new ones formulated. New systematic reviews or updates were conducted for each clinical question, Evidence to Decision frameworks developed or re-assessed and the recommendations formulated after public consultations and external review. The policy on conflicts of interest was applied throughout the process.
Conclusions. Through a broad expertise representation, the early and wide stakeholders' participation, a continual process for disclosure and management of conflict of interests and the transparency of the process, ISS standards are proving to be an efficient model for developing trustworthy clinical guidance.

INTRODUCTION

Clinical Practice Guidelines (CPGs) are "statements that include recommendations, intended to optimize patient care, that are informed by a systematic review of evidence and an assessment of the benefits and harms of alternative care options" [1]. They represent the healthcare benchmark for health workers and patients and an essential tool for making health policy decisions [2].

In Italy, the Law n. 24/2017 [3] confers CPGs an important role in the field of medical liability and identifies

Address for correspondence: Daniela D'Angelo, Centro per l'Eccellenza Clinica, la Qualità e la Sicurezza delle Cure, Istituto Superiore di Sanità, Via Giano della Bella 34, 00162 Rome, Italy. E-mail: daniela.dangelo@iss.it.

the Istituto Superiore di Sanità (ISS, Italian National Institute of Health) as methodological guarantor of the CPGs produced. CPGs have to be released by the National Guidelines System (Sistema Nazionale Linee Guida - SNLG) after a thorough review of their methodological quality, according to ISS standards based on internationally recognized CPGs development process benchmarks [4-10].

Major trauma is defined as "an injury or a combination of injuries that are life-threatening and could be life changing because it may result in long-term disability" [11]. In 2019, more than 4 million people died as a result of injuries, representing about 8% of all deaths, and 11% of YLLs worldwide [12].

Globally, according to the Global Burden of Disease [13] road injuries ranked first in the 25-49-year age group in percentage of DALY (6.6, 5.6 to 7.7). Overall, major trauma will be the third leading cause of disability by 2030 [14]. In Italy, in 2017, falls and road injuries are among the top 25 causes of DALYs [15].

The Italian Integrated System for Major Trauma Assistance established in 2015 [16] is coherent with the international evidence on the best clinical organizational models; however, there are still many critical issues, such as (i) regional variability in mortality outcomes; (ii) regional availability of Trauma Centers and Trauma Units; (iii) lack of integration between the pre-hospital and hospital emergency system; (iv) inappropriate organizational management model for major trauma in many hospitals.

To overcome these challenges, since October 2019, the ISS has been developing a guideline on major trauma, on mandate of the Ministry of Health [17].

The goal of this article is to discuss the application of the methodological standards set by the ISS for developing CPG for the integrated management of major trauma. As the guideline development is still ongoing, we focus here on how the process was applied, from the establishment of guideline development group to recommendations' formulation. The final aim is to highlight the challenges and strengths of this guideline development process, with special reference to the application of the GRADE-ADOLOPMENT approach that allows for adopting or adapting existing high-quality guidelines. This is particularly important for the Italian context where there is an urgent need of an appropriate body of trustworthy clinical guidelines in the SNLG on priority health issues, given the role of CPGs in the field of medical liability.

We will report and discuss the specific recommendations after completion of the whole guideline, in a separate paper.

METHODS

The ISS guideline on major trauma is developed according to the SNLG standards, which include GRADE-ADOLOPMENT approach for adoption, adaptation, and *de novo* development of trustworthy guidelines [18-21]. *Figure 1* summarizes the main steps of the development process as defined by the ISS methodological manual [10], while a narrative description follows below.

The establishment of guideline development group

The guideline development group is composed by several teams who work collaboratively, supported by the scientific and technical-organizational secretariat, as well as the stakeholders participating in the process. Their roles and tasks are described below.

The *ISS Steering Committee* leads and oversees the whole guideline development process, from panel members' selection to Conflict of Interest (CoI) management and strategies for patient and public participation.

The involvement of the *expert of ethics* within the guideline development group ensures that the guideline recommendations are ethical and draw upon the principles outlined by the ISS Ethics Committee.

The *expert panel* contributes to the scope and clinical questions' formulation, critically evaluates the evidence, makes judgements on the Evidence-to-Decision (EtD) framework criteria, formulates recommendations, appraises stakeholders' and external referees' comments. The *chair* and *methodological co-chair* lead the works and guide the application of the GRADE EtD framework, from the critical assessment of the evidence to the formulation of recommendations.

After literature searching by documentalists, the *Evidence Review Team* (ERT) selects, summarizes and rates the certainty of evidence and prepares the EtD framework.

The *developers* are methodologists who act as a bridge between the panellists and the ERT.

The *External Reviewers* assess the draft recommendations content and methodology.

The Quality Assurance (QA) team ensures that the guideline development process complies with the ISS methodological standards.

Scoping

Scoping aims at defining the target population, the application context, thematic areas and the economic perspective.

For this guideline, scoping was conducted through different steps, from context analysis to target population and key topics' identification and stakeholders' involvement. Unlike other ISS guidelines [22], stakeholders' opinion was collected prior to drafting the scope through a scoping workshop aimed at discussing the main thematic areas; it followed the public consultation on the draft scope through the SNLG web platform (https://piattaformasnlg.iss.it) and the guideline scope finalization by the panel.

Patient and public participation

We ensured patient and public involvement through the inclusion of a lay member in the panel, the public consultations on the draft scope and draft recommendations, and through specific searches on patients' values and preferences.

Unlike the lay member, stakeholders represent interests common to a category or organization; individuals are encouraged to participate to the public consultation through stakeholders' organizations classified as scientific societies and health professions associations, associations of citizens, patients and caregivers, industry,
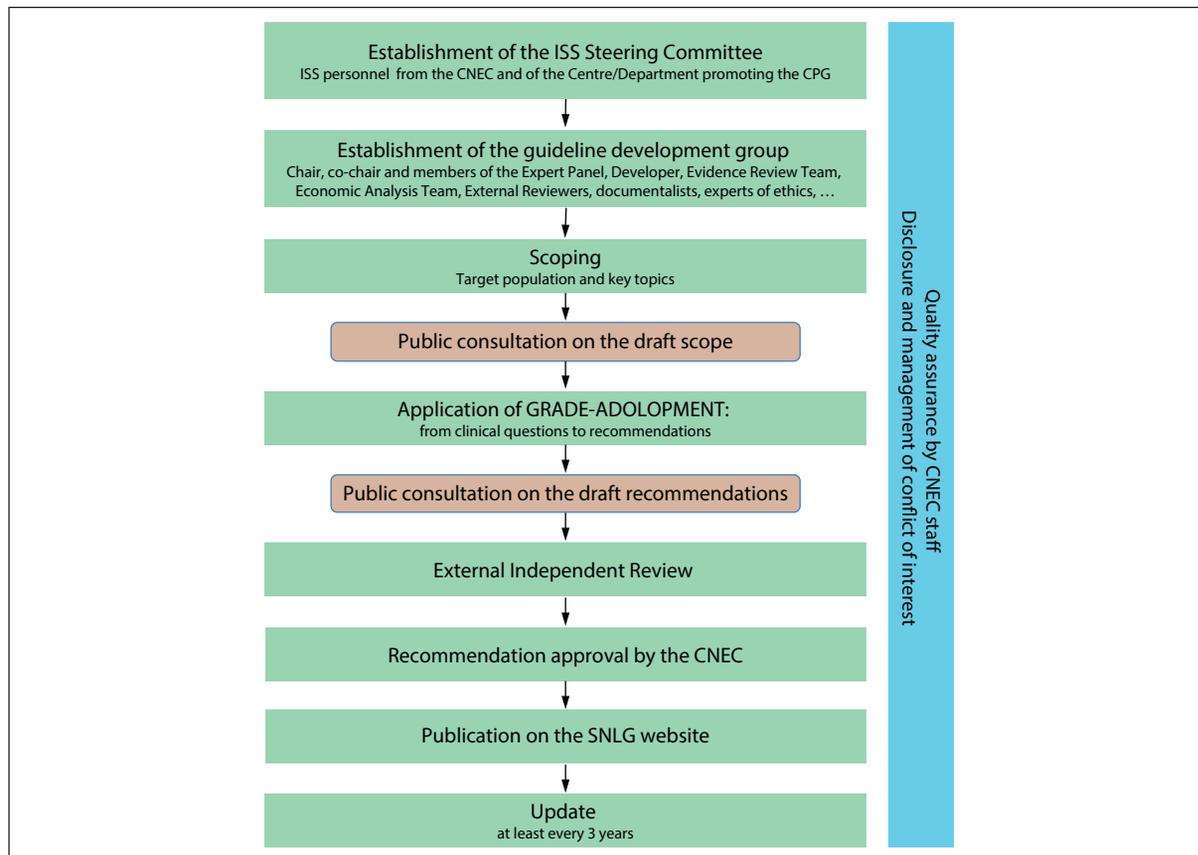
Figure 1
Guideline development process set by the Istituto Superiore di Sanità (ISS) (adapted from the methodological manual for ISS guidelines development).

national and regional public institutions, universities, public and private research institutes.

The ad hoc questionnaires used for the public consultations are reported in *Table 1*.

The GRADE-ADOLOPMENT approach

This approach combines advantages of adoption, adaptation and *de novo* guideline development and consist of the following main steps [21]:

1. identify trustworthy existing guidelines or evidence synthesis on the topic, while setting the guideline priorities, involving relevant stakeholders;

2. evaluate and complete GRADE EtD Frameworks for each recommendation by updating the systematic reviews as needed and by either developing new EtDs or reassessing existing EtD;

3. deciding on a final adoption, adaptation or *de novo* development of recommendations based on the extent of changes made or the availability of the recommendation itself in the source guideline.

Table 1
Questionnaires used for the public consultations on the draft scope and on draft recommendations

Public consultation on the draft scope
Open questions:
Does the draft scope consider aspects that are relevant to the target population of the guideline?
Does the draft scope consider key clinical topics?
Are the outcomes relevant and in adequate numbers?
Other comments on the scope

Public consultation on draft recommendations
Stakeholders were asked to express their degree of agreement/disagreement for each of the 5 statements, using a scale of 1 to 5 in which each response indicates respectively: (1) "in complete disagreement", (2) "disagreeing", (3) "uncertain", (4) "agree", (5) "completely agreed".
The recommendation is formulated in an understandable manner with regard to the intervention recommended to be used.
The recommendation is formulated in such a way that adherence to the recommendation is easy to document and measure.
The rating of the strength of recommendation is consistent with my knowledge and judgement of the supporting evidence
The rating of the certainty of evidence is consistent with my knowledge and judgement of the supporting evidence
Additional remarks provide useful information on how to implement the recommendation (if applicable).
Optional open question: "Please insert any comments here and include bibliographical references, where possible"

External review

Each draft recommendation and process documentation is submitted to two independent experts. By using the AGREE reporting checklist [23] and AGREE II [24, 25], they critically review the draft recommendations, suggest improvements, point out challenges for implementing recommendations, thus informing the guideline development and recommendations formulation.

Management of conflict of interest

Based on international standards [26, 27], the ISS policy on the management of CoI requires that all the subjects participating in the process have to declare all financial, non-financial, personal and institutional interests related to the guideline by completing a standardized CoI form (https://snlg.iss.it/wp-content/uploads/2021/02/Modulo-CdI-compilabile_feb2019.docx.), adapted from the WHO form [28]. Each declared interest is examined by the Steering Committee on the basis of its nature, type, specificity, financial value, period and duration, and then assigned a level of potential conflict, from minimal/trivial to relevant, and related actions to be taken from full participation, with public disclosure of the interest in the guideline document to a total exclusion.

RESULTS

The expert panel and external reviewers

ISS Steering Committee selected 14 panel experts, including a lay member, and two external reviewers, on the basis of their expertise, ensuring a balanced representation of relevant disciplines and health professions, as well as geographical provenience and healthcare setting *(Table 2)*.

During the inception meeting, panellists were trained on GRADE-ADOLOPMENT approach [18, 19, 21] for guideline development. Clear instructions about the disclosure and management of CoI were also given.

The guideline scope

Key-topics and target population were first identified by evaluating the evidence from existing guidelines

Table 2
Expertise of the expert panel and of the external reviewers

Role	Expertise	N.
Chair	Trauma surgeon	1
Co-chair	Emergency physician	1
Panel member	Anaesthetist	3
Panel member	Emergency physician	2
Panel member	Chief medical officer	1
Panel member	Orthopaedic traumatologist	2
Panel member	Trauma surgeon	1
Panel member	Interventional radiologist	1
Panel member	Clinical nurse	1
Panel member	Lay member	1
External reviewer	Orthopaedic traumatologist	1
External reviewer	Emergency physician	1

or evidence synthesis. The international CPGs were identified through a search via PubMed using "multiple trauma" and "trauma centers" as Mesh terms and the terms "trauma", "polytrauma", "multiple trauma", and "major trauma" as text words and using the filter "Guideline" as publication type.

In addition, the ECRI repository (https://guidelines.ecri.org/) for clinical guidelines was searched. CPGs were considered eligible if they were published after 2016 in English language, dealt with major/severe trauma, and met the guideline definition proposed by the IOM [1]. We excluded consensus conference, position statement, and any secondary publication of the guidelines. The searching process led to the identification of 6 potential CPGs *(see Supplementary Materials available online)*, among them the National Institute for Clinical Excellence (NICE) guidelines, NG39 [11] and NG40 [29] were selected as the highest quality guidelines, with an AGREE II score of 7 out of 7 [24, 25]. Moreover, the NICE guidelines allowed for covering both the clinical and organizational aspects of major trauma management in pre-hospital and hospital settings and had a detailed publicly available material (e.g., identifiable PICO elements, presence of full systematic reviews, accessible search strategy, and analysis method and evidence tables/summaries) for updating and GRADE ADOLOPMENT application.

A preliminary document with the main thematic areas thus identified, was discussed during a panel meeting and a subsequent scoping workshop with invited stakeholders. The draft scope was then finalized and ultimately commented through a public consultation on the SNLG web platform.

Trauma is a disease which starts at the time of accident, requires a support of vital functions, a timely diagnostic process, an emergency treatment of life-threatening conditions, a definitive correction of injuries and finally ends with rehabilitation process to restore function; thus, the final scope of the guideline, available at the SNLG website [17] and summarized by the infographic in *Figure 2*, is the integrated management of the condition, from the point of injury to definitive care, covering the clinical and organisational aspects of major trauma services in the Italian pre-hospital and hospital settings.

Stakeholders' involvement on the scoping phase

Nineteen out of 39 (55.8%) invited relevant stakeholders participated to the scoping workshop for discussing the main guideline thematic areas.

The draft scope formulated thereafter, was posted on the SNLG web platform for two weeks for public consultation. Fourteen scientific societies and health professions associations, registered on the web platform, submitted their comments using the standardized form shown in *Table 1*.

A total of 21 stakeholders participated both in the scoping workshop and public consultation, representing many scientific societies and health profession associations in different specialties: intensive care (n = 5); general and specialist surgery (n = 4); radiology (n = 3); forensic medicine (n=2) orthopaedics and traumatol-

ogy (n = 2); emergency medicine (n = 2) physiotherapy and rehabilitation (n=2); transfusion medicine and immunohematology (n = 1).

All the comments were discussed and responses included in a consultation report published on the SNLG website for transparency [17]. No comments were made on the target population; the key topics and relevant outcomes, instead, were revised according to the comments received.

Application of GRADE-ADOLOPMENT: from clinical questions to recommendations

The panel used a formal process to prioritise review questions from the two source guidelines NG39 [11] and NG40 [29] rating the priority of questions on a 9-point Likert scale, as follows: 7 to 9: high priority; 4 to 6: priority; 1 to 3: not a priority. As a result, 40 questions had a median value between 7 and 9 and were all included. Therefore, the panel selected the most urgent questions and those to be addressed afterwards, on the basis of considerations on uncertainty or variation in the clinical practice and new published evidence.

Following the ADOLOPMENT approach, the panel identified possibly matching recommendations of the prioritised questions on the basis of their credibility, update, acceptability and applicability to the national context and discussed whether those recommendations could be adapted, modified or developed *de novo*. Finally, the panel formulated five questions to be developed *de novo* for topics not addressed by the source guidelines.

The final list of 45 clinical questions approved by the panel addresses the following key-topics: pre-hospital triage, airway management, chest trauma assessment and management, haemorrhage assessment and management, monitoring, pain, heat loss, service organization, information, and support. These were grouped

into five macro areas, following the concept of the continuity of care for major trauma patients, from the scene to the hospital: pre-hospital triage, assessment and early management, assessment and definitive management, service organization, information, and support. The final list of the clinical questions is reported in the *Supplementary Materials* available online.

As for the outcomes to be addressed in the evidence synthesis, we maintained those considered in the PICOs (Population, Intervention, Comparison and Outcomes) of the source guidelines; for the new PICOs, the panellists listed and rated the relative importance of the outcomes on a 1 to 9 scale (7 to 9: critical for decision-making; 4 to 6: important; 1 to 3: low importance).

Since then, the ERT is updating the original systematic reviews or conducting new ones for each PICO and preparing draft EtD frameworks. Supplementary searches for evidence on acceptability, patients' values and preferences and economic analysis are being conducted to complete and contextualize the EtD frameworks. During a one-day panel meeting, on the basis of the EtDs judgements, the panel formulates recommendations through consensus or voting, when needed.

By January 2021, the experts' panel has adoloped 20 recommendations related to 10 clinical questions and published them on the SNLG website.

Stakeholders' involvement on the draft recommendations

Through public consultations, stakeholders were invited to provide their feedback on the draft recommendations by expressing their degree of agreement on 1 to 5 Likert scale, on five statements regarding recommendations' formulation, strength, rating of the certainty of evidence and implementation, through a
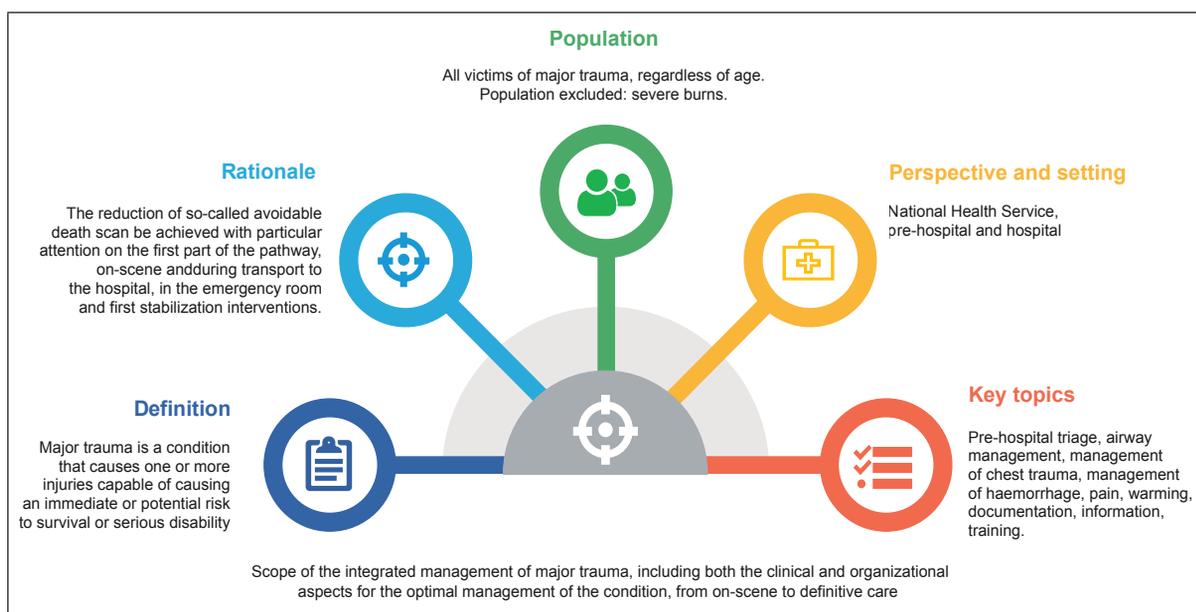
Figure 2
Scope of integrated management for major trauma clinical practice guideline by the Istituto Superiore di Sanità.

standardized form available on the SNLG platform for two-weeks *(Table 1)*. For the 9 public consultations on draft recommendations carried out so far, the average agreement score was 4, with a mean response rate of 44% (range 34-57) as reported on the SNLG website [17].

External review

The 20 draft recommendations were sent to the independent experts for the external review. The final recommendations are published on the SNLG website [17].

Management of conflict of interest

None of the declared interests deemed to represent a potentially relevant or relevant conflict related to the guideline scope, so all the candidate members participated to the panel's work. Similarly, no relevant CoIs related to specific PICOs were identified, so no experts were excluded during the recommendations' formulation. All disclosures of CoI will be publicly reported with the final guideline document.

DISCUSSION

The application of ISS standards for developing this guideline aimed at facing several challenges for ensuring a broad representation of expertise, the widest involvement and participation of all stakeholders and the maximum transparency of the process.

The panel composition sought to reflect the multidisciplinary approach that characterizes the trauma team which should include physicians expert in intensive care, emergency and trauma surgery, specially trained nurses and radiologists; in addition, it must provide for the possibility of having immediately available figures such as orthopaedist, neurosurgeon, radiology technician [30].

The SNLG web platform facilitates a transparent participative process by allowing the engagement of stakeholders at crucial stages. Their involvement through both the scoping workshop and public consultations is the very novel aspect of this guideline. This strategy was considered useful for drafting a well-focused scope and ensuring that guideline development is straightforward, easy to manage and relevant to end users [31]. The relatively low response rate to the public consultations may be due to stakeholders' limited knowledge on the guideline's highly specific topics; it nevertheless suggests that more efforts should be done to improve ISS strategies for patient and public involvement, though limited research is available for identifying strategies for successful engagement [31, 32]. Stakeholders' consultation complements the contribution of the lay panel member, and so does the external independent review that provides a further opportunity to obtain relevant and reliable inputs on both content and methodology [33, 34]. In this case, stakeholders and experts' involvement is particularly useful for ensuring that questions, evidence and recommendations are contextualised to the local needs, since specific searches for patients' values and preferences and cost effectiveness and resources use produced limited data [21].

Transparency is also guaranteed by the publication on the SNLG website of public notices on public consultations, of draft and final recommendations and attached documentation, and of consultation reports, as well as by the application of a comprehensive and continual process for the disclosure and management of CoI, which is a hallmark of a trustworthy clinical guidance [26, 27].

ISS policy on CoI conceptualizes disclosure of interest as distinct from identification and management of a CoI [35]. Indeed, the determination of a conflict is the result of a case-by-case assessment that considers the characteristics of the interest itself; only a complete and careful disclosure of all the interests allows this assessment and the adoption of appropriate measures to manage CoI in a transparent, proportional and consistent way.

The application of ISS policy on CoI is a major challenge since it requires a skill change to acknowledge that an interest does not necessarily represent a CoI and to recognize as interests to be declared professional activities or scientific production related to the guideline topic. Hence, experts need to be supported in identifying and declaring interests and encouraged to regularly review and update their declarations, as disclosure of interests is a continual process throughout the guideline development.

Last, the adoption of GRADE-ADOLOPMENT [18, 19, 21] provides panel members with a transparent and systematic approach for decision making by ensuring that all recommendations are based on the best available evidence and that all potentially important criteria are considered ; this makes guideline users aware of the rationale behind each judgment and recommendation, including the contextual factors that influence any modifications to the original recommendations. In our guideline, contextual issues such as needs, values and resources are considered as important elements for the applicability and transferability of the original recommendations.

Guideline adaptation provides an important alternative to *de novo* development by making the process more efficient, allowing to save resources and time and avoiding duplication [36]. We completed adolopment of 20 recommendations within less than one year, considering that the process has suffered a brief setback due to the Covid-19 pandemic. This is a relatively short time, compared to the time estimates of up to three years made by the NICE [9] or WHO [2] for a *de novo* guideline development. Finally, the adaptation of CPGs to the local setting is expected to improve their uptake and implementation [37].

Despite the advantage of saving resources, still the GRADE-ADOLOPMENT approach requires advanced methodological skills and further investment on methodological training [18, 21].

Preventing the participation of individuals to the public consultation, due to the lack of human resources to cope with huge amounts of comments, may be also considered a limitation, though balanced by the possibility for any individual to convey a point of view through any of the broad stakeholders' categories.

CONCLUSIONS

The development of the ISS guideline for major trauma integrated management follows a rigorous, systematic, and transparent process that allows for the application of the GRADE-methodology for adoption, adaptation and de novo development of trustworthy guidelines.

The possibility of adoloping existing high-quality guidelines is particularly important for the Italian context where there is an urgent need of trustworthy guidelines, given the role of CPGs in the field of medical liability, for reducing regional variability, providing the basis for the definition of local clinical pathways and optimizing citizens' health outcomes.

By considering this guideline development experience, the next steps for guideline development are to invest on advanced methodological training on the GRADE-ADOLOPMENT approach, to develop strategies for a major patient and public involvement and to support the experts in identifying and declaring interests, thus facilitating that key cultural change needed for developing trustworthy guidelines.

The Italian National Institute of Health Guideline Working Group on Major Trauma

Carlo Coniglio (Ospedale Maggiore, Bologna), Elvio De Blasio (Ospedaliera San Pio – G Rummo, Benevento), Andrea Fabbri (Azienda USL Romagna, Forlì), Gaddo Flego (Ospedale Evangelico Internazionale di Genova), Massimo Geraci (ARNAS Civico Di Cristina Benfratelli, Palermo), Giulio Maccauro (Università Cattolica del Sacro Cuore, Roma), Antonio Rampoldi (Ospedale Niguarda, Milano) Maria Pia Ruggieri (Azienda Ospedaliera San Giovanni Addolorata, Roma), Federico Santolini (San Martino – IST, Genova), Nino Stocchetti (Fondazione IRCCS Grande Ospedale Maggiore Policlinico, Milano), Claudio Tacconi (Ospedale Maggiore, Bologna), Gregorio Tugnoli (Azienda USL di Bologna, Bologna).

REFERENCES

1. Graham R, Mancher M, Miller Wolman D, Greenfield S, Steinberg E (Eds); Committee on Standards for Developing Trustworthy Clinical Practice Guidelines, Institute of Medicine. Clinical Practice Guidelines We Can Trust. Washington (DC): National Academies Press (US); 2011.
2. World Health Organization. WHO handbook for guideline development. WHO; 2014. Available at: https://apps.who.int/iris/handle/10665/145714
3. Italia. Legge Gelli-Bianco n. 24, 2017. Disposizioni in materia di sicurezza delle cure e della persona assistita, nonchè in materia di responsabilità professionale degli esercenti le professioni sanitarie. Gazzetta Ufficiale Serie Generale n. 64, 17 marzo 2017.
4. Iannone P, Coclite D, Fauci AJ, Graziano G, Napoletano AM. Le linee guida italiane secondo il nuovo SNLG: criticità e prospettive [Italian guidelines in accordance with the new National Guidelines System: critical issues and perspectives.]. Recenti Prog Med. 2017;108(9):360-2. Italian. doi: 10.1701/2745.27986
5. Iannone P, Coclite D, Napoletano A, Fauci A, Graziano G, Iacorossi L, D'Angelo D. The new National Guidelines System in Italy: a first evaluation. G Ital Nefrol. 2019;36(3).
6. Schünemann HJ, Wiercioch W, Etxeandia I, Falavigna M, Santesso N, Mustafa R, Ventresca, M, Brignardello-Petersen R, Laisaar KT, Kowalski S, Baldeh T, Zhang Y, Raid U, Neumann I, Norris SL, Thornton J, Harbour R, Treweek S, Guyatt G, Alonso-Coello P, Reinap M, Brozek J, Oxman A, Akl EA. Guidelines 2.0: systematic development of a comprehensive checklist for a successful guideline enterprise. Cmaj. 2014;186(3):E123-42. doi: 10.1503/cmaj.131237
7. Qaseem A, Forland F, Macbeth F, Ollenschläger G, Phillips S, van der Wees P. Guidelines International Network: toward international standards for clinical practice guidelines. Ann Intern Med. 2012;156(7):525-31. doi: 10.7326/0003-4819-156-7-201204030-00009
8. Guyatt GH, Oxman AD, Vist GE, Kunz R, Falck-Ytter Y, Alonso-Coello P, Schünemann HJ, GRADE working group. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. BMJ. 2008;336(7650):924-6. doi: 10.1136/bmj.39489.470347
9. National Institute for Health and Care Excellence. NICE Process and Methods Guides. Developing NICE Guidelines: The manual. London: NICE; 2015.
10. Iannone P, Coclite D, Napoletano A, Fauci A. Manuale

metodologico per la produzione di linee guida di pratica clinica: Centro Nazionale per l'Eccellenza Clinica, la Qualità e la Sicurezza delle Cure dell'Istituto Superiore di Sanità. Istituto Supriore di Sanità; 2019.

11. Kanani AN, Hartshorn S. NICE clinical guideline NG39: Major trauma: assessment and initial management. Arch Dis Child Educ Pract Ed. 2017;102(1):20-3. doi: 10.1136/archdischild-2016-310869

12. Kyu HH, Abate D, Abate KH. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392:1859-922.

13. GBD 2019 Diseases and Injuries Collaborators. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet. 2020;396(10258):1204-22. doi: 10.1016/S0140-6736(20)30925-9

14. Toroyan T, Peden MM, Iaych K. WHO launches second global status report on road safety. Inj Prev. 2013;19(2):150. doi: 10.1136/injuryprev-2013-040775

15. Collaborators GBDI. Italys health performance, 1990-2017: findings from the Global Burden of Disease Study 2017. Lancet Public Health. 2019;4(12):e645-e57. doi: 10.1016/S2468-2667(19)30189-6

16. Chieregato A, Volpi A, Gordini G, Ventura C, Barozzi M, Caspani MLR, Fabbri A, Ferrari AM, Ferri E, Giugni A, Marino M, Martino C, Pizzamiglio M, Ravaldini M, Russo E, Trabucco L, Trombetti S, De Palma R. How health service delivery guides the allocation of major trauma patients in the intensive care units of the inclusive (hub and spoke) trauma system of the Emilia Romagna Region (Italy). A cross-sectional study. BMJ Open. 2017;7(9):e016415. doi: 10.1136/bmjopen-2017-016415

17. SNLG. Linea guida sulla gestione integrata del trauma maggiore dalla scena dell'evento alla cura definitiva. SNLG; 2019. Available at: https://snlg.iss.it/?p=2533

18. Alonso-Coello P, Oxman AD, Moberg J, Brignardello-Petersen R, Akl EA, Davoli M, Treweek S, Mustafa RA, Vandvik PO, Meerpohl J, Guyatt GH, Schünemann HJ. GRADE Evidence to Decision (EtD) frameworks: a systematic and transparent approach to making well informed healthcare choices. 2: Clinical practice guidelines. Gaceta sanitaria. 2018;32(2):167.e1-.e10. doi: 10.1016/j.gaceta.2017.03.008

19. Alonso-Coello P, Schünemann HJ, Moberg J, Brignardello-Petersen R, Akl EA, Davoli M, Treweek S, Mustafa RA, Rada G, Rosenbaum S, Morelli A, Guyatt GH, Oxman AD.GRADE Evidence to Decision (EtD) frameworks: a systematic and transparent approach to making well informed healthcare choices. 1: Introduction. BMJ. 2016;353:i2016. doi: 10.1136/bmj.i2016

20. Andrews JC, Schünemann HJ, Oxman AD, Pottie K, Meerpohl JJ, Coello PA, Rind D, Montori VM, Brito JP, Norris S, Elbarbary M, Post P, Nasser M, Shukla V, Jaeschke R, Brozek J, Djulbegovic B, Guyatt G. GRADE guidelines: 15. Going from evidence to recommendation-determinants of a recommendation's direction and strength. J Clin Epidemiol. 2013;66(7):726-35. doi: 10.1016/j.jclinepi.2013.02.003

21. Schünemann HJ, Wiercioch W, Brozek J, Etxeandia-Ikobaltzeta I, Mustafa RA, Manja V, Brignardello-Petersen R, Neumann I, Falavigna M, Alhazzani W, Santesso N, Zhang Y, Meerpohl JJ, Morgan RL, Rochwerg B, Darzi A, Rojas MX, Carrasco-Labra A, Adi Y, AlRayees Z, Riva J, Bollig C, Moore A, Yepes-Nuñez JJ, Cuello C,

Waziry R, Akl EA. GRADE Evidence to Decision (EtD) frameworks for adoption, adaptation, and de novo development of trustworthy recommendations: GRADE-ADOLOPMENT. J Clin Epidemiol. 2017;81:101-10. doi: 10.1016/j.jclinepi.2016.09.009

22. Morgano GP, Fulceri F, Nardocci F, Barbui C, Ostuzzi G, Papola D, Fatta LM, Fauci AJ, Coclite D, Napoletano A, De Crescenzo F, D'Alò GL, Amato L, Cinquini M, Iannone P, Schünemann HJ, Scattoni ML; Italian National Institute of Health guideline working group on Autism Spectrum Disorder. Introduction and methods of the evidence-based guidelines for the diagnosis and management of autism spectrum disorder by the Italian National Institute of Health. Health Qual Life Outcomes. 2020;18(1):81. doi: 10.1186/s12955-020-01320-4

23. Brouwers MC, Kerkvliet K, Spithoff K; AGREE Next Steps Consortium. The AGREE Reporting Checklist: a tool to improve reporting of clinical practice guidelines. BMJ. 2016;352:i1152. doi: 10.1136/bmj.i1152. Erratum in: BMJ. 2016;354:i4852.

24. Brouwers MC, Kho ME, Browman GP, Burgers JS, Cluzeau F, Feder G, Fervers B, Graham ID, Hanna SE, Makarski J; AGREE Next Steps Consortium. Development of the AGREE II, part 2: assessment of validity of items and tools to support application. CMAJ. 2010;182(10):E472-8. doi: 10.1503/cmaj.091716

25. Brouwers MC, Kho ME, Browman GP, Burgers JS, Cluzeau F, Feder G, Fervers B, Graham ID, Grimshaw J, Hanna SE, Littlejohns P, Makarski J, Zitzelsberger L; AGREE Next Steps Consortium. AGREE II: advancing guideline development, reporting, and evaluation in health care. Prev Med. 2010;51(5):421-4. doi: 10.1016/j.ypmed.2010.08.005

26. Schünemann HJ, Al-Ansary LA, Forland F, Kersten S, Komulainen J, Kopp IB, Macbeth F, Phillips SM, Robbins C, van der Wees P, Qaseem A; Board of Trustees of the Guidelines International Network. Guidelines International Network: Principles for disclosure of interests and management of conflicts in guidelines. Ann Intern Med. 2015;163(7):548-53. doi: 10.7326/M14-1885

27. Lo B, Field MJ (Eds); Institute of Medicine (US) Committee on Conflict of Interest in Medical Research, Education, and Practice. Conflict of interest in medical research, education, and practice. Washington (DC): National Academies Press (US); 2009.

28. World Health Organization. Declaration of interests for WHO experts – forms for submission.WHO; 2021.

29. National Clinical Guideline Centre (UK). Major trauma: Service delivery. London: National Institute for Health and Care Excellence (UK); 2016.

30. Tiel Groenestege-Kreb D, van Maarseveen O, Leenen L. Trauma team. Br J Anaesth. 2014;113(2):258-65. doi: 10.1093/bja/aeu236

31. Petkovic J, Riddle A, Akl EA, Khabsa J, Lytvyn L, Atwere P, Campbell P, Chalkidou K, Chang SM, Crowe S, Dans L, Jardali FE, Ghersi D, Graham ID, Grant S, Greer-Smith R, Guise JM, Hazlewood G, Jull J, Katikireddi SV, Langlois EV, Lyddiatt A, Maxwell L, Morley R, Mustafa RA, Nonino F, Pardo JP, Pollock A, Pottie K, Riva J, Schünemann H, Simeon R, Smith M, Stein AT, Synnot A, Tufte J, White H, Welch V, Concannon TW, Tugwell P. Protocol for the development of guidance for stakeholder engagement in health and healthcare guideline development and implementation. Syst Rev. 2020;9(1):21. doi: 10.1186/s13643-020-1272-5

32. Armstrong MJ, Mullins CD, Gronseth GS, Gagliardi AR. Recommendations for patient engagement in guideline development panels: A qualitative focus group study of

guideline-naïve patients. PloS one. 2017;12(3):e0174329. doi: 10.1371/journal.pone.0174329

33. Armstrong MJ, Gronseth GS, Gagliardi AR, Mullins CD. Participation and consultation engagement strategies have complementary roles: A case study of patient and public involvement in clinical practice guideline development. Health expectations. 2020;23(2):423-32. doi: 10.1111/hex.13018

34. Shekelle P, Woolf S, Grimshaw JM, Schünemann HJ, Eccles MP. Developing clinical practice guidelines: reviewing, reporting, and publishing guidelines; updating guidelines; and the emerging issues of enhancing guideline implementability and accounting for comorbid conditions in guideline development. Implement Sci. 2012;7:62. doi: 10.1186/1748-5908-7-62

35. Qaseem A, Wilt TJ. Disclosure of interests and management of conflicts of interest in clinical guidelines and guidance statements: Methods from the Clinical Guidelines Committee of the American College of Physicians. Ann Intern Med. 2019;171(5):354-61. doi: 10.7326/m18-3279

36. Burgers JS, Anzueto A, Black PN, Cruz AA, Fervers B, Graham ID, Metersky M, Woodhead M, Yawn BP; ATS/ERS ad hoc Committee on Integrating and Coordinating Efforts in COPD Guideline Development. Adaptation, evaluation, and updating of guidelines: article 14 in Integrating and coordinating efforts in COPD guideline development. An official ATS/ERS workshop report. Proc Am Thorac Soc. 2012;9(5):304-10. doi: 10.1513/pats.201208-067ST

37. Harrison MB, Legare F, Graham ID, Fervers B. Adapting clinical practice guidelines to local context and assessing barriers to their use. CMAJ. 2010;182(2):E78-84. doi: 10.1503/cmaj.081232

Book Reviews, Notes and Comments

Edited by
Federica Napolitani Cheyne
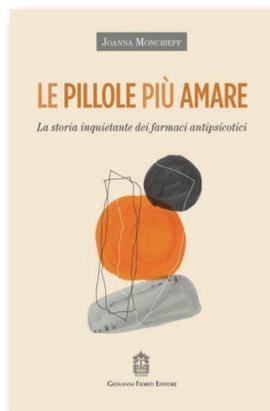
Joanna Moncrieff's essay, published in the Italian edition in 2020, is an accurate scientific investigation that can be read like a novel based on real facts: psychotropic drugs with their peculiar history and their use (and abuse). Although it is a pharmacology textbook, purposefully limited to brief descriptions of the characteristics and development of hypotheses about the action of some of the most prescribed psychotropic drugs (from haloperidol to clozapine and the more recent "atypical" ones), the work of the Author helps the reader reach an improved, broad awareness about prescribing (if the reader is a medical doctor) or recruitment (if the reader is a potential user).

There is an impalpable common thread that runs through the book: "Although there is a variation in the response of individuals to all drugs, psychoactive ones produce their characteristic range of effects in anyone who takes them, regardless of whether or not they have psychological problems. Most psychoactive drugs also have physical effects, and physical and mental effects are often inextricably linked" (p. 10).

The Author, page after page, makes us witness the sunset of the "drug's model of action based on the drug itself" (the drug is a chemical that alters the functioning of the brain and the entire body) and dawn of a "model based on the disease" (the drug acts on the biological processes that cause a disease). All the arguments are supported by compelling evidence about causes (including marketing actions and the absence of evidence in some cases) and clinical-cultural consequences: "The idea that psychiatric therapies, including medications, worked by inducing other diseases, was no longer an acceptable basis for treatment (...)" and "When textbooks began to present the disease-centered view, there was little recognition that there was an alternative explanation of how antipsychotics might work (...)" (p. 49).

The essay is also a historical investigation into the change of psychiatric approaches over time. Such a change has led to treatments that are applied on the basis of labels or diagnoses derived from Manuals ("The 'bipolar' epidemic began in the United States in the 90s, when some academics began to suggest that the disorder was poorly recognized (Ghaemi *et al.*, 1999)" (p. 188). Also, this change is precisely connected to the spread of the disease-centered model, which is well suited to approaches based on economic, rapid therapies, which, in some cases, are only on-the-surface grounded in real evidence: "No research has ever been conducted, or at least published, that has shown that Valproic Acid reduces mood variability and there remains no evidence that it modifies the biological basis of mood (...) Despite the evidence, its sales skyrocketed when the concept of mood instability and the idea that there was a specific treatment for it were introduced into mental health services (Ilyas and Moncrieff, 2012)" (p. 190).

The indirect protagonists are Mental Health Users, Psychiatrists, and Researchers – the various voices, through a "(...) critical debate on the huge volume of literature and marketing that describe these drugs as a godsend for humanity (...)" (p. 18), will gradually lead the reader to ask himself "(...) questions about the consequences of long-term treatment and why, sixty years after their introduction, we still have no certainty whether antipsychotics help or harm those who take them for a long time" (p. 18).

The importance of psychiatric drugs used in a critical and targeted way is not denied at all. On the contrary, the Author provides a well-round reflection on the need to maintain "humane" psychiatric care, by arguing against the logic of renewed, "total institutions" that embrace excessively agile and ready-to-use diagnostic packages associated with standardized psychopharmacological treatments: "It must be recognized that in many circumstances antipsychotics are not taken because the individual finds them useful but, in fact, because other people or society in general cannot tolerate this person's behavior". And, if it is not wrong "to change people's behavior if it is seriously antisocial, threatening or dangerous (...) yet as a society we must feel a sense of awareness and responsibility in trying to do so and be ready to think objectively about the methods we use to do so" (p. 215).

In its final analysis, the essay provides an indirect invitation for the reader to ponder on therapeutic relationship as reciprocity between its actors and on the fact that even the future of psychiatry cannot be separated from its biological, cultural, psychodynamic, phenomenological, social, and relational facets. Leveraging the dialogue between different disciplines and fusing the resulting knowledge as a single whole, it will be possible

to achieve the most effective and efficient "recovery" in Mental Health.

Psychic care aimed at alleviating mental suffering is certainly not a mere and stereotyped application of algorithms or rigid schemes to follow, rather, it is an action that focuses on the very life of the human being: care is a taking care. We must not be distracted by such core mission, whereby "although there is no evidence to suggest that early intervention is responsible for a better prognosis for individuals diagnosed with schizophrenia and psychosis (...) emotional advertising is meant to convince doctors that not starting antipsychotic treatment at the earliest possible opportunity leads people to an empty life and ruin. (...)". An advertisement for a Long Acting Injectable antypsychotic "read: 'prescribe early, because what he/she loses, could lose forever' (...)" (p. 179).

An exclusively quantitative model centered on illness aims to quell mental pain, making it deaf and invisible. As such, it might empty human existence and create humans devoid of pain and its meaning, prompting societies without pain, passive, and at risk of totalitarianism.

The essay does not definite solutions to the reader, but it is a successful solicitation to examine mental health also according to a drug-centered model. As such, it constitutes a valuable viewpoint on the deceptive equivalence between the person and her\his biology and an energizing basis to support an evidence-based, constructive, critical attitude that is mindful of the homologation of behaviors crushed by technicalities.

Extrapolating to the Italian Mental Health model, the reading of this very well translated volume places at the center of the scene those public services that, basing their operations on a bio-psycho-social model, are able to respond to the multiple variables of help demand to offer an effective response even, and above all, to serious mental disorders.

Based on the data released by the recent Report of the Ministry of Mental Health in Italy, the essay could advocate for the continuous implementation and strengthening of the network of public mental health services, which, equipped with suitable resources, must be increasingly able to intercept the youth psychic discomfort that represents a real emergency, lately exacerbated by the COVID-19 pandemic.

Alongside new resources, new forms of collaboration between the territorial mental health services and the universities could contribute to the progressive development of innovative training projects. By giving centrality to public mental health services, these projects could contribute to reducing gaps and promoting global health. Relocating the "person" at the center of the therapeutic scene is an act of defense of democracy and a memory of the future.

Emanuele Caroppo
Department of Mental Health -
Local Health Unit ASL Roma 2
emanuele.caroppo@aslroma2.it

HOTEL PENICILLINA
Storia di una grande fabbrica diventata rifugio per invisibili
Anna Ditta, Marco Passaro, Andrea Turchi
Con testi di Matteo Balduzzi, Luigi Cerruti, Mauro Palma
Formigine (MO): Infinito Edizioni; 2020. 255 p.
ISBN 9788868614416
€ 14,00

[The story of a big factory that became a shelter for the homeless]

This is a strange book. It is made of science, of local and global politics, of the past and the present, of large-scale economics and neighborhood solidarity. All these issues are concentrated in one (large) place: the abandoned penicillin factory created after WWII at the Eastern periphery of Rome. It was named Leo – after the Danish company Løvens that licensed the production process, and it was built between 1947 and 1950 by Giovanni Armenise, who acquired the license from another Italian company – Cisitalia, a former car-maker – that went bankrupt soon after the war. Penicillin, the miracle drug, was at the time an Anglo-American monopoly, except for the Danish pharmaceutical company: most of the antimicrobial drug was produced in the USA and UK, and other countries merely filled the vials. The first half of the book – written by Andrea Turchi (a chemist by training, with a passion for history and politics) – details the early history of penicillin in Italy. Drawing on published sources and archival material, the book outlines the trajectory of the molecule and the associated technology, together with the political history of Armenise who, with the help of Danish engineers and technicians, togheter with Italian scientists and politicians, managed to open the "fabbrica" in 1950, when it was inaugurated by the universal symbol of the new pharmaceutical era, sir Alexander Fleming.

To understand in full the history of the Leo, the reader should also remember the greater picture of the introduction of penicillin research and production in Italy. While Armenise was dealing with the Danes to obtain the patent and build the "fabbrica", the director of the Istituto Superiore di Sanità (just a few kilometers from the Leo plant) Domenico Marotta hired the biochemical mind behind the wonder drug: Ernst Boris Chain, the German-born British chemist awarded with the Nobel prize in 1945 for the discovery, together with Alexander Fleming and Howard Florey. For 15 years, the ISS became a global center of innovation in the biochemistry and engineering of antibiotics production, with a research laboratory, a pilot plant and a small factory (built with American aids) to supply hospitals and the Italian Army. Leo and the whole Italian pharmaceutical sector greatly benefitted from Marotta's vision of the ISS as a support to the development of Italian industry. Scientists and technicians from private com-

panies often turned to Chain and the ISS for help and advice, and international connections (for example, the ISS was a WHO reference center for antibiotics); while the ISS could count on the help of Leo and other companies when special and expensive needs arose. The physical proximity between Leo and ISS was obviously a bonus, with frequent exchanges between the private "fabbrica" and the public establishment.

The narrative of the first half of the book is multifold. Turchi mixes the detailed reconstruction of the techno-scientific history of the Leo (how the fermentation process was gradually perfected, the emergence of semi-synthetic penicillin, etc.) with the history of the area where the factory was built, and the history of the people working inside the factory. Over the years, Turchi developed several connections with former technicians, clerks and engineers of the Leo, and their voices are often heard in the book. They speak not only of the interaction of science, technology and industry, but they also remember an almost forgotten past of working-class pride and paternalistic capitalism. The factory was the core of the area, with hundreds of families living in the neighborhood relying on the Leo as the main source of income, so that the evolution of the company is mirrored by the evolution of the urban context. In the 1970s, the Armenise family leaves the company, and in 1985 the factory is sold to a global player, Smith Kline & French. However, the pharmaceutical market has completely changed, and despite some efforts for innovation, the large establishment is gradually dismantled: in 1987 penicillin production by fermentation is definitely halted, and the buildings are slowly but steadily abandoned as production decreases and scandals sweep Italian pharmaceutical market. The factory finally ended its operations in 2006, and every trace of activity disappears in 2008.

Here starts the second life of the factory, and the second part of the book, written by the journalist Anna Ditta. The immense building became a shelter for dozens of homeless, and some of its structures were predated for scrapping metals, glasses, and whatever could be of some use. The factory, in its decline, followed the gradual failure of industrial development of the area, so that a working-class neighborhood became an underclass district. The Leo factory became a house to many asylum-seekers, immigrants *sans papier*, or simply working poors who could not afford a rent. Once again, the fate of the factory is intertwined to Italian politics: according to the authors, the failure of restrictive immigration policies and the lagging economy led to the birth of large ghettos in hidden corners of most Italian cities, often ending with forced removal of people, as in the case of the Leo.

The book is thus interesting for several reasons: the reader interested in the history of science and medicine will appreciate the pharmaceutical development of an industry in the context of post-WWII Italy; the sociologically versed reader will find interesting insights about the connections between a factory and its urban context; a political reading is also possible, especially when the book deals with the workers' struggles in the 1960s and 1970s, and in its second part about contemporary Italian politics. The three authors (with Turchi and Ditta, Marco Passaro contributed to a chapter and shot the photographs documenting the lives of the people living in the "Hotel Penicillina") successfully turned the building of the Leo Penicillina and its trajectory in an interesting and stimulating case study relevant to several academic disciplines. Unfortunately, the factory is now a ghost haunting the surrounding area, a decaying monument that reveals more of the bleak present than of the great past.

Mauro Capocci
Dipartimento di Civiltà e Forme del Sapere
Università degli Studi di Pisa, Pisa, Italy
mauro.capocci@unipi.it

PICCOLI PICCOLI.
Storie di neonati nell'Italia di oggi
Mario De Curtis, Sarah Gangi
Roma: Laterza; 2021. 184 p.
€ 16,00

[Little little ones. Stories of newborns in Italy today]

Mario De Curtis and Sarah Gangi have been working together for a long time in the Neonatalogy and Neonatal Intensive Care Unit (NICU) of the Umberto I Polyclinic in Rome, Italy. The former is a neonatologist, who was head of the NICU and full professor of Pediatrics at the University of Rome La Sapienza; the latter is a psychologist and psychotherapist, in charge of the Psychology Service in the same structure. After years of work in such a delicate and complex environment, they decided to share with a wider audience both the passion needed to face its daily challenges and the lessons they have drawn, on a professional and human level. They did this mainly by writing two books: the first *Voglia di vivere. Storie di piccoli guerrieri*, published in 2015 by Hoepli, and the one in review here, *Piccoli Piccoli. Storie di neonati nell'Italia di oggi,* published this year, 2021, by Laterza.

Through seven different stories of newborns and of their parents and families, the authors bring us into what they call the "microcosm" of NICU and illustrate some complex medical problems (by definition in a neonatal intensive care!), the challenging social background that often accompanies them and the related ethical dilemmas.

The book unfolds on two parallel levels, according to the different professional specializations of the two authors.

For each of the stories told, at first Dr. Gangi takes us inside the ward and vividly recreates the difficulties and the emotions that the families involved and the health personnel face on a daily basis. The result is a competent and empathic portrait of every single situation.

Then follows Professor De Curtis, who offers the medical background of the story, the related health care aspects, and gives, whenever necessary or useful, the epidemiological framework of reference. He often broadens his description by touching delicate issues, whose common thread is the fragility of the newborns and their dependence on the choices of adults – aspects with which De Curtis became well acquainted during his long tenure within the National Bioethics Committee.

All the stories told are examples of situations that recur in the hospital practice involving premature babies. The authors' desire is to offer parents, who might unexpectedly face difficulties with their newborn kids, a little perspective to feel less alone, and to help them find the energies that the situation requires, however unobtainable they might appear.

What remains after reading this book is a choral image of the NICU microcosm, involving infants and their families, the health personnel, doctors and nurses, who fight every day together with premature babies to get them out of the emergency. We are outside this microcosm, blissfully unaware of it, but we could be thrown inside it, utterly unprepared. And this is a good reason for reading this book.

Antonia Stazi
Istituto Superiore di Sanità, Rome, Italy
antonia.stazi@iss.it

REALTÀ
Mario De Caro
Torino: Bollati Boringhieri;
2020 p. 128.
ISBN 9788833928074.
€ 13,00

[Reality]

In this little book that once would have been deemed as "golden" for the outstanding clarity and the unparalleled lucidity of its argumentation, Mario De Caro faces the most philosophical and at the same time the most common-sensical problem, that of reality. His main assumption is tantamount to maintain that "no one has succeeded in convincingly demonstrating that we can give up the idea of a reality independent from us, that is, the idea of an internally structured reality even before the mind gives it a conceptual framework" (p. 14).

De Caro has multifariously collaborated with some exponents of the so-called new realism (such as Maurizio Ferraris and Markus Gabriel), but this book does not represent a sort of manifesto of that philosophical standpoint: it is rather a defence of a naturalistic perspective on reality. In the first chapter, the author, starting from the general scepticism that characterized most of the philosophers of the last decades of the twentieth century about the idea of reality, observes how, apart from two important exceptions such as Karl R. Popper and John Searle, authentically realist options were to be found almost only in Australia, so much so that in the ocean of anti-realism those who could be defined as the "marsupials of philosophy" found themselves swimming in the opposite direction, with great difficulty. Anti-realism found its champions both among the eminent philosophers of the Anglo-Saxon tradition (like Davidson or Feyerabend), and among the so-called continental philosophers (from Foucault to Gadamer).

Moreover, none of these philosophers went so far as to explicitly deny the existence of an extramental reality, but rather maintained that it was impossible to understand it as something already given, as if it were pre-packaged independently of the structuring of the categories of mind and language. Without them, reality would be unstructured and amorphous. This point of view had then led to the rehabilitation of various traditional forms of anti-realism, from nominalism to phenomenalism, from classical idealism to radical empiricism, ending with more or less pronounced forms of scepticism, often declined with disguised clothes (postmodernism, "weak thought" or deconstructionism)[1].

However, De Caro observes, just as in the last act of Mozart's *Don Giovanni* the Commendatore's ghost appears to remind us of those truths that cannot be erased, so in recent years reality has once again firmly

---

[1] Even a historian of science like Thomas Kuhn defended a rather radical relativism, according to which we live in a world determined by the paradigms of the dominant sciences: our world, or rather the Galilean world, differs from the Aristotelian world because a body oscillating above us is a pendulum, whereas for the ancients it is only a body tending towards its *locus naturalis*. And even Hilary Putnam, along with the seemingly distant Gadamer and Davidson, came to argue that it is impossible to speak of a world without the participation of the mind or of reality data presupposed to the mind and not interpreted. Putnam, in the phase in which he defended the so-called internal realism, argued that it is the mind and the world, jointly, that generate the mind and the world, in a "transitive" way, so to speak, as Spinoza would have said; there cannot be, therefore, a "ready-made" world, without the participation of our intuitive and cognitive functions. In a not dissimilar vein, the American philosopher Richard Rorty, in an epoch-making book entitled *The Philosophy and the Mirror of Nature* (1979), argued that external reality is always shaped by our conceptual framework. Given the impossibility of standing outside our conceptual apparatus to understand what reality really is before it is conceptualised, we cannot form any non-contradictory representation of a reality that pre-exists in our minds. From Rorty, bridge-builder between analytic philosophy and continental philosophy, the passage to postmodernism, deconstructionism and weak thought is short: these conceptions are characterised precisely by the explicit denial of the idea of an independent objective reality, in the name of the thesis that it makes no sense to speak of a prelinguistic reality.

and decisively occupied the philosophical scene, so that the number of philosophers who call themselves realists has been increasing, both within analytical philosophy and continental philosophy. The marsupials, far from being an endangered species, have returned, with their realism, to occupy the philosophical continents.

But De Caro does not intend to prepare a survey of the new realist philosophies, but rather to give a convincing answer to the questions that arise once the correctness of the realist point of view has been assumed. Here is a brief overview: 1) Should we rely more on the senses or on what science tells us about the world as a parameter for making judgements about external reality? 2) If the answer were to assign more value to science, how would we cope if the various scientific disciplines talk about entities that cannot be perceived either with the senses or with instruments that amplify the senses (such as telescopes or microscopes)? 3) Do colours, sounds, smells – i.e. what the tradition from Galileo to Locke called secondary qualities and which, on the basis of the testimony of the senses, seem to us to be located in the external world – really exist outside us or are they projections fashioned by our mind? 4) Apart from material objects, do non-material entities such as disembodied minds, numbers, missing acts and universals exist? 5) Do unobservable entities in physics, such as electrons or black holes, exist objectively or are they merely theoretical constructs with an "economic" value, as Ernst Mach once claimed? 6) In the non-hard sciences, do collective entities, such as multinational corporations, exist as independent entities that are responsible for their actions, or are they fictitious entities that, precisely because they are fictitious, cannot be held responsible for what seems to everyone to be happening because of their intervention? 7) What status of reality do mental illnesses have? Are they cultural constructs or pathologies objectively located in the biological world? 8) Are moral and aesthetic judgments able to identify objective aspects of reality or do they have a merely subjective status? 9) Does time exist as we think it does or is its nature entirely illusory, as even some physicists claim? 10) Do causal phenomena really exist in the world or is causation a mere projection of our mind?

For De Caro, all these questions can only be plausibly answered once it has been determined which is the best form of philosophical realism available today. De Caro, moreover, starts from the hypothesis that no serious philosopher has ever been completely realist or completely anti-realist[2]. Therefore, the author argues that

all attempts to solve the problem of realism are a matter of degree: for him, it is a matter of assessing which doses of realism should be adopted on a case-by-case basis, specifying which entities are real with respect to the various fields.

However, there are at least two basic forms of philosophical realism:

1. Ontological realism, whereby certain kinds of things are real, whether they are concrete entities (the computer I am writing with, the table, the Alpha Centauri double star or Woody Allen), abstract entities (disembodied minds, numbers, aliens and the Schumann cello concerto I am listening to) or properties (the red being, goodness and free will), events or processes (the Big Bang, transubstantiation and the Middle Ages). These theories can go so far as not only to affirm the reality of the external world as a whole but also to determine in what sense time, including the future, or space, as a container of entities, according to Isaac Newton, is real. Two parallel questions then arise: whether something really exists, or, if it does, whether it exists independently of the minds that think it up. The first question is usually asked of atoms, and the second is asked of colours.

2. Epistemological realism. According to the proponents of this conception, there are facts that go far beyond our ability to verify them. Let us imagine that it is true, for instance, that there are no life forms in the universe outside the solar system. This clearly seems to be a fact that cannot be definitively ascertained, because it would imply the possibility of scouring the entire universe, which is beyond the reach of today's technologies. An anti-realist might object that this is a fact that can be ascertained under "ideal epistemic conditions", i.e. without spatio-temporal constraints placed on the knowing subject. In turn, then, the epistemological realist might reply that accepting such a possibility would mean appealing to a "divine" point of view, a hypothesis that is little considered today even by believers.

De Caro deals especially with the first form of realism (p. 19), ontological realism, assuming, however, that in all serious philosophical theories elements of realism and anti-realism are combined. For him, the two basic forms of ontological realism are (a) ordinary realism, which attributes reality exclusively to entities we can experience, whether directly (through introspection or the senses) or indirectly (by means of instruments that extend the reach of the senses, such as microscopes and telescopes), and (b) scientific realism, i.e. the conception according to which the world contains only those entities and events (both observable and unobservable) that the natural sciences are able to describe and explain. According to a version of this perspective that was already called physicalism in the Wiener Kreis, physics becomes a fundamental science, because all other sciences are reducible to it: in this perspective, therefore, physics delimits in principle the whole of our knowledge and ontology.

---

2   Not even the Austrian Alexius Meinong, perhaps the most radical of realists, attributed to the round square a real existence, but if anything a hypothetical existence. Or Bishop George Berkeley, champion of anti-realism in the form of subjective idealism and defender of immaterialism: he became a convinced realist when he discussed the divine mind. As Benedetto Croce had already argued, in his somewhat old-fashioned prose: "not even Berkeley, by denying matter, denied reality, which was for him the will and reality of God; and for Hegel the Idea was not mere knowing, but the unity of knowing and willing, capable of producing the sun, the earth and the other stars, and executing the programme of all the seven days of creation; and, even for the most vacuous of today's idealists, the act they call the act of thinking is more than the act of knowing, so that they fall, if ever, into mysticism or ir-

rationalism or phenomenism, but not into 'solipsism', which is a bogey-man of something that no one has ever seriously thought of proposing and supporting" (*La Critica*, 1937, 35, p. 153).

De Caro turns to be rather sceptical about the possibility of admitting the reducibility of biology and biological taxonomy to physics and physical taxonomy (p. 19). In his view (which he shares with his colleague David Macarthur), evolutionary biology, for example, aims to provide a causal explanation for a highly specific sequence of *actual* historical events. It is not concerned about laws for a *possible* sequence, but about historical events that are represented by the evolution of single organisms under determined circumstances. But the same is true of physics itself, if we look at cosmology, that aims at describing the actual development of the universe, i. e. another specific sequence of historical events. Another biological example that could fit this standpoint is represented by the Mendelian genetics, insofar as it involves predictions through statistically discovered patterns of phenotypic variations in populations of biological entities. Furthermore, in the philosophy of biology we can find the supporters of final causes (the so-called teleosemanticists, biomedical Nobel laureate Jacques Monod, Fred Dreske, Garrett Millikan), who have variously endorsed the irreducibility of biological functions to the entities of physics or chemistry. According to these scholars, biology provides examples of authentic scientific explanations and predictions that don't necessarily become general laws of nature. Therefore we are obliged to recognize the failure of the deductive-nomological conception of science in some fields of investigation: if we recognize biology as an autonomous science we can go on in the process of liberalizing the philosophical conception of the sciences within a naturalist perspective. And De Caro stresses this standpoint when he deals with ethics: it can be argued that moral properties are reducible to non-moral natural properties, but it does not necessarily imply that the property of a certain action to be good, for example, could simply mean that that action conforms to a system of instructions – hardwired into our brains by virtue of natural selection – that results in a benefit to humanity. Moral properties can be studied by the natural sciences, but not identical to some non-moral property: that is, they are natural properties of a specific kind (p. 47)[3].

De Caro then undertakes extensive historical excursus that take us first of all to the time of Galileo: the great scientist is seen as a defender of the Platonic theses, i.e. of the idea that it should be mathematics to determine

the ontological sphere, as the Platonists claimed, and not perception, as the Aristotelians claimed: for him the ontological and epistemological primacy belongs to physics. According to physical-mathematical Platonism, there are only physical properties, the nature of which is intrinsically mathematical and, more precisely, geometrical[4].

De Caro eventually arrives at a form of "liberalised naturalism", which admits the existence (and necessity) of a plurality of keys to access a reality that is irreducibly complex and varied[5]. In order to demonstrate the feasibility of such a path, he dwells on a subject that is very dear to him, namely that of free will (which Hume called "the most controversial question that philosophy and science have to face"). He first discusses some common misconceptions (such as the idola of Baconian memory), which are frequently found in discussions on the concept of free will or freedom of the will and which risk leading any discussion into an impasse. He then analyses the two main current challenges to the concept of free will, the neuroscientific deterministic one and the epiphenomenalist one.

According to the former, our behaviour is entirely determined by factors beyond our control so that free will is impossible. To support this thesis, reference was once made to physics (whether Newtonian mechanics or the theory of relativity) or to social sciences such as sociology and anthropology (in their deterministic versions centred on the notion of social and cultural context). Today, genetics and neuroscience are preferred, in their more deterministic declinations. The latter emphasises the discrepancy between the (explanatory) motives that the subject adduces to explain his actions and the unconscious motivational factors (i.e. the real causes) that would actually determine the actions themselves. In this case, there would be conditions in which, beyond appearances, conscious states would not be causally relevant in the generation of actions: the consequence is that, at least in some cases, the conscious mind would not have causal powers, i.e. it would be epiphenomenal. This thesis would also put out of play the classic compatibilism, i.e. the classic conception developed by Locke, Hume and Leibniz according to which determinism is, at least in part, reconcilable with free will.

Another interesting point is the challenge set up by the so called mysterianism (p. 65). In fact, we have, on the one side, philosopher who contend that the features of the manifest image of nature have a place in the world as it is described by natural science and want

3 These versions of realism can then be combined with a third version, which is more sophisticated from a philosophical point of view, although unfamiliar to non-philosophers: this is realism with respect to abstract entities (i.e entities that by definition cannot be located from a spatio-temporal point of view, such as universals, numbers, sets, species and meanings): realism with respect to these classes of entities is often labelled "ontological Platonism", because it can be compared in many ways to Plato's so-called "theory of ideas". According to this form of realism, abstract entities exist independently of the concrete exemplifications that occur in the space-time continuum. For example, the species Canis lupus exists as an abstract entity beyond its individual concrete exemplifications, such as my neighbour's German shepherd or the dog-actor playing Commissioner Rex. Similarly, according to ontological Platonists (e.g. Gottlob Frege), the meaning of the utterance "Greenland is an island" exists independently of the concrete manifestations of that utterance (and of similar utterances in languages other than Italian).

4 He then dwells on Husserl, according to whom phenomenological investigations prove that the only real world is the "life-world" (Lebenswelt): this is the world of human experience, in which secondary values and properties are real. It is the "forgotten foundation of meaning of natural science". Scientific concepts become mere idealisations with practical ends, such as measurement and prediction, without referring to real entities. From this perspective, science must be interpreted instrumentally, i.e. in ontological and anti-realistic terms.

5 He does not forget to compare his position with the one of some authoritative contemporary analytical philosophers, such as van Fraassen (for whom he uses the felicitous label of "constructive empiricism"), Quine, and Putnam.

those features to be reduced to scientifically acceptable ones. Others, instead, argue that these features are mere delusions and, consequently, should be taken away from our ontology. The two ontological choices, i.e. reductionism and eliminativism are deemed by others as something between the devil and the deep blue sea, that is both equally unpleasant or not convenient. Consequently, they opt for 'mysterianism', the view according to which we cannot renounce the naïve and non-scientific features of the manifest image even if we are not able to understand the ways (which certainly exist) in which they could be reduced to the scientific ones. De Caro thinks that mysterianism is only a way to explain away the problems, without providing any solution: in the last analysis, reductionism, eliminativism and mysterianism fail in their own so that the best tenet is that the scientific image and the manifest image of the world are essential and mutually irreducible but not incompatible with each other, as only a genuine philosophical outlook can highlight. That's why it is absurd to give credit to some scientist that sentence philosophy to death, just because are simply philosophizing on their own without having the appropriate tools to do so. And this is an error symmetrical to that of philosophers – still quite numerous, unfortunately – who claim to discuss scientific issues without having the slightest idea of what they are talking about (p. 78).

At this point, according to De Caro, it is necessary for philosophy and science to proceed jointly, each according to its competences and prerogatives, in order to find a space in which it is legitimate to speak of free will: for him, this is exactly the perspective of liberalised naturalism that allows human beings to be considered, at the same time, as free agents and as natural entities. In the first sense, we belong to the normative sphere of the space of reasons; in the second sense, to the sphere of natural legality: only a poorly justified scientistic metaphysics can lead us to think that there is one explanation that is more correct and fundamental than others. There are cases in which we must resort to the explanations proper to the natural sciences; then we will gradually move on to the level proper to the human and social sciences; and finally we will arrive at the level of normative explanations. As Hilary Putnam has written, there are "as many kinds of cause as there are senses of 'because'".

Why to suggest such a book to the biomedical audience? Cognitive sciences with their increasing association with neuroscientific approaches and the relevance of neurodegenerative diseases in our Western aging population find a cross-pollinating relationship, e.g. on p. 71, where brain scientists melt with philosophers. The problems of language, the pathophysiology of communication and their "mystery" are presented under a Chomskyan perspective. In the paragraph "Problems of scientific realism", reductionist straitjackets are concisely reviewed beyond Descartes and Kant. The most provoking point could be "the mistake of the neuromaniac", the average scientist who brandishes theories and neuroscientific experiments to reinforce with weak evidence his feeble tenets and arrogant statements (p. 98). Libet's electro-encephalographic experiments and their

reflection on awareness do represent another intriguing challenge.

The author, Mario De Caro, full professor of moral philosophy at University Roma Tre, has also been holding a course in Boston (USA) for several years. He recently participated into a series of joint seminars at ISS, in collaboration with the Accademia Nazionale dei Lincei and the Imperial College, London, on the issue of community and health. This agile contribution may indeed provide a lively interaction between biomedicine and the humanities.

Teodosio Orlando
Università degli Studi Roma Tre, Rome Italy
teodosio.orlando@uniroma3.it

COME PENSANO LE FORESTE
Eduardo Kohn
Prefazione di
Emanuele Coccia
Milano: Nottetempo; 2021
439 p.
ISBN 9788874528943
€ 20,00

[How forests think: Toward an anthropology beyond the human]

A PIEDI NUDI NEL VERDE
Giocare per imparare a vivere
Albertina Oliverio,
Anna Oliverio Ferraris
Milano: Giunti;
2011
223 p.
ISBN 978-88-09-76624-2
€ 10,00

[Bare foot in the green: Play to learn how to live]

An emerging research sector, with an exploding variety of biomedical studies and analytical approaches, deals with the beneficial effects of spending the entire lifespan in a natural environment, or at least within an ecological niche characterized by a sufficient amount of natural-type stimuli, those enough to trigger appropriate mental and bodily reactions. Experience of a natural environment is relevant during infancy. During such sensitive or critical periods, the developing brain is prepared to be moulded by stimulus sets of a sufficient complexity.

In general, it is suggested that environment characterized by scarce natural stimuli, a variety of unnatu-

ral stimulus sets, or stimuli which for frequency and\or duration are far from those expected by *Homo sapiens*, might not support wellbeing and resilience. Even exposure to plant essence or walking under a tree canopy covering reportedly ameliorate psychophysical discomfort. Vulnerable patients are a particular target.

Very recently, the policy paper for the G20 "Mental health" included this statement, formally inspired by the UN Environmental Programme: "Environmental issues should not be neglected, given that factors such as pollution, climate change, and ecosystem degradation negatively impact mental health" (*COVID-19 and the need for action on mental health 2021*, pag. 2, par. 1).

The volume by Kohn (a recognized anthropologist interested in climate change effects based at McGill University, Montréal, awarded the Gregory Bateson Prize in 2014 for this present book) is an elegant, sometimes poetic narration about a kind of individual (the ecology of selves) ecology, an "aboutness" which reveals a generalized *telos* which initiates with interhuman relationships to eventually delineate an interactive creative effort which envelops and permeates all the living organisms: where the local human populations have spent, are spending, and will spend their existence?

"Distributed selfhood" is a central concept here (p. 210), as "The ecology of selves" (p. 156). In fact, One health is a third Millennium revelation in contemporary biomedicine [1-4].

In the Kichwa population (inhabiting the Ecuadorian forests around Àvila), the *kawsanguichu* greeting sounds "Are you still alive?" (p. 80). Such a bonding, empathic ceremony is the occasion for Kohn's reflections and speculations about the inevitable networking which envelopes all the living beings that populate the forest. The paragraph "Of and into the world" again represents a pivot for elegantly disentangling the subtle, yet very robust bond between all forest inhabitants, as a generalized approach explaining the difficulties to maintain a decent level of wellness in a variety of anthropogenically-modified contexts. The urban, inner cities well represent a special case, not rarely hitting the psychophysical equilibrium of people, in particular in the case of vulnerable subjects with a biography of mental suffering.

Dogs seem indeed a special case. Dreaming dogs, dogs warmly empathic with forest humans, dogs predated by jaguars. "The canine imperatives" (p. 252) represent "a delicate interspecific negotiation". The neuroscientific approaches by Terrence W. Deacon, an author of paramount importance or human brain evolution, embedded in a narrative, fluid text, reveal a truly original perspective.

The interaction of the human mind in those forest population is not at all limited to humans. India rubber and especially "green venoms" extracted by local plants all represent a complex network of survival, maintained in equilibrium by an intricated ensemble of living "Like traders, Amazonian dolphins congregate at the confluence of rivers".

Kohn based his essay on four years of field research in one of the world's most complex forest ecosystems. As the author recalls in the fascinating concluding chapter, the main objective of this complex essay is to build an anthropology "beyond the human" that includes the possibility of thinking with images beyond the semiotic mode of human thought. In the ecology of the Amazon rainforest the complexity of the relationships between all living beings and their interdependence put into action a "thinking mind" endowed with harmony and inclusive of the phyletic history of each living species. A food for thought perhaps for those eager of understanding the healing power of nature on the human mind.

The nice, little essay by Oliverio (Associate Professor of Logic and Philosophy of Science, University of Chieti) and Oliverio-Ferraris (Full Professor of Developmental Psychology, Sapienza University of Rome) starts with an apparently trivial consideration, i.e., that until a few decades ago our cities provided open spaces where children could freely meet and play. Green zones, streets, squares. It was indeed sufficient to go down to the courtyard or to go out from home to find playmates. Then, slowly but inexorably this picture mutated and a variety of social and "green" stimuli evaporated for the subsequent generations. Natural times and developmental times (p. 22) are implicitly suggested along the text. The volume enlists some reflections on the actual dangers for urban children by the well-known British architect, town planner and anarchic thinker Colin Ward. Charles A. Lewis, author in 1996 of *Green nature, human nature* is present along with Maria Montessori and John Dewey (p. 128). Proposals to regularly cordon off some streets to allow children playgrounds or outdoor kindergartens or forest kindergartens are already a reality, mostly in selected areas of Northern Italy or around Rome (Ostia). Such a selected, yet wide, series of readings make this book a quite excellent introduction to the matter. A few agile schemes (list of commonest urban pollutants, Natural elements for outdoor play activities, etc.) represent a very useful complement.

Chapters and paragraphs summarize "Bird fountain and tv laboratory", "Naturalistic mind", "Attention deficit or deficit of nature?", "Play: evolutionary advantages, Hidden significance", Green cities and transition towns". They all touch relevant and emerging issues, stimulating readers of different sectors to elaborate on.

These, and similar books, may profitably attract professionals interested in both children and\or adult mental health as well town or hospital planners, architects, municipality, or citizens worried by the increasing loss of urban biodiversity and local and global change of the environments where they spend their lives. It is likely that new lines of research will be launched, therefore young investigators could benefit from considering those two and similar books.

Gemma Calamandrei, Enrico Alleva
Centro di Riferimento per le Scienze comportamentali e la Salute mentale, SCIC Istituto Superiore di Sanità, Rome, Italy
enrico.alleva@iss.it

REFERENCES

1. Bertinato L, et al. How can we manage the COVID-19 in-fodemics? A case study targeted to health workers in Italy. Ann Ist Super Sanità 2021;57(2):121-7. Available from: https://annali.iss.it/index.php/anna/article/view/1250/737.

2. Casà C, et al. COVID-19 and digital competencies among young physicians: are we (really) ready for the new era? A national survey of the Italian Young Doctors Associa-tion. Ann Ist Super Sanità. 2021;57(1):1-6. Available from: https://annali.iss.it/index.php/anna/article/view/1170/707.

3. Petrini C, Minghetti L, Brusaferro S. A few ethical issues in translational research for medicinal products discovery and development. Ann Ist Super Sanità 2020;56(4):487-91. Available from: https://annali.iss.it/index.php/anna/article/view/1169/662.

4. Petrini C, Brusaferro S. Ethics committees and research in Italy: seeking new regulatory frameworks (with a look at the past). Ann Ist Super Sanità 2019;55(4):314-8. Available from: https://annali.iss.it/index.php/anna/ar-ticle/view/958.

Publications from International Organizations on Public Health

Edited by
Annarita Barbaro

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO)

A guide to forest-water management. FAO Forestry Paper No. 185. Rome: Food and Agriculture Organization of the United Nations, International Union of Forest Research Organizations (IUFRO), U.S. Department of Agriculture (USDA) 2021; 184 p. ISBN 978-92-5-134851-2. A Guide to Forest-Water Management is the product of a collaboration among numerous experts worldwide supported by FAO, the European Commission, the United States Forest Service, the International Union of Forest Research Organizations' Task Force for Forests and Water, and the European Commission Joint Research Centre. This Guide is the first comprehensive global publication on the monitoring, management, and evaluation of forest–water interactions. It was developed to stimulate discussions on strategic forest management and governance for water and to provide general guidance on forest–water monitoring, management, and valuation at multiple scales. Because of the importance of context in forest–water relationships, this publication does not provide comprehensive and detailed guidance for all situations. It does, however, examine certain specific forest ecosystem types as examples to illustrate how sustainable forest management can support hydrologic functions and services at different scales, from local to landscape. The purpose of A Guide to Forest-Water Management is to improve the global information base on the protective functions of forests for soil and water. It reviews emerging techniques and methodologies, provides guidance and recommendations on how to manage forests for their water ecosystem services, and offers insights into the business and economic cases for managing forests for water ecosystem services. Intact native forests and well-managed planted forests can be a cheap approach to water management while generating multiple co-benefits. Water security is a significant global challenge, but this paper argues that water-centred forests can provide nature-based solutions to ensuring global water resilience.

Smits CHM, Li D, Patience JF, den Hartog LA. **Animal nutrition strategies and options to reduce the use of antimicrobials in animal production.** FAO Animal Production and Health Paper No. 184. Rome: Food and Agriculture Organization of the United Nations 2021; 98 p. ISBN 978-92-5-34670-9. This publication focuses on dietary strategies aiming to reduce the risk of enteric health problems during critical transition periods where antibiotic use is high. In fact, nutrition is one of the pillars of gastrointestinal health and contributes to minimizing antimicrobial use in farm animals. The main tools available for diet formulation, and feed and drinking water management, are described. This publication discusses in more detail the practical application of dietary tools during critical transition periods in the lives of swine, poultry, and ruminants, with an emphasis on the species categories for which antibiotic use is highest (piglets, broilers, and calves). Dietary measures adopted alongside biosecurity, genetics, animal health care, animal welfare and farm management are the keys to success in improving animal health and welfare.

Climate-smart agriculture case studies 2021 – Projects from around the world. Rome: Food and Agriculture Organization of the United Nations 2021; 98 p. ISBN 978-92-5-134616-7. This publication describes climate-smart agriculture (CSA) case studies from around the world, showing how the approach is implemented to address challenges related to climate change and agriculture. These case studies are grouped into chapters according to the five action points for CSA implementation: expanding the evidence base for CSA, supporting enabling policy frameworks, strengthening national and local institutions, enhancing funding, and financing options, and implementing CSA practices at field level. The aims of this publication are to demonstrate the relevance of all these five action points of CSA implementation, inspire stakeholders to implement CSA actions in response to climate change, show how recent CSA projects are contributing to the SDGs, and formulate recommendations for future projects based on the five action points approach. To do so, this publication provides examples of the innovative roles that farmers, researchers, government officials, private sector agents and civil society actors can play to transform food systems and help meet the Sustainable Development Goals; it also demonstrates how these actors can collaborate.

INTERNATIONAL SCIENCE COUNCIL (ISC)

Unleashing science. Delivering missions for sustainability. Paris: International Science Council (ISC) 2021; 50 p. This report presents a framework of ideas on how science, along with science funders, policymakers, civil society, and the private sector, could rise to the occasion of acting effectively in the face of urgent and existential risks to humanity. The report offers a Framework to Unleash Mission-Oriented Science,

highlighting the need to focus on a limited number of Sustainability Science Missions asking for a major step change in the approach to science and science funding by delivering specific missions for science as they relate to the critical areas of food, energy and climate, health and wellbeing, water, and urban areas.

A synthesis of research gaps for science to enable societies to accomplish the Sustainable Development Goals by 2030. Paris: International Science Council (ISC) 2021; 39 p. This Document collects the inputs highlighted by the report *Unleashing science. Delivering missions for sustainability*. They have been distilled into five topical areas: Sustainable planet for a dignified human future; Economies for the People and the Planet; Towards integrated and inclusive governance and capable institutions at all levels; Digital transformations for humanity and inclusive sustainable development; and Understanding the processes of societal transformations in different contexts. In addition to these topical research areas, the inputs from the report provided valuable insights on how science systems, including science funding, need to evolve to support societal transformations required to achieve these Sustainable Development Goals (SDGs). These key findings are provided in the second section of this report, Reforming Science Systems. This section outlines five broad areas (strengthening the directionality of science, strategic collaboration and governance; changing the practice of science through new incentives and awards; boosting research capacity in the Global South; advancing Open Science globally; and strengthening trust in science and relevance for policy) with specific reform actions required for science systems, including science funding, to become more effective in supporting societal transformations towards sustainability.

UNITED NATIONS ENVIRONMENTAL PROGRAMME (UNEP)

Advancing the transition to an inclusive green economy – A policy review manual. Nairobi: United Nations Environmental Programme 2020; 44 p. This manual provides policymakers with a methodology for conducting a review of a country's Inclusive Green Economy Policy framework, to take the pulse of the concept and related policies 10 years after the work on UNEP's Green Economy report was launched. The Green Economy Policy Review manual provides a step-by-step guide on how to conduct a review of an existing policy framework according to the following criteria: coherence with other policy frameworks, particularly the Sustainable Development Goals and the Paris Agreement of the UN Framework Convention on Climate Change (UNFCCC), including Nationally Determined Contributions (NDCs), as well as existing national frameworks; and effectiveness. The manual hence looks at two levels of achievement by analysing if the policies are aligned with national and international frameworks, and if the outcomes of the policies correspond to the intended objectives. The methodology in the manual was pilot tested in Hainan Province, China, Mongolia, and South Africa, which helped refine the methodology.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA)

EFSA Panel on Animal Health and Welfare (AHAW), Nielsen SS, Bicout DJ, Calistri P, et al. **Scientific Opinion on the assessment of animal diseases caused by bacteria resistant to antimicrobials: dogs and cats.** EFSA 2021;19(6):6680, 58 p. In this opinion the antimicrobial-resistant bacteria responsible for transmissible diseases that constitute a threat to dog and cat health have been assessed. The assessment has been performed following a methodology based on information collected via an extensive literature review and expert judgement. Details of the methodology used for this assessment are explained in a separate opinion. A global state of play of antimicrobial resistant Staphylococcus pseudintermedius, Staphylococcus aureus, Staphylococcus schleiferi, Escherichia coli, Proteus mirabilis, Klebsiella spp., Enterobacter spp., Pseudomonas aeruginosa, Clostridium perfringens, Clostridioides difficile, Enterococcus faecalis and Enterococcus faecium has been provided. Among those bacteria, EFSA identified S. pseudintermedius, E. coli and P. aeruginosa with >90% certainty as the most relevant antimicrobial resistant bacteria in the EU based on the available evidence. The animal health impact of these most relevant bacteria, as well as their eligibility for being listed and categorised within the animal health law framework will be assessed in separate scientific opinions.

EFSA Scientific Committee, More S, Bampidis V, Benford D, et al. **Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health.** EFSA Journal 2021;19(8):6768, 111 p. The EFSA has updated the Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain, human and animal health. It covers the application areas within EFSA's remit, including novel foods, food contact materials, food/feed additives and pesticides. The updated guidance, now Scientific Committee Guidance on nano risk assessment (SC Guidance on Nano-RA), has taken account of relevant scientific studies that provide insights to physico-chemical properties, exposure assessment and hazard characterisation of nanomaterials and areas of applicability. Together with the accompanying Guidance on Technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (Guidance on Particle-TR), the SC Guidance on Nano-RA specifically elaborates on physico-chemical characterisation, key parameters that should be measured, methods and techniques that can be used for characterisation of nanomaterials and their determination in complex matrices. The SC Guidance

on Nano-RA also details aspects relating to exposure assessment and hazard identification and characterisation. Nanospecific considerations relating to *in vitro/in vivo* toxicological studies are discussed and a tiered framework for toxicological testing is outlined. Furthermore, *in vitro* degradation, toxicokinetics, genotoxicity, local and systemic toxicity as well as general issues relating to testing of nanomaterials are described. Depending on the initial tier results, additional studies may be needed to investigate reproductive and developmental toxicity, chronic toxicity, and carcinogenicity, immunotoxicity and allergenicity, neurotoxicity, effects on gut microbiome and endocrine activity. The possible use of read-across to fill data gaps as well as the potential use of integrated testing strategies and the knowledge of modes or mechanisms of action are also discussed. The Guidance proposes approaches to risk characterisation and uncertainty analysis.

WORLD HEALTH ORGANIZATION (WHO)

Global status report on the public health response to dementia. Geneva: World Health Organization 2021; 137 p. ISBN 978-92-4-003324-5 (electronic version) ISBN 978-92-4-003325-2 (print version). Halfway into the implementation of the Global dementia action plan (2017-2025, representing the international commitment to meaningfully improve the lives of people with dementia), the Global status report on the public health response to dementia takes stock of actions driven by Member States, WHO and civil society since the adoption of the global action plan, identifies barriers to its implementation especially in light of the COVID-19 pandemic, and highlights areas where urgent, accelerated action is required. The report, which proposes recommendations across seven key action areas: dementia policy; awareness and friendliness; risk reduction; diagnosis, treatment, care, and support; support for carers; health information systems; and research and innovation, includes updated estimates on dementia burden and costs globally based on WHO's Global Health Estimates 2019 and the Global Burden of Disease study 2019. It also uses data submitted by 62 of WHO Member States to the Global Dementia Observatory. The report, written for national and state policy-makers, health-sector planners, academics and researchers, organizations involved in dementia education and service provision, as well as people living with dementia, their careers, and families, shows that while some progress is being made, urgent increased efforts are needed globally to reach the dementia targets by 2025.

WHO global air quality guidelines. Particulate matter (PM2.5 and PM10), ozone, nitrogen dioxide, sulfur dioxide and carbon monoxide. Geneva: World Health Organization 2021; 290 p. ISBN 978-92-4-003422-8 (electronic version) ISBN 978-92-4-003421-1 (print version). These Guidelines are the updated version of The WHO *Air quality guidelines – global update 2005. Particulate matter, ozone, nitrogen dioxide and sulfur dioxide*. The overall objective of the updated global guidelines is to offer quantitative health-based recommendations for air quality management, expressed as long- or short-term concentrations for several key air pollutants. Exceedance of the Air Quality Guideline (AQG) levels is associated with important risks to public health. These guidelines are not legally binding standards; however, they do provide WHO Member States with an evidence-informed tool that they can use to inform legislation and policy. The goal of these guidelines is to provide guidance to help reduce levels of air pollutants to decrease the enormous health burden resulting from exposure to air pollution worldwide. These guidelines, issued to protect populations from the adverse effects of air pollution, are designed to serve as a global reference for an audience of diverse groups of end-users, including those involved in policy-making, research, and advocacy.

WHO guidance on research methods for health emergency and disaster risk management. Geneva: World Health Organization 2021; 584 p. ISBN 978-92-4-003228-6 (electronic version) ISBN 978-92-4-003229-3 (print version). This Guidance is for the policy-makers, practitioners and community actors involved in health emergency and disaster risk management (Health EDRM). The main driver for this book – which arose from the work of the WHO Thematic Platform for Health Emergency and Disaster Risk Management Research Network (Health EDRM RN) – is the shared aim of Health EDRM stakeholders to reduce the risks and consequences for the many millions of people worldwide whose health is affected by emergencies and disasters each year. The 43 chapters contained in this book provide straightforward, practical guidance on how to plan, do and report a wide variety of studies that can answer quantitative and qualitative questions in different settings, with specific emphasis on health-related disasters. Case studies of direct relevance to Health EDRM provide real-life examples of research, to illustrate the methods and their impact. The Guidance begins with an overview of the Health EDRM framework and the role of research to explain the context, followed by an historical review of the impact of emergencies and disasters on public health and the development of Health EDRM policies, focusing on Japan as a case study. Sections 2, 3 and 4 cover three major aspects of the research process: identifying and understanding the problem that needs to be studied; determining the research question and developing a scoping study; and designing and conducting the main study. The book ends with a section on the practicalities of becoming a researcher and a glossary to explain terms that might be unfamiliar to some readers.

REFEREES

Acknowledgements to Referees

Instructions to Authors

Annali dell'Istituto Superiore di Sanità is a peer reviewed quarterly science journal which publishes research articles in biomedicine, translational research and in many other disciplines of the health sciences. The journal includes the following material: original articles, reviews, commentaries, editorials, brief and technical notes, book reviews. The publication of Monographic Sections on Annali ISS has been discontinued. In case you wish to present a limited number of coordinated contributions on specific themes concerning priorities in public health, please contact the Editorial office. *Annali* follows the Recommendations for the Conduct, Reporting, Editing, and Publications of Scholarly Work in Medical Journals, issued by the International Committee of Medical Journal Editors (ICMJE) www.icmje.org.

MANUSCRIPT SUBMISSION

Manuscripts should be submitted online to https://annali.iss.it/. The submission should include:
• cover letter where the authors declare that the manuscript has not been published or submitted for publication elsewhere;
• manuscript;
• tables and figures;
• author's contribution statement (individual contribution to the manuscript);
• conflict of interest statement (a conflict of interest exists when authors or their institutions have financial or personal relationship with other people or organizations that could inappropriately bias conduct and findings of the study);
• permission to reproduce figures, if appropriate.
Receipt of author's paper will be acknowledged by an e-mail containing an identification number which should be used in future correspondence.

REVIEW PROCEDURE

Each paper submitted to *Annali* is subjected to the following procedures:
• it is reviewed by the Editor-in-Chief for general suitability;
• if it is judged suitable, qualified referees are selected and a peer review process takes place;
• based on the recommendations of the referees and replies of the authors, the Editor-in-Chief decides whether the article should be accepted, modified or rejected;
• once the paper has been accepted, authors will receive proofs from the editorial office, which should be corrected and returned (usually within three working days).

MANUSCRIPT PRESENTATION

Please ensure that your manuscript follows these guidelines.
Manuscripts should be written in good English, as concisely as possible to allow a clear understanding of the text. The title should be followed by the complete name of the authors, their affiliations – in the original language – town and country. The name of the Working Group should appear at the end of the by-line; its composition should be reported before the References, names and affiliations of each member are required. The name and address, telephone and e-mail of the corresponding author should also be indicated. On the same page a running head of no more than 40 characters (including spaces) should be included. Original articles should normally be organized into different sections (*i.e.*: Introduction, Materials and methods, Results, Discussion, Conclusions). In the Methods section a specific paragraph on the adopted statistical analysis should necessarily be included.
Each article should be accompanied by:
• an abstract of about 150 words; the abstract should be structured when required (such as in original articles);
• key words up to a maximum number of five (MeSH headings, whenever possible. Refer to: www.nlm.nih.gov/mesh/meshhome.html).
Tables and figures should be kept to a minimum and be presented only if necessary.
Authors should deal responsibly and effectively with security issues that might be raised by their papers (see: Statement on Scientific Publication and Security *Science* 2003;299:1149).
This journal has adopted the SAGER reporting Guidelines for Sex and Gender Equity in Research.
These guidelines apply to original research articles and review papers. Authors should use the terms sex and gender carefully in order to avoid confusing both terms. Where subjects can also be differentiated by gender (shaped by social and cultural circumstances), the research should be conducted similarly at this additional level of distinction. Where the subjects of research comprise organisms capable of differentiation by sex, the research should be designed and conducted in a way that can reveal sex-related differences in the results, even if these were not initially expected.
Please consult the guidelines (https://researchintegrity-journal.biomedcentral.com/articles/10.1186/s41073-016-0007-6).
Authors are also encouraged to use fair, accurate and respectful language, but preferences can change and vary across groups and individuals and can also evolve overtime. The following guidelines may help in use of a correct terminology in the area of HIV: https://www.cdc.gov/stophivtogether/library/stop-hiv-stigma/fact-sheets/cdc-lsht-stigma-factsheet-language-guide.pdf
https://www.hptn.org/resources/HIVLanguageGuide
https://unesdoc.unesco.org/ark:/48223/pf0000144725
The name of the bioresource (and identifier, if available) which provided samples/data useful for the conduct of the study should be reported in extense, either in the Material and methods section or in the Acknowledgements.

LENGTH OF THE TEXT

To provide a text that meets the requirements of our publication:

• the *letter* to the Editor should be about 450 words; it does not need an abstract;

• the *editorial* should be no longer than 1000 words; editorials are submitted on invitation. Please contact the editorial office in advance if you wish to submit an editorial;

• the *commentary,* 2000 words; the commentary is an opinion piece or reflection on recent papers previously published on *Annali ISS* or elsewhere; an abstract is required; please contact in advance the editorial office;

• the *brief note*, 3000 words, including about 15 references, one table and one figure;

• the *article,* 6000 words, including about 40 references, three tables and two figures;

• the *review* should be no longer than 10 000 words, including no more than 100 references up to a maximum of four tables and three figures.

FORMATTING GUIDELINES
Text

• Use Times New Roman font, 10 point, single spaced;

• do not use the automated features of your application (endnotes, headers, footers, especially for references);

• avoid using bold characters to emphasise words or sentences within the text;

• indicate clearly titles of chapters and subchapters avoiding numbering.

Tables and figures

They should be understandable also without reference to the text and should be numbered in Arabic numerals in a consecutive and independent way according to their citation within the paper.

Tables should be presented on a separate sheet and preceeded by a title. Each column within the table should have a heading. Abbreviations should be reported in full in the legend.

Figures should be loaded as separate files. The following file formats are acceptable: JPEG, TIFF or EPS. Vectorial images (graphs, flow charts, schemes, and other non bitmap material) should be in Excel, Adobe Illustrator, Microsoft Power Point so as to allow the editorial formatting of the material.

Figures are redrawn into the *Annali* style by our in-house illustrators.

Photographs must have a minimum resolution of 300 dpi. Captions should be presented on a separate sheet and contain a sufficient explanation of their object. They should be concise but comprehensive.

REFERENCES

All references in the text must be numbered in square brackets, *i.e.* [1, 2, 3-6], and mentioned at the end of the article in the order in which they are quoted. They should conform to the "Recommendations for the Conduct, Reporting, Editing, and Publications of Scholarly Work in Medical Journals" (www.icmje.org), according to the following examples.

Titles of periodicals should be abbreviated in accordance with the Medline abbreviation of the US National Library of Medicine (www.nlm.nih.gov/bsd/aim.html). Online journal articles can be cited using, in addition to the complete citation, the DOI number.

Articles in journal

Bozzuto G, Ruggieri P, Molinari A. Molecular aspects of tumor cell migration and invasion. Ann Ist Super Sanità. 2010;46(1):66-80. doi: 10.4415/ANN_10_01_09

Books and chapters in a book

Godlee F, Jefferson T. Peer review in health sciences. London: BMJ Books; 1999.

Van Weely S, Leufkens HGM. Background paper: orphan diseases. In: Kaplan W, Laing R (Eds). Priority medicines for Europe and the world – a public health approach to innovation. Geneva: World Health Organization; 2004.

Proceedings

Fadda A, Giacomozzi C, Macellari V. Comparative measurements to validate a new telemetric pressure insoles system. In: 2. International Symposium on measurement, analysis and modelling of human functions. 1. Mediterranean Conference on measurement. Workshop on evaluation check of traceability. Proceedings. Genova: June 14-16, 2004. p. 425-7.

Technical reports

Della Seta M, Di Benedetto C, Leone L, Pizzarelli S, Siegmund U. ETHICSWEB technical guides. Manual for the creation of standards and guidelines for sharing information about knowledge organization systems on ethics and science. Roma: Istituto Superiore di Sanità; 2011. (Rapporti ISTISAN, 11/32).

Legislation

Italia. Decreto legislativo 29 ottobre, n. 419. Riordinamento del sistema degli enti pubblici nazionali, a norma degli articoli 11 e 14 della legge 15 marzo 1997, n. 59. Gazzetta Ufficiale – Serie Generale n. 268, 15 ottobre 1999.

US Social Security Administration. Evidentiary requirements for making findings about medical equivalence. Final rules. Fed Reg. 2006 Mar 1;71(40):10419-33.

The authors should check that each reference cited in the text appears in the reference list and viceversa. References should not include works submitted for publication but not yet accepted or unpublished results, etc. These can be mentioned in the text in parentheses.

CONVENTIONS

All Latin or foreign words should be in italics. The authors should use internationally accepted abbreviations. All abbreviations should be spelled out in full the first time they occur in the text, followed by the shortened term in parentheses; afterwards use the abbreviation only.

Avoid abbreviations in the title of the manuscript.

For writing symbols, quantities and units of measurements refer to the International Systems of Units (SI) and the ISO standards.