

# BMJ Open Assessment of interferon gamma and indoleamine 2,3-dioxygenase 1 analysis during respiratory syncytial virus infection in infants in Italy: an observational case-control study

Francesco Savino <sup>1</sup>, Valentina Daprà,<sup>2</sup> Andrea Savino,<sup>3</sup> Cristina Calvi,<sup>2</sup> Paola Montanari,<sup>2</sup> Ilaria Galliano,<sup>2</sup> Massimiliano Bergallo<sup>2</sup>

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<sup>1</sup>Early Infancy Special Care Unit, Department of Pediatric care, Regina Margherita Children's Hospital, AOU, Città della Salute e della Scienza di Torino, 10126 Torino, Italy

<sup>2</sup>Department of Public Health and Pediatric Sciences, Pediatric Laboratory, University of Turin, Medical School, 10126 Turin, Italy

<sup>3</sup>Post graduate School of Pediatrics, University of Turin. Piazza Polonia, 94 Turin, Italy

## Correspondence to

Dr Francesco Savino;  
francesco.savino@unito.it

## ABSTRACT

**Objectives** The aim of this study was to measure interferon gamma (IFN- $\gamma$ ) and indoleamine 2,3-dioxygenase 1 (IDO1) values in the White blood cells of infants during respiratory tract infections and to compare these with healthy age-matched controls.

**Design** This was a prospective, observational case-control study conducted in 2019–2020.

**Setting** The study took place at Regina Margherita Children's Hospital, Turin, Italy.

**Participants** The study comprised 63 infants, including 26 patients hospitalised for bronchiolitis due to a respiratory syncytial virus (RSV) infection and 37 age-matched controls. The inclusion criteria included a positive RSV test for an infant with bronchiolitis.

**Methods** We collected peripheral blood and measured the relative quantification of messenger RNA (mRNA) expression of IFN- $\gamma$  and IDO1 with TaqMan real-time PCR amplification. The data were collected on the first day of admission.

**Results** The mean age of the 26 patients with RSV bronchiolitis (53.8% female) was 85 (9–346) days when they were admitted to the hospital. Their mean gestational age at birth was 38 weeks and their mean birth weight was 3100 (2780–3730) g. The expression of IFN- $\gamma$  was significantly reduced in patients with bronchiolitis RSV compared with healthy controls ( $p=0.0132$ ). However, there was no significant difference between the two groups when the IDO1 mRNA expression values in their WCC were measured ( $p=0.0642$ ).

**Conclusion** Our findings did not clarify whether IDO1 expression was related to the early stage of the disease or to the young age of the infants. The data provide evidence that IFN- $\gamma$  was significantly reduced in infants with bronchiolitis due to RSV, compared with age-matched healthy controls, but the IDO1 was not different. New investigations that focus on subjects infected with RSV at different stages of infancy would help to clarify whether IDO1 expression can be related to age.

## INTRODUCTION

Respiratory syncytial virus (RSV) is the main cause of respiratory infections and hospital

## Strengths and limitations of this study

- We used TaqMan real-time PCR amplification to assess the interferon gamma (IFN- $\gamma$ ) and indoleamine 2,3-dioxygenase 1 (IDO1) values in the white cell count of 26 infants with respiratory syncytial virus (RSV) bronchiolitis at a mean age of 85 days.
- The expression of IFN- $\gamma$  was significantly reduced in patients with RSV bronchiolitis when compared with 37 healthy age-matched controls.
- The IDO1 messenger RNA expression values in infants with RSV bronchiolitis were not significantly different from the healthy controls.
- Our findings did not clarify whether IDO1 expression was related to the early stage of the disease or to the young age of the infants.
- We collected our data on the first day of hospital admission and future studies are needed to assess IFN- $\gamma$  and IDO1 at the end of the RSV infectious episode to overcome the limitations.

admissions for bronchiolitis among infants under 12 months of age.<sup>1</sup> Global estimates suggest that RSV causes nearly 34 million lower respiratory tract infections (LRTIs) a year and that 3.4 million infants and children under 5 years of age are hospitalised. An estimated annual increase of 10% has been reported.<sup>2–3</sup> RSV is a public health issue in many countries, including Italy.<sup>4</sup> It was formerly considered a subfamily of Paramyxoviridae, but was recently reclassified as belonging to the Pneumoviridae family.<sup>5</sup> The virus is characterised by a large envelope and negative-sense RNA coding for 11 glycoproteins. RSV infections are seasonal, with peaks throughout the winter months in temperate regions,<sup>6,7</sup> and young children, the elderly and those with chronic medical conditions face the greatest risk for severe RSV

infections.<sup>8,9</sup> However, some evidence has been presented that suggests that individuals who develop severe LRTIs have a compromised ability to develop sufficient type I-like immune responses during primary RSV infections.<sup>10</sup>

Indoleamine 2,3-dioxygenase 1 (IDO1) is an interferon-stimulated gene and interferon gamma (IFN- $\gamma$ ) has been recognised as the most important inducer of IDO in several cell types, as mentioned above.<sup>11,12</sup> In vitro experiments with epithelial A549 cells have shown that IFN- $\gamma$  is a more potent inducer of IDO than type I IFN.<sup>12</sup> Hundreds of IFN- $\gamma$ -stimulated genes have been described, but only a handful of them have displayed antiviral activity in vivo. We know that there are not many cells in the human body that are able to express IDO1. However, the expression of this molecule can be modulated by IFN- $\gamma$ -induced signalling pathways in numerous cell types,<sup>13,14</sup> such as dendritic cells and macrophages.<sup>15–17</sup> This is because the promoter region of the IDO1 gene contains two Interferon Gamma-stimulated response elements and three IFN- $\gamma$ -activated sites that respond to the IFNs that are often produced to control viral infections.<sup>18</sup> Although type I IFNs can also induce IDO1 expression, IFN- $\gamma$  remains the most potent inducer of IDO1 expression.<sup>19</sup>

IFN- $\gamma$  is a type II cytokine which acts directly against viruses, promotes antigen presentation through induction of Major Histocompatibility Complex (MHC) molecule expression and stimulates cytotoxic activity of natural killer cells and virus-specific T cells.<sup>20</sup> This early response is able to influence the clinical course of RSV bronchiolitis as it leads to a lower production of cytokines, which influences the duration of the disease and the damage caused to the lungs. Furthermore, the clinical pictures presented by severe RSV infections have been associated with reduced IFN- $\gamma$  secretions in both the blood and respiratory system.<sup>21,22</sup>

Multiple factors influence the magnitude of the body's response to a viral infection, such as genetics, immunological maturation, age of the subject and viral agent.

The aim of this study was to measure IFN- $\gamma$  and IDO1 levels in the white bloods cell of infants with bronchiolitis due to RSV infections and to compare these with the levels in healthy age-matched controls.

## MATERIALS AND METHODS

### Patients

This prospective case-control study was conducted in Turin, Italy, between 1 September 2019 and 31 January 2020. The cohort comprised healthy full-term infants below 12 months of age who were hospitalised at Regina Margherita Children's Hospital, Turin, Italy, for a first episode of bronchiolitis. We selected patients who were diagnosed with bronchiolitis and RSV infections and tested them for IFN- $\gamma$  and IDO1 on the day that they were admitted to the hospital.

The controls were age-matched healthy infants below 12 months of age who attended an outpatient clinic at the

hospital's Department of Pediatrics for routine postnatal checks.

Bronchiolitis was diagnosed by trained paediatricians using clinical signs that included wheezing with or without cough, rales, dyspnoea and increased respiratory rate and retractions of the respiratory muscles. Hospitalised infants with bronchiolitis underwent routine blood tests on admission to the hospital.

The parents of the enrolled infants were informed about the purpose, benefits and possible risks of the study and written informed consent was obtained.

The mean age of the 26 patients with RSV bronchiolitis (53.8% female) was 85 (9–346) days when they were admitted to the hospital. Their mean gestational age at birth was 38 weeks and their mean birth weight was 3100 (2780–3730) g. The 37 infants in the control group (51.6% male) were seen at a mean age of 98 (14–336) days and 41.9% were still being exclusively or predominantly breast fed. Their mean gestational age at birth was 38 weeks and their mean birth weight was 3020 (2500–4000) g. The controls had not been hospitalised for bronchiolitis or any other infections. WCC, neutrophils and eosinophils were recovered from their medical records.

RSV was diagnosed using Xpert Xpress Flu/RSV real-time PCR technology (Cepheid, Milan, Italy). The inclusion criteria included a positive RSV test for infants with bronchiolitis.

The exclusion criteria for the patients and controls included known or suspected impairment of immunological function, congenital malformations and being born premature at less than 37 weeks of gestation. A paediatric investigator collected personal data from the parents or guardians and clinical data were gathered during physical examinations.

Verbal informed consent was obtained from the parents of the study participants and recorded in their clinical records in accordance with Italian good clinical practice guidelines and the hospital's clinical investigation guidelines. The samples were anonymised before processing.

### Messenger RNA isolation, complementary DNA synthesis and real-time PCR

RNA were extracted from each heparinised blood sample using the RNA Blood Kit protocol, without any modifications, in the Maxwell 16 system (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. RNA was eluted in a final volume of 50  $\mu$ L. RNA purity and concentration were evaluated by spectrophotometry using NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). Absorbance ratios of 260/230 and 260/280 were used to assess the presence of contaminants: peptides, phenols, aromatic compounds or carbohydrates and proteins. We reverse-transcribed 400 ng of total RNA with 2  $\mu$ L of 10X buffer, 4.8  $\mu$ L of MgCl<sub>2</sub> 25 mM, 2  $\mu$ L Improm-II (Promega), 1  $\mu$ L of RNase inhibitor 20 U/L, 0.4  $\mu$ L random hexamers 250  $\mu$ M (Promega), 2  $\mu$ L mix Desossinucleotides 100 mM (Promega) and double distilled water in a final volume

of 20 µL. The reaction mix was carried out in GeneAmp PCR System 9700 Thermal Cycle (Applied Biosystems, Foster City, California, USA) under the following conditions: 5 min at 25°C, 60 min at 42°C and 15 min at 70°C for inactivation of the enzymes. Complementary DNA were then stored at -80°C until use. We controlled for genomic DNA contamination by directly amplifying the RNA extracts without reverse transcription.

After the reverse transcription step was carried out, we achieved relative quantification of messenger RNA (mRNA) expression of IFN-γ and IDO1 using TaqMan real-time PCR amplification and glyceraldehyde-3-phosphate dehydrogenase using ABI PRISM 7500 Real-Time System (Life Technologies, California, USA). We amplified 40 ng of complementary using the IFN-γ and IDO1 gene mRNA expression kits, PP-BioMole 001 and 030 (BioMole, Turin, Italy), respectively, in a 20 µL total volume reaction. The amplifications were performed on ABI 7500 Real-Time PCR System (Life Technologies, Carlsbad, California, USA) in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate. The delta quantification cycle (Cq) was calculated by subtracting the glyceraldehyde-3-phosphate dehydrogenase RNA Cq value from the Cq value of the mRNA of interest.

### Statistical analysis

Differences in age and WCC were examined using Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism V.5 (GraphPad Software, California, USA). We used the non-parametrical Mann-Whitney U test to compare IFN-γ and IDO1 mRNA expressions in the two patient groups we analysed. Significant differences were indicated by  $p < 0.05$ .

### Patient and public involvement

Patients and the public were not directly involved in the design, conduct, reporting or dissemination of this study.

## RESULTS

All patients were screened for RSV infection on enrolment using the rapid antigen Xpert Xpress Flu/RSV (Cepheid, California, USA). The study comprised 26 infants with bronchiolitis who were positive for RSV infection and 37 healthy controls with negative results. WCC were higher in the RSV group, but not significantly. However, lymphocytes and monocytes were significantly higher in the RSV group than the healthy controls (table 1).

The IFN-γ and IDO1 mRNA expression levels in the peripheral blood were compared in the RSV-positive bronchiolitis and healthy controls based on relative quantification.

The IDO1 mRNA expression levels in patients with RSV bronchiolitis were not significantly different from those in the WCC of the healthy controls ( $p = 0.0642$ ) (figure 1). However, the expression of IFN-γ was significantly reduced

**Table 1** Characteristics of the study population in terms of age and White blood cells count

| Variable  | Bronchiolitis* (n=26) | Healthy controls* (n=37) | P value† |
|---|-----------------------|--------------------------|----------|
| Age (days)  | 85±25.20              | 98±38.40                 | 0.285    |
| White blood cells count (cells ×10 <sup>9</sup> /L) | 9750±780              | 8580±1010                | 0.286    |
| Neutrophils (cells ×10 <sup>9</sup> /L)             | 2712±1296             | 3325±1185                | 0.645    |
| Lymphocytes (cells ×10 <sup>9</sup> /L)             | 5305±2182             | 4190±1683                | 0.021    |
| Eosinophils (cells ×10 <sup>9</sup> /L)             | 332±90                | 460±95                   | 0.315    |
| Monocytes (cells ×10 <sup>9</sup> /L)               | 1401±1077             | 605±256                  | <0.001   |

\*Data are reported as mean and SD.

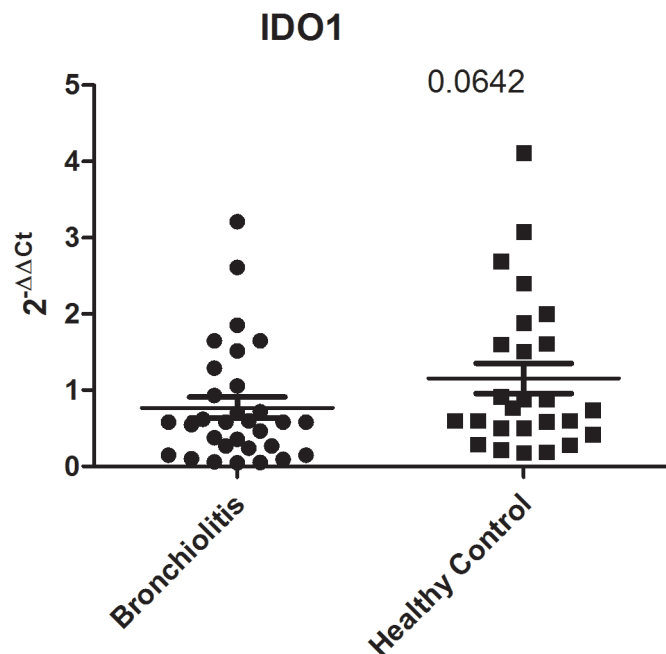
†Mann-Whitney U test.

in patients with RSV bronchiolitis ( $p = 0.0132$ ), as shown in figure 2.

## DISCUSSION

RSV is the most important aetiological agent of acute LRTI in infants and young children worldwide and the leading cause of hospitalisation in childhood. This causes considerable problems for global healthcare services.<sup>23</sup> It has been estimated that RSV infects 50% of children during the first year of life and affects 100% of children under 3 years old.<sup>24 25</sup> It has been reported that 30%–75% of infants infected before 12 months of age will be reinfected before 2 years of age.<sup>26</sup> This effect may be due to the incomplete immune response of the host to the virus, which can alter the response of the CD8+ T lymphocytes in the lungs.<sup>3 27–29</sup>

IFN-γ has significant antiviral activity and is related to the modulation of Th-like or Th2-like immune responses, as IFN-γ affects the differentiation of naïve T cells into either Th1 or Th2 cells.<sup>30</sup> Decreased IFN-γ cytokine levels have been found in respiratory samples from infants with severe RSV disease.<sup>21 31–35</sup> Similarly, a negative correlation between IFN-γ mRNA levels and RSV disease severity has been reported in nasopharyngeal samples,<sup>36</sup> indicating a suppressed type II IFN (IFN-γ) response. Studies of cytokine responses have shown conflicting evidence, probably due to marked heterogeneity in study designs and sample sizes.<sup>32 34 35</sup> Although the data suggest predominantly decreased IFN-γ production in nasal samples, the data are conflicting in blood, with several studies reporting either positive associations<sup>37</sup> or negative associations<sup>38–40</sup> or a lack of any association. Our study observed decreased IFN-γ production in infants with RSV bronchiolitis. All the infants we investigated were hospitalised with a first LRTI, were of similar age and had no family history of atopy.

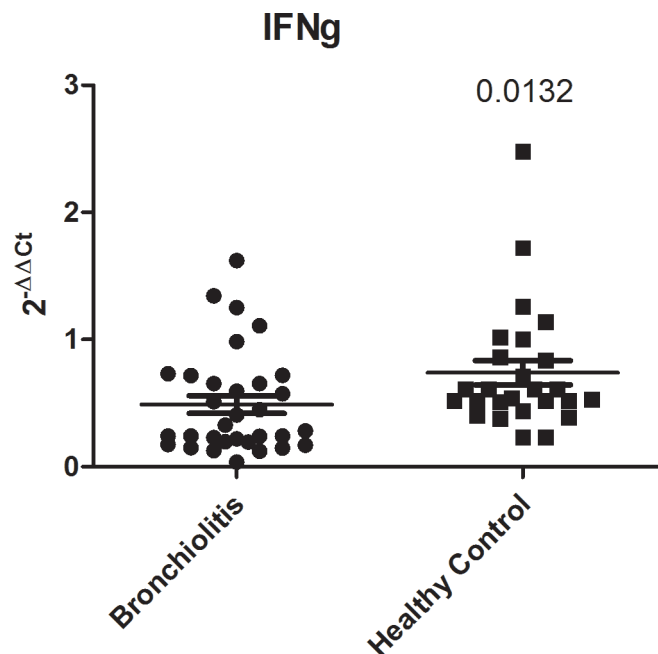


**Figure 1** IDO1 mRNA levels normalised with GAPDH expression analysed in the peripheral blood swab of patients with RSV bronchiolitis versus healthy controls. Each sample was run in triplicate.  $\Delta Ct$  was calculated by subtracting the GAPDH RNA Ct value from the Ct value of the mRNA of interest to obtain  $\Delta Ct$ . Essentially,  $\Delta\Delta Ct$  is the difference between the  $\Delta Ct$  values of the bronchiolitis samples and the healthy control samples. Fold gene expression was obtained with the formula  $2^{-\Delta\Delta Ct}$ . GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IDO1, indoleamine 2,3-dioxygenase 1; mRNA, messenger RNA; RSV, respiratory syncytial virus.

Therefore, they were comparable with healthy controls. Significantly reduced IFN- $\gamma$  production was found in both RSV-infected infants with severe LRTI and those with a relatively mild illness, in contrast to infants not affected by RSV. This suggests that virus-specific factors may affect the ongoing immune response, in addition to probable intrinsic disposition. This was also supported by a recent study on IFN- $\gamma$  responses from infants with upper respiratory tract infections.<sup>41</sup>

The authors of that study found that acute RSV infections were associated with reduced levels of IFN- $\gamma$  in nasopharyngeal aspirates compared with rhinoviruses or other respiratory viral infections. However, only prospective studies can determine the temporal sequence of events in individual infants, namely low IFN- $\gamma$  predisposing to RSV infections and/or RSV infections with deteriorating ongoing immune responses. Due to the low incidence of severe LRTI in infants, any study would have to include a substantial number of newborn infants for the results to be sufficiently meaningful.

We did not see decreased production of IDO1 in infants with RSV bronchiolitis in comparison with healthy controls in our study, in contrast to IFN- $\gamma$ . IDO1 was IFN- $\gamma$ -stimulated. However, it was reported for the first time in 2015 that RSV induced the expression of IDO in human



**Figure 2** IFN- $\gamma$  levels normalised with GAPDH expression analysed in the peripheral blood of patients with RSV bronchiolitis versus healthy controls. Each sample was run in triplicate.  $\Delta Ct$  was calculated by subtracting the GAPDH RNA Ct value from the Ct value of the mRNA of interest to obtain  $\Delta Ct$ . Essentially,  $\Delta\Delta Ct$  is the difference between the  $\Delta Ct$  values of the bronchiolitis samples and the healthy control samples. Fold gene expression was obtained with the formula  $2^{-\Delta\Delta Ct}$ . GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN- $\gamma$ /IFNg, interferon gamma; mRNA, messenger RNA; RSV, respiratory syncytial virus.

monocyte-derived dendritic cells, which depended on viral replication but were independent of IFN- $\gamma$ .<sup>42</sup> This alternative pathway of IDO1 stimulation could partly explain our data because we performed assays as soon as the infants were admitted to the children's hospital. Gene expression of IDO can also be induced by a mechanism that is independent of IFN- $\gamma$ , which depends on the activity of the transcription factors Nuclear factor KappaB (NF- $\kappa$ B) and p38 Mitogen-activated protein Kinase (MAPK). Interestingly, Fujigaki *et al*<sup>43</sup> showed that inhibitors of p38 MAPK and NF- $\kappa$ B could inhibit the activity of IDO. Both p38 MAPK and NF- $\kappa$ B are also necessary for the production of IFN- $\gamma$  and the subsequent transcriptional regulation of IDO1. However, the precise mechanism that mediates this regulation is still not fully understood.<sup>44–46</sup> Some reports have shown that IDO activity had both antiviral and immunosuppressive effects during RSV infections.<sup>42</sup> Studies have also demonstrated that the immunoregulatory pathway of tryptophan catabolism, initiated by IDO, played a critical role in allergic inflammation.<sup>47</sup>

However, there is still not enough information in the literature about the relationship between IDO expression during the whole course of RSV infection. Therefore, in vivo studies need to be carried out to provide better clarification of the immunomodulatory effect of IDO1 so that researchers can provide more adequate conclusions.

We observed during the course of this study that lymphocytes and monocytes were significantly higher in infants with bronchiolitis. This could suggest that the RSV replication rate in the monocytes could be involved in the levels of IDO1. There is very little knowledge about the contribution that monocytes make to the antiviral responses to RSV infections. A recently published animal study,<sup>48</sup> which investigated the pathway and cytotoxic T cell response against mucosal RSV infections, is of interest in this respect. It investigated the kinetic analysis of IFN- $\gamma$ -producing cells in lung cells infected with RSV in vivo and this indicated that monocytes were recruited to the inflamed lung during the early phase of the infection.<sup>48</sup>

Our study concluded that RSV significantly reduced IFN- $\gamma$  in infants with bronchiolitis when compared with age-matched healthy controls, but IDO1 did not change. However, these results must be interpreted with caution and a number of limitations should be borne in mind.

### Study limitations

One limitation was that the level of IDO transcription was maintained, despite the lack of IFN- $\gamma$ , and the source of the IDO activity could have been the RSV infecting the monocytes. Unfortunately, this study was not designed to study this mechanism and this is a limitation that could be explored by further research.

It is important to take into account that we assessed the parameters of the infants with an RSV infection on the first day of their hospital admission. We can speculate that RSV replication may not have been high as expected and that this may have been insufficient to influence IDO induction.

To overcome these limitations, subjects with an RSV infection need to be investigated at different stages of the disease so that we can clarify if the IDO1 expression can be related to the stage of the infection. This is because it could be higher at the end of the infectious episode than it is at the start. This is why it is important to carry out new investigations, with different study designs and larger sample sizes, in order to provide more valid research results.

**Contributors** All authors have made substantial, direct and intellectual contribution to the work and approved it for publication. MB and FS designed the study. VD and IG performed the RNA extractions. CC and PM performed the RNA extraction and statistical analyses. AS enrolled the patients, visited the infants, collected the blood and faecal samples, analysed the data and compiled the references. PM performed the genomic RNA extractions. MB revised the paper critically for important intellectual content and performed the PCR assays and data analysis. MB and FS revised the manuscript and all authors contributed to critical revision of the manuscript. FS is guarantor.

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**Competing interests** None declared.

**Patient and public involvement** Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

**Patient consent for publication** Parental/guardian consent obtained.

**Ethics approval** This study involves human participants. The protocol was approved by the Ethics Committee of Azienda Ospedaliera University, Turin, Italy. The study and data collection procedures were approved by the Ethics and Research Committee of Città della Salute e della Scienza di Torino on 24 November 2014 (protocol number 116918). Participants gave informed consent to participate in the study before taking part.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** Data are available upon reasonable request. The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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### ORCID iD

Francesco Savino <http://orcid.org/0000-0001-9163-1016>

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