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Cohort profile. Protocol for a longitudinal birth cohort study in three contaminated sites in southern Italy: the Neonatal Environment and Health Outcome (NEHO) cohort

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Keywords:	birth cohort study, maternal exposure, fetal exposure, polluted areas, Developmental Origins of Health and Disease

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3 **Cohort profile. Protocol for a longitudinal birth cohort study in three contaminated sites in**
4
5 **southern Italy: the Neonatal Environment and Health Outcome (NEHO) cohort**
6

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Abstract

Introduction: Exposure to environmental contaminants during pregnancy is one of the determinants of the health outcomes of future child. The influence of environmental pollution on pregnant women living in heavily polluted areas is of special interest and, in this context, the Neonatal Environment and Health Outcome (NEHO) cohort will focus on the investigation of: i) toxicants transferred from the environment to the mother and from the mother to the developing fetus; ii) the influence of toxicants on pregnancy outcomes, fetal development, and health status during infancy. Because human placenta is positioned at the interface between maternal/external environment and embryo, it can be considered a highly informative matrix regarding many pregnancy key events that could shape babies' future health.

Methods and analysis: NEHO Cohort estimates to enroll a total of 800 pregnant women in three selected National Priority Contaminated Sites (NPCS) of southern Italy. Epidemiological data collection, concerning maternal health status, lifestyle, and pregnancy is obtained through survey questionnaires provided to the mother. At the time of the delivery, maternal blood, umbilical cord blood, and placenta tissue will be collected in order to assess contaminant levels and to clarify how toxicants interact with placental domain. Furthermore, placental transcriptome will be studied in order to explore the interferences of toxicant on the maternal/fetal interplay role of the placenta. Regular follow-up is planned at 6, 12, and 24 months.

Ethics and dissemination: The study has been approved by all the Ethics Committees of the three involved NPCS: Ethics Committee of the University Hospitals of Messina (September 18, 2017, n. 9/2017); Ethics Committee "Catania 2" (July 11, 2017, n. 38/2017/CECT2); Ethics Committee of Regione Calabria (July 20, 2017, n. 173). Findings will be disseminated in the scientific community and on a regional basis for appropriate policy actions.

Keywords: birth cohort study, maternal exposure, fetal exposure, highly polluted areas, Developmental Origins of Health and Disease

Strengths and limitations of this study:

- NEHO Cohort is the first cohort in Italy with an experimental design specifically aimed at evaluating the environment/health relationship in heavily polluted areas.
- In the context of NEHO Cohort the exposure will be assessed by means of the biomonitoring of pollutants in the biological samples from mother and child, along with an extensive and multidisciplinary evaluation of pollution in all the environmental matrices, including food chain.
- NEHO will enroll mother-child pairs resident in areas disadvantaged by poor socio-economic status thus representing a peculiar condition in the context of Italian and European birth cohorts.
- NEHO Cohort requires voluntary participation: this could constitute a bias due to the self-selection of enrolled women.
- The enrollment is limited to the catchment areas of public hospitals.

INTRODUCTION

Exposures occurring during early-life, such as environmental pollutants, dietary habits and parental lifestyles may have an effect on growth and development in fetal life and in childhood and on health across the life course.[1] Early childhood provides elements that may affect many outcomes in later life[2]. Consequently, epidemiologic studies on health effects of environmental pollution try to focus on the more vulnerable subjects: thus, in the last years considerable effort was made in the evaluation of the possible effects of environmental contaminants on children's health.

From Barker's postulate of "*intrauterine origins of health and disease susceptibility*",[4] growing evidence has highlighted how the early stage of fetal development can alter the health trajectory throughout life.[5-8] This provides a better understanding of the cause of many multifactorial disorders. In fact, adult diseases may have an in utero origin, when suboptimal intrauterine conditions – including exposure to environmental contaminants – induce irreversible changes, which manifest themselves in post-natal and adult life. Birth cohorts provide an opportunity to monitor and to study associations between early-life environmental exposures and child development and health.[9] By means of a long-term follow-up, cohorts help to evaluate possible effects of exposure to environmental pollutants on development of adult diseases, also allowing the identification of risk factors, taking into account genetics, epigenetics, socio-economic factors, and lifestyles.

The number of studies linking maternal exposure to environmental pollutants during fetal period to various adult health outcomes is gradually increasing. Some Italian pregnancy and birth cohort studies investigate the health effects of environmental contaminant exposure during early-life with a specific interest on prenatal exposures, air pollution, growth, neurocognitive development, and respiratory health: NINFEA cohort,[10] NACII,[11] MUBICOS,[12] *Piccolipiù*. [13] GASPII and Co.N.ER.,[14] and *Mamma & Bambino*. [15]

The CISAS project (International Centre of advanced Study in Environment, ecosystem and human Health), funded by the Italian Ministry of Education, Universities and Research, aims at understanding the chemical-physical processes that regulate the distribution of contaminants in the

1
2 various environmental matrices and their transfer to the ecosystem and the human compartment.
3
4 CISAS Project is developed in three selected National Priority Contaminated Sites (NPCS) of
5
6 southern Italy: in its context the influence of environmental pollution on pregnant women is of special
7
8 interest.
9

10
11 The three selected NPCS are: Milazzo-Valle del Mela, Augusta-Priolo, and Crotone. Those of
12
13 Milazzo and Augusta are wide industrialized coastal areas located in eastern Sicily, in which large
14
15 production sites are present, mainly refineries, petrochemical complexes, power plants, and cement
16
17 plants, numerous hazardous waste dumps and the former Eternit plant in Syracuse where asbestos
18
19 was processed.[16] In Crotone area, located in the region of Calabria, the most relevant environmental
20
21 impact is due to three disused industrial areas (ex Pertusola, ex Fosfotec, and ex Agricoltura) which
22
23 operated between the 1920s and the 1990s, mainly in the field of production of zinc, phosphoric acid,
24
25 and complex fertilizers.[17]
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28
29 During the course of CISAS project, the birth cohort NEHO (Neonatal Environment and Health
30
31 Outcomes) will enroll pregnant women living in the three NPCS, along with pregnant women living
32
33 in surrounding areas, outside the NPCS. NEHO cohort is aimed at understanding processes and
34
35 mechanisms for the transfer of heavy metals and POPs (e.g., PBDEs, PCBs, chlorinated pesticides)
36
37 from the environment to the ecosystem and to humans. In the context of CISAS Project, the same
38
39 toxicants will be evaluated in all the environmental matrices (atmosphere, soil, sediment, inland
40
41 waters and sea) and in the food chain (fish, meat, eggs, milk and dairy products, sampled from local
42
43 producers of each studied area).
44
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47
48 Epidemiologic evidences support the concept that early life exposure to pollutants – as measured
49
50 during pregnancy and/or during childhood – is detrimental to health outcomes of the child. Previous
51
52 works indicate that polybrominated diphenyl ethers (PBDE) and polychlorinated biphenyls (PCB)
53
54 bioaccumulate in human placenta tissue possibly contributing to prenatal exposures to the
55
56 environmental contaminants.[18-20] PBDEs – largely used as flame retardants in electronic
57
58 equipment, carpet, and in the polyurethane foam used in furniture – have been detected in umbilical
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2
3 cord blood.[21-22] Attention will be focused on the investigation of toxicants that are transferred
4
5 from the mother's blood to the developing fetus and on the influence of toxicants on both pregnancy
6
7 outcome and fetal development and late onset consequences. Because human placenta represents the
8
9 interface between maternal/external environment and embryo, it can be taken as an environmental
10
11 monitoring system. In fact, placental examination constitutes an useful tool for estimating both
12
13 maternal and fetal exposures.[20, 23, 24] Moreover, placenta has an active role in the homeostasis of
14
15 the intrauterine environment and also mediates signal transmission form the fetus to the mother and
16
17 vice versa. Nutrition supply, endocrine/immune regulation, and gas exchange are orchestrated by the
18
19 placenta. All these evidences pone the placenta as highly informative organ in the study of
20
21 pregnancy.[25]
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25
26 The presence of toxicants in the placenta can cause alterations of its structure and function along with
27
28 fetal development interference. An example is provided by exposure to cigarette smoking during
29
30 pregnancy: a modification of the gene expression of placental and fetal cells has been demonstrated
31
32 in relation to both direct and indirect tobacco smoke exposure.[26-28] High concentrations of
33
34 mercury in fetal tissues are associated with the reduction of hormone synthesis and the consumption
35
36 of oxygen by the placenta.[29,30]
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39
40 Persistent organic compounds have been measured in fetal tissues and in particular in the placenta.
41
42 Exposure to PCBs and PBDEs has been shown to interfere with fetal development resulting in
43
44 significant weight reduction at birth.[31]
45

46
47 Umbilical cord blood and placenta are also noninvasive indicators for exposure to heavy metals, and
48
49 may be easily collected along with maternal blood.[32] Cadmium level in placenta is also a valuable
50
51 biomarker of metal dietary exposure related to specific dietary habits and soil characteristics. Lead
52
53 and mercury have been shown to be easily transferred through the placental and blood barriers.[30,
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3 Monitoring pollutants concentration in human tissues along with the extensive characterization of all
4 the environmental matrix proposed in CISAS project will provide new insights on the toxicants'
5 transfer routes from environment to human fetus.
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9 Particular attention will be paid to the possible interaction between the environmental exposures and
10 the low socioeconomic status which often characterizes the investigated population.
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18 **METHODS AND ANALYSIS**

19 **Study population and recruitment**

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21 From January 2018, NEHO cohort started to recruit pregnant women living in the three selected
22 NPCS of Crotone, Milazzo-Valle del Mela and Augusta-Priolo, in southern Italy (Figure 1), along
23 with pregnant women living in surrounding areas presenting similar geographic and socio-economic
24 characteristics.
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32 The NEHO study involves twenty-six cities subdivided in study areas and local reference areas. Table
33 1 shows the selected cities in Sicily and Calabria and their respective distance from industrial areas.
34
35 Local reference areas were identified as "local control" by ISTISAN reports[16, 34] and by
36 SENTIERI Project, an epidemiological study on the epidemiological evidence on the association
37 between causes of death and environmental exposures,[35-36] coordinated by the Italian National
38 Institute of Health and supported by the Ministry of Health. Aimed at maximizing the recruitment
39 efficiency, in each NPCS, maternal units were selected on the basis of both deliveries/population ratio
40 and available resources.
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50 NEHO study recruits pregnant women in four selected maternal units located in the public hospitals
51 of four cities: "G. Fogliani" Hospital in Milazzo (for Milazzo-Valle del Mela NCPS), General
52 Hospital in Lentini and "Umberto I" Hospital in Siracusa (for Augusta-Priolo NCPS), and "San
53 Giovanni di Dio" Hospital in Crotone.
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All the pregnant women presenting at one of the maternal units during the last two months of pregnancy are requested to participate – on voluntary basis – to the study to be followed-up until delivery. Thereafter, children will be followed from birth to 24 months of age to assess their exposure to toxicants through their own diet, and their physical environment, as well as their cognitive and behavioral development. In order to limit the possible bias due to the self-selection of enrolled women and the exclusive recruitment in public hospitals, we planned periodic meeting in birthing classes and with general practitioners.

Table 1 - The communities selected for the project in study and local reference areas with their respective distance from industrial areas.

National Priority Contaminated Sites	Study areas		Local reference areas	
	Name	km	Name	km
Milazzo - Valle del Mela	Milazzo	-	Barcellona P.d.G.	8
	Pace del Mela	6	Spadafora	13
	San Filippo del Mela	5	Terme Vigliatore	12
			Villafranca Tirrena	22
Augusta - Priolo	Augusta	9	Avola	27
	Floridia	8	Canicattini Bagni	19
	Melilli	5	Carlentini	22
	Priolo Gargallo	-	Lentini	22
	Solarino	8		
Crotone	Crotone	-	Botricello	28
			Cariati	49
			Crucoli and fractions	39
			Cirò Marina	32
			Isola di C. Rizzuto	14
			Melissa e frazioni	26
			S. Leonardo di Cutro	28
			Strongoli	20
Marina di Strongoli	23			

1
2
3 Figure 2 shows the selected cities and the distribution of the hospitals where the pregnant women are
4 recruited in each NPCS.

5
6 Because the recruitment of NEHO cohort is carried out in a heavily polluted area, detailed information
7 is also collected on daily commuting to work, use of electronic devices at home, dietary habits
8 (validated food frequency questionnaires are used) also including data on type of consumed water
9 and place of purchase of fish, meat, and vegetables, food packaging, use of plastic crockery and
10 detergents, etc.

11
12 After recruitment, mothers are asked to fill out the second part of baseline questionnaire by means a
13 web-based interface. The second part includes questions on health status, smoking habits, diet,
14 occupational exposures, and any other possible chemical exposures in the periconceptual period.
15
16

17 18 19 20 21 22 23 24 25 26 27 **Inclusion criteria**

28
29 The general criteria for combined residential and hospital-based recruitment of healthy pregnant
30 women are:
31

- 32 • to be resident in a study area or in a local reference area for at least one year;
- 33 • to be able to speech and understand Italian language;
- 34 • to be aged 18-40 years old at the time of delivery;
- 35 • to not have followed any program of assisted reproduction;
- 36 • absence of serious chronic diseases, such as diabetes, hypertension, etc.;
- 37 • absence of any evident complications during pregnancy diagnosed previously of the signature of
38 informed consent.
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51 52 53 **Questionnaire**

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55 Similarly to previous studies, NEHO questionnaire collects comprehensive information on pre-
56 pregnancy health status such as physical activity, lifestyle, stress factors, socio-demographic
57 characteristics, use of medication and information about previous births (including stillbirths). The
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1
2 same data are also collected as concerns gestational periods, along with smoking habits (including e-
3 cigarette use), possible chemical exposures, and maternal health characteristics.
4

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6
7 Subsequently, after delivery information is collected on newborn along two years: use of medicine,
8
9 nutritional outcomes (including growth and breastfeeding), neurocognitive development, infections
10
11 and injuries, hospitalizations, and characteristics of domestic environment.
12

13
14 In addition to the above listed variables, the NEHO cohort collects detailed information about diet:
15
16 origin of the food (if local or unknown) and where it is bought (local markets or organised large-scale
17
18 distribution), drinking water. Information on domestic and/or working environmental exposures is
19
20 also collected.
21
22

23 24 25 **Collection of biologic samples**

26
27 Biological material is collected, managed and briefly cryopreserved in the recruiting centers and
28
29 periodically transferred to the NEHO biobank, located at the Institute of Biomedicine and Molecular
30
31 Immunology (Palermo, Italy), to be stored at -80°C . All four maternity units follow the same protocol
32
33 for sample collection, processing, and storage. All the involved personal is periodically trained and
34
35 all protocols are updated at regular intervals.
36
37

38
39 Blood samples (15ml) are drawn from mother's cubital vein at enrollment and after delivery and from
40
41 the child's umbilical cord immediately after delivery (5ml in EDTA and 10ml in serum separator
42
43 tubes). Blood tubes are stored at 4°C and centrifuged within 24 hours for 10 minutes at 2,000
44
45 gravities. Serum is divided into 8 aliquots of approximately 0.5ml. Fractionated EDTA blood is
46
47 dispensed in 4 aliquots of plasma (0.5ml each). One aliquot of at least 0.1ml of white blood cells
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49 (buffy coat) is stored in cryotubes.
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52
53 In addition, 12 sections of placenta are systematically collected from central and peripheral region by
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55 means of a biopsy punch, stored in cryotubes with RNAlater and frozen at -20°C , within 2 hours from
56
57 delivery. Cryotubes with blood and cord samples are periodically transported in dry ice to the NEHO
58
59 biobank where they are stored in -80°C freezers. Placenta samples are also transferred to the central
60

1
2 biobank for long-term storage at -80°C. Incomplete sample collection is not considered as exclusion
3
4
5 criteria for follow up.

6
7 Figure 3 provides a schematic overview of the project's time course.
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10 11 **Biobank**

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14 The long-term perspective of the project and the possibly delayed evidence of children's impairment
15
16 in physical, psychological, social and cognitive health require the need for the creation of a biobank
17
18 for the storage of maternal and fetal tissues.

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21 Preservation of high-quality placental tissue specimens will enable the search for new biomarkers of
22
23 prenatal exposure to pollutants also promoting better understanding of the mechanisms through which
24
25 potential disruptors are transmitted from mother to fetus. This will possibly suggest interventions to
26
27 be taken during pregnancy for the prevention of some adult diseases.
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29

30 31 32 **Transcriptomics**

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34
35 Placenta has an active role on fetal development. Impairment in placental formation, differentiation
36
37 and/or function, severely affects fetal development and is associated with a wide range of pregnancy
38
39 complications, also resulting in pregnancy loss.[37] Other complications, linked with placental
40
41 dysfunction, including gestational diabetes, hypertension, pre-eclampsia and intrauterine growth
42
43 restriction could irreversibly result in greater susceptibility to multifactorial disorders during the
44
45 entire span of life.[38-40] It is known that toxic substances may interfere with placental signaling
46
47 cascades involved in metabolism, transport of nutrients and waste products, production and release
48
49 of steroid hormones and enzymes.[41] Therefore, in the context of NEHO cohort we will investigate
50
51 the relationship between exposure to environmental toxic compounds (both heavy metals and POPs)
52
53 and shift in gene expression by means of a whole transcriptome analysis. RNA microarray analysis
54
55 will be performed by Microarray SurePrint G3 Human Gene Expression v3 8x60k acquired by
56
57 G2565CA Microarray scanner Agilent. The raw data will be analysed using R biostatistical
58
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60

1
2 computing platform RStudio GUI. Gene set enrichment analysis will be performed to visualise
3 regulated biological processes.
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9 **Outcome assessment**

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11 At the time of delivery, a clinical evaluation is performed: delivery details, birth outcomes, infant's
12 anthropometry including birth weight, head circumference and APGAR score at 5 and 10 minutes are
13 recorded. Presence of any possible congenital defect is noted for each newborn.
14
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18 Children will be followed-up in the first two years of life through questionnaires via web at 6, 12, and
19 24 months: breastfeeding, nutritional supplements and vaccinations data are recorded by regular
20 parental surveys conducted via web.
21
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23
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26

27 Similarly to the second part of baseline questionnaire, the follow-up questionnaires (6 and 12 months)
28 are self-administered. If requested, telephone helpline service is provided to those experiencing
29 difficulties in on-line self-administered questionnaires.
30
31
32
33

34 Finally, at 24 months from birth, all participant will be invited to an infant clinical evaluation. A
35 trained psychologist will administer the Bayley-III test, including cognitive, language, and motor
36 scales. Physiological and behavioral development will be evaluated by means of CAT/CLAMS test
37 [43].
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43 Women are regularly contacted by phone or via email and invited to follow the timeline for
44 questionnaire submission.
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47

48 All the collected data are organized in a database and submitted to procedure for pseudonymization.

49
50 Table 2 shows the major outcome that will be evaluated in NEHO cohort.
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Table 2 – Outcomes of the study, separately for age, with their description.

Project stage	Outcome description	
At the birth	<ul style="list-style-type: none"> • Gestational age, • Weight, • Height, • Head Circumference • Apgar score (5', 10'), • Type of Delivery • Congenital Birth defects 	➤ Clinical evaluation
6 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • CAT/CLAMS part 1 	➤ Self-reported
12 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • CAT/CLAMS part 2 	➤ Self-reported
24 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • Barkley's scale of infant development 	➤ Clinical evaluation

Statistics

Primary analyses will concern the evaluation of possible differences in toxicant concentration in maternal blood, placental tissue and cord blood, by exploring the correlation between the toxicant concentrations in the different biological matrices. Moreover, the possible effect of confounders/effect modifiers will be evaluated for each toxicant by means of generalized linear models. Clinical outcomes will be evaluated by means of logistic models (for dichotomous variables) or generalized linear models (for continuous variables). Finally, subsamples of placental specimens will be evaluated for transcriptome analysis.

1
2 NEHO estimates to enroll a total of 800 mother-child pairs from the three highly polluted areas, within
3
4 January 2021. Sorkun et al (2007) found increased (0.048 ± 0.014 vs 0.038 ± 0.012) cadmium
5
6 concentration in placenta of Turkish women living in a highly polluted area with respect to women
7
8 living in a control rural area.[42] Founded on these data, we estimated that a sample of 38 subjects
9
10 for each study and control areas (76 subjects as total sample) will be adequate for detecting such
11
12 differences in placental heavy metals, with an alpha level of 0.05 and a power ($1-\beta$) of 0.95.
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18

19 **Aims**

20
21 NEHO is the first cohort in Italy to study and analyze pollutants blood levels in pregnant women
22
23 living nearby high polluted area and fetal exposure. NEHO will attempt to identify the influence of
24
25 risk factors on fetal genetics and on newborn outcomes.
26
27

28 Finally, NEHO will enroll mother-child pairs resident in areas disadvantaged by poor socio-economic
29
30 status thus representing a peculiar condition in the context of Italian and European birth cohorts.
31

32 Thus, on the basis of the collected data, NEHO cohort is aimed at:

- 33
34
35 • evaluating the risk of heavy metals and emerging contaminants during pregnancy in a cohort of
36
37 mother-child pairs resident in highly polluted areas characterized by different levels of environmental
38
39 pollution;
- 40
41
42 • evaluating the bioaccumulation features and patterns of toxicants by examining their distribution
43
44 among maternal, placental and fetal tissues;
- 45
46
47 • determining whether the bio-accumulation of toxicants might impact placental mRNA expression;
- 48
49
50 • understanding whether prenatal exposure to contaminants may cause negative pregnancy
51
52 outcomes and/or long-term effects on children health and disease predisposition;
- 53
54
55 • defining the associations of placental contamination and gene expression patterns with long-term
56
57 infant health outcomes, to evaluate the validity of placental analyses in predicting future infant health
58
59 outcomes.
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Patient and public involvement

The recruitment is based on healthy pregnant volunteers. Patients and public were neither involved in the study design nor in the establishment of questions and/or outcome definition. No individual result will be provided to participants unless possible impact for participants' health.

Ethics and dissemination

The collection of human tissues and the creation of a biobank for medical research involve important ethical and legal issues:[44] consequently, a complete explanation to participants of the details and the aims of the research project is a prerequisite before enrollment. To promote and guarantee respect for the pregnant woman's free choice, during a routine visit pregnant women receive a great deal of information about the collection and the storage of biologic samples until the child will be eighteen years old. The participants are required to sign a consent form to confirm complete understanding of project scope and to indicate their agreement to take part in the project. This includes knowing how to withdraw from the project at any time if they change their mind.

Qualified project staffs (biologists, midwives, nurses, gynecologists) provide complete information about the project and manage the completion of baseline questionnaire and the collection of blood samples. Aimed at making data totally comparable with previous large Italian birth cohorts, the questionnaire was developed from *Piccolipiù* questionnaire.[14]

After completing the questionnaire, a unique identification code is automatically assigned to each woman to identify the questionnaires and the biological samples collected during the study also preserving complete pseudonymization. The study is conducted following the Declaration of Helsinki. All the adopted procedures comply the General Data Protection Regulation (UE 2016/679) and the Italian law concerning data protection.

The results from the study will be disseminated to participants, to the local Regional Health Agency and to clinical professionals only on an aggregated basis through *ad hoc* meetings. The Researchers will also communicate results by means of peer-reviewed journals and scientific conferences.

DECLARATIONS

Ethics approval and consent to participate

The NEHO study protocol has been approved by the Ethics Committees which have responsibility the three involved NPCS: Ethics Committee of the University Hospitals of Messina for the NPCS of Milazzo-Valle del Mela (September 18, 2017, n. 9/2017); Ethics Committee “Catania 2” for the NPCS of Augusta-Priolo (July 11, 2017, n. 38/2017/CECT2); Ethics Committee of Regione Calabria for the NPCS of Crotona (July 20, 2017, n. 173). Each participant read the information sheet and signed the informed consent. Copies of the participants’ information sheet are available at the website: www.neho.it.

Availability of data and materials

Documentation on the study, on information sheet, and on informed consent are available at www.neho.it.

Competing interest

The authors declare that they have no competing interests.

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Authors’ contributions

SR, GD, PC, and FC made substantial contributions to conception and design of the study and are involved in study monitoring. They drafted and critically revised the manuscript for its intellectual

1
2 content, gave final approval of the version to be published, and agreed to be accountable for all aspects
3 of the work.
4

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6 AA, PA, ABi, ABu, PD, RDS, VLR, AL, LLP, BM, FP, GP, and AZC make substantial contributions
7 to acquisition of data and were involved in drafting the manuscript. Each of the authors read and
8 approved the final version of the manuscript.
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FIGURE LEGENDS

Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy.

Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. In red the National Priority Contaminated Sites, in green the surrounding areas.

Figure 3 - Schematic overview of the project's time course.

For peer review only



Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy

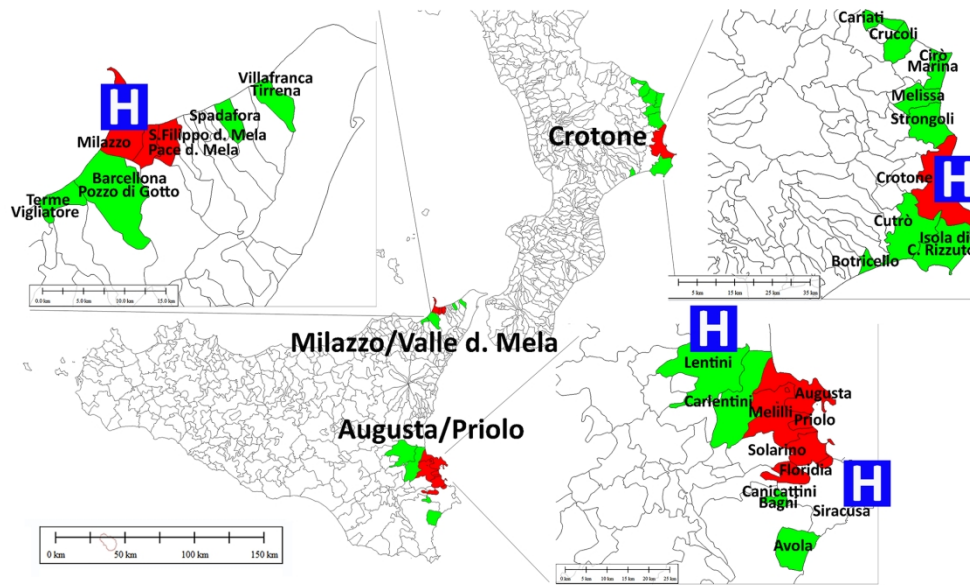


Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. In red the National Priority Contaminated Sites, in green the surrounding areas

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	7-9
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	9
		(b) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	12-13
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10-11
Bias	9	Describe any efforts to address potential sources of bias	8
Study size	10	Explain how the study size was arrived at	14
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	13
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	13
		(b) Describe any methods used to examine subgroups and interactions	13
		(c) Explain how missing data were addressed	
		(d) If applicable, explain how loss to follow-up was addressed	
		(e) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Protocol for a longitudinal birth cohort study in three contaminated sites in southern Italy: the Neonatal Environment and Health Outcome (NEHO) cohort

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Primary Subject Heading:	Public health

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Secondary Subject Heading:	Epidemiology
Keywords:	birth cohort study, maternal exposure, fetal exposure, polluted areas, Developmental Origins of Health and Disease



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3 **Protocol for a longitudinal birth cohort study in three contaminated sites in southern Italy:**
4
5 **the Neonatal Environment and Health Outcome (NEHO) cohort**
6

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Abstract

Introduction: Exposure to environmental contaminants during pregnancy is one of the determinants of the health outcomes of future child. The influence of environmental pollution on pregnant women living in heavily polluted areas is of special interest and, in this context, the Neonatal Environment and Health Outcome (NEHO) cohort will focus on the investigation of: i) toxicants transferred from the environment to the mother and from the mother to the developing fetus; ii) the influence of toxicants on pregnancy outcomes, fetal development, and health status during infancy. Because human placenta is positioned at the interface between the maternal/external environment and the embryo, it can be considered a highly informative matrix regarding many pregnancy key events that could shape babies' future health.

Methods and analysis: NEHO Cohort estimates to enroll a total of 800 pregnant women in three selected National Priority Contaminated Sites (NPCS) of southern Italy. Epidemiological data collection, concerning maternal health status, lifestyle, and pregnancy is obtained through survey questionnaires provided to the mother starting from the last two months of pregnancy. At the time of delivery, maternal blood, umbilical cord blood, and placenta tissue will be collected to assess contaminant levels and to clarify how toxicants interact with placental domain. Furthermore, placental transcriptome will be studied in order to explore the interferences of toxicant on the maternal/fetal interplay role of the placenta. Regular follow-up is planned at 6, 12, and 24 months.

Ethics and dissemination: The study has been approved by all the Ethics Committees of the three involved NPCS: Ethics Committee of the University Hospitals of Messina (September 18, 2017, n. 9/2017); Ethics Committee "Catania 2" (July 11, 2017, n. 38/2017/CECT2); Ethics Committee of Regione Calabria (July 20, 2017, n. 173). Findings will be disseminated in the scientific community and on a regional basis for appropriate policy actions.

Keywords: birth cohort study, maternal exposure, fetal exposure, highly polluted areas, Developmental Origins of Health and Disease

Strengths and limitations of this study:

- NEHO Cohort is a birth cohort study which, for the first time in Italy, was specifically aimed at evaluating the environment/health relationship in heavily polluted areas.
- In the context of NEHO Cohort the exposure will be assessed by means of the biomonitoring of pollutants in the biological samples from mother and child, along with an extensive and multidisciplinary evaluation of pollution in all the environmental matrices, including food chain.
- NEHO will enroll mother-child pairs resident in areas disadvantaged by poor socio-economic status thus representing a peculiar condition in the context of Italian and European birth cohorts.
- NEHO Cohort requires voluntary participation: this could constitute a bias due to the self-selection of enrolled women.
- The enrollment is limited to the catchment areas of public hospitals.

INTRODUCTION

Exposures occurring during early-life, such as environmental pollutants, dietary habits and parental lifestyles may have an effect on growth and development in fetal life and in childhood as well as on health across the life course. [1] Early childhood provides elements that may affect many outcomes in later life. [2] Consequently, epidemiologic studies on health effects of environmental pollution try to focus on the more vulnerable subjects: thus, in the last years considerable effort was made in the evaluation of the possible effects of environmental contaminants on children's health.

From Barker's postulate of "*intrauterine origins of health and disease susceptibility*", [3, 4] growing evidence has highlighted how the early stage of fetal development can alter the health trajectory throughout life. [5-8] This provides a better understanding of the cause of many multifactorial disorders. In fact, adult diseases may have an in utero origin, when suboptimal intrauterine conditions – including exposure to environmental contaminants – induce irreversible changes, which manifest themselves in post-natal and adult life. Birth cohorts provide an opportunity to monitor and to study associations between early-life environmental exposures and child development and health.[9] By means of a long-term follow-up, cohorts help to evaluate possible effects of exposure to environmental pollutants on development of adult diseases, also allowing the identification of risk factors, taking into account genetics, epigenetics, socio-economic factors, and lifestyles.

The number of studies linking maternal exposure to environmental pollutants during fetal period to various adult health outcomes is gradually increasing. Some Italian pregnancy and birth cohort studies investigate the health effects of environmental contaminant exposure during early-life with a specific interest on prenatal exposures, air pollution, growth, neurocognitive development, and respiratory health: NINFEA cohort,[10] NACII,[11] MUBICOS,[12] *Piccolipiù*, [13] GASPII and Co.N.ER., [14] and *Mamma & Bambino*. [15]

Epidemiologic evidence supports the concept that exposure to pollutants during early life – as measured during pregnancy and/or during childhood – has a detrimental impact on the health outcomes of the child. Previous works indicate that polybrominated diphenyl ethers (PBDE) and

1 polychlorinated biphenyls (PCB) bioaccumulate in human placenta tissue possibly contributing to
2 prenatal exposures to the environmental contaminants. [16-18] PBDEs – largely used as flame
3 retardants in electronic equipment, carpet, and in the polyurethane foam used in furniture – have been
4 detected in umbilical cord blood. [19-20] Because human placenta represents the interface between
5 maternal/external environment and embryo, it can be used as an environmental monitoring system.
6
7 In fact, placental examination constitutes an useful tool for estimating both maternal and fetal
8 exposures. [18, 21, 22] Moreover, placenta has an active role in the homeostasis of the intrauterine
9 environment and also mediates signal transmission from the fetus to the mother and vice versa.
10 Nutrition supply, endocrine/immune regulation, and gas exchange are orchestrated by the placenta.
11 All these evidences pone the placenta as a highly informative organ in the study of pregnancy.[23]
12
13 The presence of toxicants in the placenta can cause alterations of its structure and function along with
14 fetal development interference. An example is provided by exposure to cigarette smoking during
15 pregnancy: a modification of the gene expression of placental and fetal cells has been demonstrated
16 in relation to both direct and indirect tobacco smoke exposure.[24-26] High concentrations of
17 mercury in fetal tissues are associated with the reduction of hormone synthesis and the oxygen
18 consumption of by the placenta.[27,28]
19
20 Persistent organic compounds have been measured in fetal tissues and in particular in the placenta.
21 Exposure to PCBs and PBDEs has been shown to interfere with fetal development resulting in
22 significant weight reduction at birth.[29]
23
24 Umbilical cord blood and placenta are also noninvasive indicators for exposure to heavy metals, and
25 may be easily collected along with maternal blood.[30] Cadmium level in placenta is also a valuable
26 biomarker of metal dietary exposure related to specific dietary habits and soil characteristics. Lead
27 and mercury have been shown to be easily transferred through the placental and blood barriers.[28,
28
29 31]
30
31 Monitoring pollutants concentration in human tissues along with the extensive characterization of all
32 the environmental matrix proposed in CISAS project will provide new insights on the toxicants'

1
2 transfer routes from environment to human fetus. Attention will be focused on the investigation of
3
4 toxicants that are transferred from the mother's blood to the developing fetus and on the influence of
5
6 toxicants on fetal development, pregnancy outcomes, and late onset health consequences.
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9 Particular attention will be paid to the possible interaction between the environmental exposures and
10
11 the low socioeconomic status which often characterizes the investigated population.
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18 **METHODS AND ANALYSIS**

19 **Experimental context**

20
21 The CISAS project (International Centre of advanced Study in Environment, ecosystem and human
22
23 Health), funded by the Italian Ministry of Education, Universities and Research, aims at
24
25 understanding the chemical-physical processes that regulate the distribution of contaminants in the
26
27 various environmental matrices and their transfer to the ecosystem and the humans. CISAS Project is
28
29 developed in three selected National Priority Contaminated Sites (NPCS) of southern Italy: in its
30
31 context the influence of environmental pollution on pregnant women is of special interest.
32
33
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36 The three selected NPCS are: Milazzo-Valle del Mela, Augusta-Priolo, and Crotona. Those of
37
38 Milazzo and Augusta are wide industrialized coastal areas located in eastern Sicily, in which large
39
40 production sites are present: mainly refineries, petrochemical and cement plants, power plants,
41
42 numerous hazardous waste dumps and the former Eternit plant in Syracuse where asbestos was
43
44 processed.[32] In Crotona area, located in the region of Calabria, the most relevant environmental
45
46 impact is due to three disused industrial areas (former Pertusola, former Fosfotec, and former
47
48 Agricoltura) which operated between the 1920s and the 1990s, mainly in the field of production of
49
50 zinc, phosphoric acid, and complex fertilizers.[33]
51
52
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54

55 During the course conduct of CISAS project, the birth cohort NEHO (Neonatal Environment and
56
57 Health Outcomes) will enroll pregnant women living in the three NPCS, along with pregnant women
58
59 living in surrounding areas, outside the NPCS. NEHO cohort is aimed at understanding processes and
60

1
2 mechanisms for the transfer of heavy metals and POPs (e.g., PBDEs, PCBs, chlorinated pesticides)
3
4 from the environment to the ecosystem and to humans. In the context of CISAS Project, the same
5
6 toxicants will be evaluated in all the environmental matrices (atmosphere, soil, sediment, inland
7
8 waters and sea) and in the food chain (fish, meat, eggs, milk and dairy products, sampled from local
9
10 producers of each studied area).
11
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16 **Study population and recruitment**

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18 From January 2018, NEHO cohort started to recruit pregnant women living in the three selected
19
20 NPCS of Crotona, Milazzo-Valle del Mela and Augusta-Priolo, in southern Italy (Figure 1), along
21
22 with pregnant women living in surrounding areas presenting similar geographic and socio-
23
24 demographic characteristics.
25
26

27
28 The NEHO study involves twenty-six cities subdivided in study areas and local reference areas. Table
29
30 1 shows the selected cities in Sicily and Calabria and their respective distance from industrial areas.
31
32 Local reference areas were identified as “local control” by ISTISAN reports[32, 34] and by
33
34 SENTIERI Project, a study on the epidemiological evidence on the association between causes of
35
36 death and environmental exposures,[35-36] coordinated by the Italian National Institute of Health
37
38 and supported by the Ministry of Health. Aimed at maximizing the recruitment efficiency, in each
39
40 NPCS, maternal units were selected on the basis of both deliveries/population ratio and available
41
42 resources.
43
44

45
46 NEHO study recruits pregnant women in four selected maternal units located in the public hospitals
47
48 of four cities: “G. Fogliani” Hospital in Milazzo (for Milazzo-Valle del Mela NCPS), General
49
50 Hospital in Lentini and “Umberto I” Hospital in Siracusa (for Augusta-Priolo NCPS), and “San
51
52 Giovanni di Dio” Hospital in Crotona.
53

54
55 All the pregnant women attending one of the maternal units during the last two months of pregnancy
56
57 are requested to participate – on voluntary basis – to the study to be followed-up until delivery.
58
59 Thereafter, children will be followed from birth to 24 months of age to assess their exposure to
60

1
2 toxicants through their own diet, and their physical environment, as well as their cognitive and
3 behavioral development. In order to limit the possible bias due to the self-selection of enrolled women
4 and the exclusive recruitment in public hospitals, we planned periodic meeting in birthing classes and
5 with general practitioners.
6
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14 **Table 1** - The communities selected for the project in study and local reference areas with their
15 respective distance from industrial areas.
16

National Priority Contaminated Sites	Study areas		Local reference areas	
	Name	km	Name	km
Milazzo - Valle del Mela	Milazzo	-	Barcellona P.d.G.	8
	Pace del Mela	6	Spadafora	13
	San Filippo del Mela	5	Terme Vigliatore	12
			Villafranca Tirrena	22
Augusta - Priolo	Augusta	9	Avola	27
	Floridia	8	Canicattini Bagni	19
	Melilli	5	Carlentini	22
	Priolo Gargallo	-	Lentini	22
	Solarino	8		
Crotona	Crotona	-	Botricello	28
			Cariati	49
			Crucoli and fractions	39
			Cirò Marina	32
			Isola di C. Rizzuto	14
			Melissa e frazioni	26
			S. Leonardo di Cutro	28
			Strongoli	20
Marina di Strongoli	23			

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Figure 2 shows the selected cities and the distribution of the hospitals where the pregnant women are recruited in each NPCS.

Because the recruitment of NEHO cohort is carried out in a heavily polluted area, detailed information is also collected on daily commuting to work, use of electronic devices at home, dietary habits

(validated food frequency questionnaires are used) also including data on type of consumed water and place of purchase of fish, meat, and vegetables, food packaging, use of plastic crockery and detergents, etc.

After recruitment, mothers are asked to fill out the second part of baseline questionnaire by means a web-based interface. The second part includes questions on health status, smoking habits, diet, occupational exposures, and any other possible chemical exposures in the periconceptual period.

Inclusion criteria

The general criteria for combined residential and hospital-based recruitment of healthy pregnant women are:

- to be resident in a study area or in a local reference area for at least one year;
- to be able to speech and understand Italian language;
- to be aged 18-40 years old at the time of delivery;
- to not have followed any program of assisted reproduction;
- absence of serious chronic diseases, such as diabetes, hypertension, etc.;
- absence of any evident complications during pregnancy diagnosed previously of the signature of informed consent.

Questionnaire

Similarly to previous studies, NEHO questionnaire collects comprehensive information on pre-pregnancy health status such as physical activity, lifestyle, stress factors, socio-demographic characteristics, use of medication and information about previous births (including stillbirths). The same data are also collected as concerns gestational period, along with smoking habit (including e-cigarette use), possible chemical exposures, and maternal health characteristics.

1
2 Subsequently, after delivery, information is collected on newborn along two years: use of medicine,
3
4 nutritional outcomes (including growth and breastfeeding), neurocognitive development, infections
5
6 and injuries, hospitalizations, and characteristics of domestic environment.
7
8

9 In addition to the above listed variables, the NEHO cohort collects detailed information about diet:
10
11 origin of the food (if local or unknown) and where it is bought (local markets or organised large-scale
12
13 distribution), drinking water. Information on domestic and/or working environmental exposures is
14
15 also collected.
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20 **Collection of biological samples**

21
22 Biological material is collected, managed and briefly cryopreserved in the recruiting centers and
23
24 periodically transferred to the NEHO biobank, located at the Institute of Biomedicine and Molecular
25
26 Immunology (Palermo, Italy), to be stored at -80°C . All four maternity units follow the same protocol
27
28 for sample collection, processing, and storage. All the personnel involved is periodically trained and
29
30 all protocols are updated at regular intervals.
31
32

33
34 Blood samples (15ml) are drawn from mother's cubital vein at enrollment and after delivery and from
35
36 the child's umbilical cord immediately after delivery (5ml in K2-EDTA and 10ml in serum separator
37
38 tubes). Blood tubes are stored at 4°C and centrifuged within 24 hours for 10 minutes at 2,000
39
40 gravities. Serum is divided into 8 aliquots of approximately 0.5ml. Fractionated K2-EDTA blood is
41
42 dispensed in 4 aliquots of plasma (0.5ml each). One aliquot of at least 0.1ml of white blood cells
43
44 (buffy coat) is stored in cryotubes.
45
46

47
48 In addition, 12 samples of placenta are systematically collected from central and peripheral region by
49
50 means of a biopsy punch, stored in cryotubes with RNAlater and frozen at -20°C , within 2 hours from
51
52 delivery. Cryotubes with maternal and cord blood samples are periodically transported in dry ice to
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54 the NEHO biobank where they are stored in -80°C freezers. Placenta samples are also transferred to
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56 the central biobank for long-term storage at -80°C . Incomplete sample collection is not considered as
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58 exclusion criteria for follow up.
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3 Figure 3 provides a schematic overview of the project's time course.
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7 **Biobank**

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9 The long-term perspective of the project and the possibly delayed evidence of children's impairment
10 in physical, psychological, social and cognitive health require the need for the creation of a biobank
11 for the storage of maternal and fetal tissues.
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16 Preservation of high-quality placental tissue specimens will enable the search for new biomarkers of
17 prenatal exposure to pollutants also promoting better understanding of the mechanisms through which
18 potential disruptors are transmitted from mother to fetus. This will possibly suggest interventions to
19 be taken during pregnancy for the prevention of some adult diseases.
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26 **Transcriptomics**

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28 Placenta has an active role on fetal development. Impairment in placental formation, differentiation
29 and/or function, severely affects fetal development and is associated with a wide range of pregnancy
30 complications, also resulting in pregnancy loss.[37] Other complications, linked with placental
31 dysfunction, including gestational diabetes, hypertension, pre-eclampsia and intrauterine growth
32 restriction could irreversibly result in greater susceptibility to multifactorial disorders during the
33 entire span of life.[38-40] It is known that toxic substances may interfere with placental signaling
34 cascades involved in metabolism, transport of nutrients and waste products, production and release
35 of steroid hormones and enzymes.[41] Therefore, in the context of NEHO cohort we will investigate
36 the relationship between exposure to environmental toxic compounds (both heavy metals and POPs)
37 and shift in gene expression by means of a whole transcriptome analysis. RNA microarray analysis
38 will be performed by Microarray SurePrint G3 Human Gene Expression v3 8x60k acquired by
39 G2565CA Microarray scanner Agilent. The raw data will be analysed using R biostatistical
40 computing platform RStudio GUI. Gene set enrichment analysis will be performed to visualise
41 regulated biological processes.
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Outcome assessment

At the time of delivery, a clinical evaluation is performed: delivery details, birth outcomes, infant's anthropometry including birth weight, head circumference and APGAR score at 5 and 10 minutes are recorded. Presence of any possible congenital defect is noted for each newborn.

Children will be followed-up during the first two years of life through web-administered questionnaires at 6, 12, and 24 months: breastfeeding, nutritional supplements and vaccinations data are recorded by regular parental surveys conducted via web.

Similarly to the second part of baseline questionnaire, the follow-up questionnaires (6 and 12 months) are self-administered. If requested, telephone helpline service is provided to those experiencing difficulties in on-line self-administered questionnaires.

Women are regularly contacted by phone or via email and invited to follow the timeline for questionnaire submission.

Finally, at 24 months from birth, all participant will be invited to an infant clinical evaluation. A trained psychologist will administer the Bayley-III test, including cognitive, language, and motor scales. Physiological and behavioral development will be evaluated by means of CAT/CLAMS test[42].

Long-term outcomes will be evaluated by means of regional health records providing information on causes of hospitalization and death.

All the collected data are organized in a database and submitted to procedure for pseudonymization.

Table 2 shows the major outcomes that will be evaluated in NEHO cohort.

Table 2 – Outcomes of the study, separately for age, with their description.

Project stage	Outcome description	
At the birth	<ul style="list-style-type: none"> • Gestational age, • Weight, • Height, • Head Circumference • Apgar score (5', 10'), • Type of Delivery • Congenital Birth defects 	➤ Clinical evaluation
6 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • CAT/CLAMS part 1 	➤ Self-reported
12 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • CAT/CLAMS part 2 	➤ Self-reported
24 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • Barkley's scale of infant development 	➤ Clinical evaluation

Statistics

Primary analyses will concern the evaluation of possible differences in toxicant concentration in maternal blood, placental tissue and cord blood, by exploring the correlation between the toxicant concentrations in the different biological matrices. Moreover, the possible effect of confounders/effect modifiers will be evaluated for each toxicant by means of generalized linear models. Clinical outcomes will be evaluated by means of logistic models (for dichotomous variables) or generalized linear models (for continuous variables). Finally, subsamples of placental specimens will be evaluated for transcriptome analysis.

1
2 NEHO estimates to enroll a total of 800 mother-child pairs from the three highly polluted areas, within
3
4 January 2021. Sorkun et al (2007) found increased (0.048 ± 0.014 vs 0.038 ± 0.012) cadmium
5
6 concentration in placenta of Turkish women living in a highly polluted area with respect to women
7
8 living in a control rural area.[43] Founded on these data, we estimated that a sample of 38 subjects
9
10 for each study and control areas (76 subjects as total sample) will be adequate for detecting such
11
12 differences in placental heavy metals, with an alpha level of 0.05 and a power ($1-\beta$) of 0.95. Moreover,
13
14 García-Esquinas et al (2013) found significantly reduced 5-minutes Apgar score (9.13 ± 0.6 vs
15
16 9.40 ± 0.5) in newborns with umbilical cadmium levels >0.30 $\mu\text{g/l}$.[44] From these data, we estimated
17
18 that a sample of 129 subjects for each study and control areas (258 subjects as total sample) will be
19
20 adequate for detecting such differences in 5-minutes Apgar score, with an alpha level of 0.05 and a
21
22 power ($1-\beta$) of 0.95.
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31 **Aims**

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33 NEHO is the first cohort in Italy to study and analyze pollutant blood levels in pregnant women living
34
35 nearby high polluted area along with fetal exposure. NEHO will attempt to identify the influence of
36
37 environmental risk factors on placental function and on pregnancy outcomes and newborns' health
38
39 outcomes.
40

41
42 Finally, NEHO will enroll mother-child pairs resident in areas disadvantaged by poor socio-economic
43
44 status thus representing a particular condition in the context of Italian and European birth cohorts.
45

46
47 Thus, on the basis of the collected data, NEHO cohort is aimed at:

- 48 • evaluating the risk of heavy metals and emerging contaminants during pregnancy in a cohort of
49
50 mother-child pairs resident in highly polluted areas characterized by different levels of environmental
51
52 pollution;
- 53 • evaluating the bioaccumulation features and patterns of toxicants by examining their distribution
54
55 among maternal, placental and fetal tissues;
- 56 • determining whether the bio-accumulation of toxicants might impact placental mRNA expression;
- 57
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- understanding whether prenatal exposure to contaminants may cause negative pregnancy outcomes and/or long-term effects on children health and disease susceptibility;
- defining the associations of placental contamination and gene expression patterns with long-term infant health outcomes, to evaluate the validity of placental analyses in predicting future infant health outcomes.

Patient and public involvement

The recruitment is based on healthy pregnant volunteers. Patients and public were neither involved in the study design nor in the establishment of questions and/or outcome definition. No individual result will be provided to participants unless possible impact for participants' health.

Ethics and dissemination

The collection of human tissues and the creation of a biobank for medical research involve important ethical and legal issues:[45] consequently, a complete explanation to participants of the details and the aims of the research project is a prerequisite before enrollment. To promote and guarantee respect for the pregnant woman's free choice, during a routine visit pregnant women receive a great deal of information about the collection and the storage of biological samples until the child will be eighteen years old. The participants are required to sign a consent form to confirm complete understanding of project scope and to indicate their agreement to take part in the project. This includes knowing how to withdraw from the project at any time if they change their mind.

Qualified project staffs (biologists, midwives, nurses, gynecologists) provide complete information about the project and manage the completion of baseline questionnaire and the collection of blood samples. Aimed at making data totally comparable with previous large Italian birth cohorts, the questionnaire was developed from *Piccolipiù* questionnaire.[14]

After completing the questionnaire, a unique identification code is automatically assigned to each woman to identify the questionnaires and the biological samples collected during the study also

1
2 preserving complete pseudonymization. The study is conducted following the Declaration of
3
4 Helsinki. All the adopted procedures comply the General Data Protection Regulation (UE 2016/679)
5
6 and the Italian law concerning data protection.
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9 The results from the study will be disseminated to participants, to the local Regional Health Agency
10
11 and to clinical professionals only on an aggregated basis through *ad hoc* meetings. The Researchers
12
13 will also communicate results by means of peer-reviewed journals and scientific conferences.
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21 **DECLARATIONS**

22 **Ethics approval and consent to participate**

23
24 The NEHO study protocol has been approved by the Ethics Committees which have responsibility
25
26 for the three involved NPCCS: Ethics Committee of the University Hospitals of Messina for the NPCCS
27
28 of Milazzo-Valle del Mela (September 18, 2017, n. 9/2017); Ethics Committee “Catania 2” for the
29
30 NPCCS of Augusta-Priolo (July 11, 2017, n. 38/2017/CECT2); Ethics Committee of Regione Calabria
31
32 for the NPCCS of Crotona (July 20, 2017, n. 173). Each participant read the information sheet and
33
34 signed the informed consent. Copies of the participants’ information sheet are available at the website:
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37 www.neho.it.
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44 **Availability of data and materials**

45
46 Documentation on the study, information sheet, and informed consent are available at www.neho.it.
47
48
49

50 **Competing interest**

51
52 The authors declare that they have no competing interests.
53
54
55
56

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1
2
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5 Committee for Economic Planning (CIPE) – body of the Italian Government – with Resolution no.
6
7 105/2015 of December 23, 2015.
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13 14 **Authors' contributions**

15
16 SR, GD, PC, and FC made substantial contributions to conception and design of the study and are
17 involved in study monitoring. They drafted and critically revised the manuscript for its intellectual
18 content, gave final approval of the version to be published, and agreed to be accountable for all aspects
19 of the work.
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25 AA, PA, ABi, ABu, PD, RDS, VLR, AL, LLP, BM, FP, GP, and AZC make substantial contributions
26 to acquisition of data and were involved in drafting the manuscript. Each of the authors read and
27 approved the final version of the manuscript.
28
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32

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35
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37 questionnaires and structure of the study.
38
39

40
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42 and behavioral development.
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For peer review only

FIGURE LEGENDS

Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=2ca75003ef9d477fb22db19832c9554f>. Last accessed March 29, 2019.

Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. In red the National Priority Contaminated Sites, in green the surrounding areas. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=61145ee86375431f9c54762de4ccd9e7>

Figure 3 - Schematic overview of the project's time course.

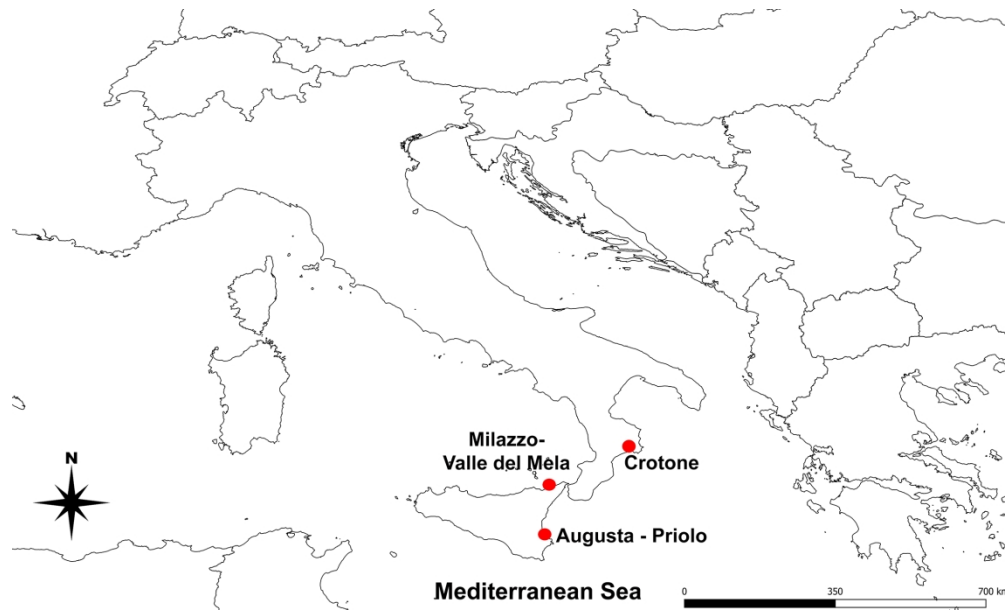


Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=2ca75003ef9d477fb22db19832c9554f>. Last accessed March 29, 2019

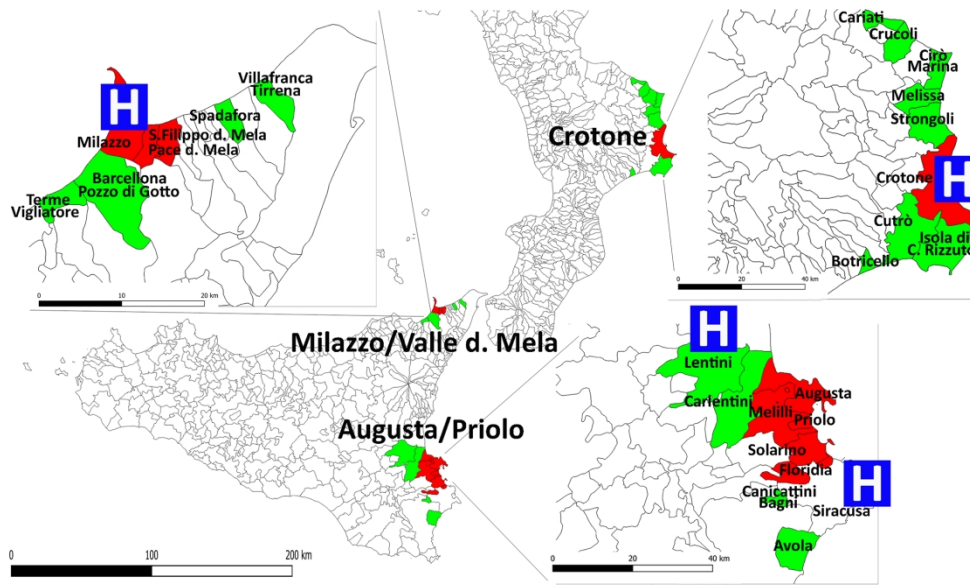


Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. In red the National Priority Contaminated Sites, in green the surrounding areas. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=61145ee86375431f9c54762de4ccd9e7>

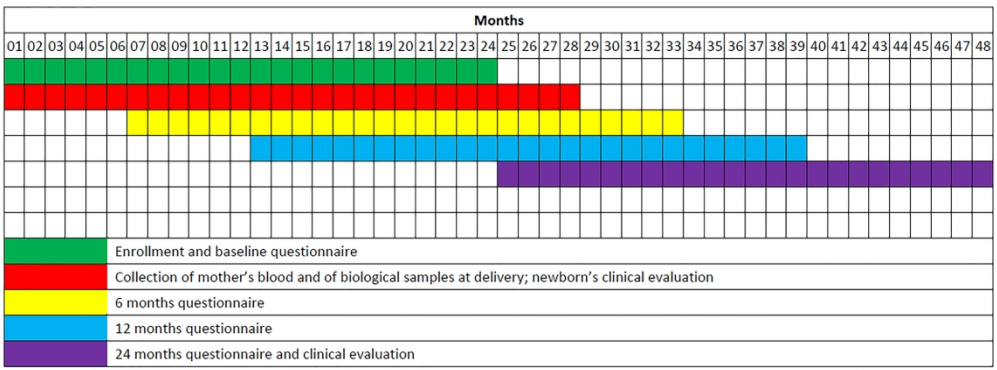


Figure 3 - Schematic overview of the project's time course

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	7-9
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	9
		(b) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	12-13
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10-11
Bias	9	Describe any efforts to address potential sources of bias	8
Study size	10	Explain how the study size was arrived at	14
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	13
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	13
		(b) Describe any methods used to examine subgroups and interactions	13
		(c) Explain how missing data were addressed	
		(d) If applicable, explain how loss to follow-up was addressed	
		(e) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Protocol for a longitudinal birth cohort study in three contaminated sites in southern Italy: the Neonatal Environment and Health Outcomes (NEHO) cohort

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Primary Subject Heading:	Public health

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Secondary Subject Heading:	Epidemiology
Keywords:	birth cohort study, maternal exposure, fetal exposure, polluted areas, Developmental Origins of Health and Disease



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3 **Protocol for a longitudinal birth cohort study in three contaminated sites in southern Italy:**
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5 **the Neonatal Environment and Health Outcomes (NEHO) cohort**
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Abstract

Introduction: Exposure to environmental contaminants during pregnancy is one of the determinants of child's future health outcomes. The effects of environmental pollution on pregnant women living in heavily polluted areas is of special interest and, in this context, the Neonatal Environment and Health Outcomes (NEHO) cohort will focus on the investigation of: i) toxicants transferred from the environment to the mother and from the mother to the developing fetus; ii) the influence of toxicants on pregnancy outcomes, fetal development, and health status during infancy. Because the human placenta is positioned at the interface between the maternal/external environment and the embryo, it can be considered a highly informative matrix regarding many key pregnancy events that can shape infant's future health.

Methods and analysis: The NEHO cohort will enroll an estimated total of 800 pregnant women in three selected National Priority Contaminated Sites in southern Italy. Epidemiological data collection, concerning maternal health status, lifestyle, and pregnancy are obtained through questionnaires provided to the mother starting from the last two months of pregnancy. At delivery, maternal blood, umbilical cord blood, and placenta tissue are collected to assess contaminant levels and to clarify how toxicants interact with the placental domain. Furthermore, placental transcriptome is studied in order to explore the interferences of toxicants on the role of the placenta in maternal/fetal interplay. Regular follow-up is planned at 6, 12, and 24 months.

Ethics and dissemination: The study has been approved by all the Ethics Committees of the three NPCSSs involved: the Ethics Committee of the University Hospitals of Messina (September 18, 2017, n. 9/2017); the Ethics Committee "Catania 2" (July 11, 2017, n. 38/2017/CECT2); the Ethics Committee of the Region of Calabria (July 20, 2017, n. 173). Findings will be disseminated in the scientific community and on a regional basis for appropriate policy actions.

Keywords: birth cohort study, maternal exposure, fetal exposure, highly polluted areas, Developmental origins of health and disease

Strengths and limitations of this study:

- The NEHO Cohort is a birth cohort study which, for the first time in Italy, is specifically aimed at evaluating the environment/health relationship in heavily polluted areas.
- In the context of the NEHO cohort exposure is assessed by means of the biomonitoring of pollutants in biological samples from mother and child, along with an extensive and multidisciplinary evaluation of pollution in all environmental matrices, including the food chain.
- NEHO enrolls mother-child pairs residing in disadvantaged areas of low socio-economic status thus representing a peculiar condition in the context of Italian and European birth cohorts.
- The NEHO cohort requires voluntary participation, which could constitute a bias due to the self-selection of enrolled women.
- The enrollment is limited to the catchment areas of public hospitals.

INTRODUCTION

Early-life exposure to factors such as environmental pollutants, dietary habits and parental lifestyles may affect growth and development during fetal life and childhood as well as influence health over a person's entire lifetime.[1] Early childhood is particularly important as it is a period which involves contact with factors that may affect many outcomes in later life.[2] Consequently, epidemiological studies on the health effects of environmental pollution typically focus on the most vulnerable subjects: thus, in recent years, considerable effort has been made to evaluate the possible effects of environmental contaminants on children's health.

From Barker's postulate of the "*intrauterine origins of health and disease susceptibility*",[3, 4] growing evidence has highlighted how the early stage of fetal development can alter the health trajectory throughout life.[5-8] This provides a better understanding of the causes of many multifactorial disorders. In fact, adult diseases may have an in utero origin, when suboptimal intrauterine conditions—including exposure to environmental contaminants—induce irreversible changes which manifest themselves in post-natal and adult life. Birth cohorts provide an opportunity to monitor and study associations between early-life environmental exposures and child development and health.[9] By means of a long-term follow-up, cohorts help to evaluate the possible effects of exposure to environmental pollutants on the development of adult diseases, also allowing the identification of risk factors, taking into account genetics, epigenetics, socio-economic factors, and lifestyles.

The number of studies linking maternal exposure to environmental pollutants during fetal gestation to various adult health outcomes has been gradually increasing. Several Italian pregnancy and birth cohort studies have investigated the health effects of environmental contaminant exposure during early-life, with specific attention to prenatal exposures, air pollution, growth, neurocognitive development, and respiratory health: NINFEA cohort,[10] NACII,[11] MUBICOS,[12] *Piccolipiù*,[13] GASPII and Co.N.ER.,[14] and *Mamma & Bambino*. [15]

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Epidemiological evidence supports the concept that exposure to pollutants during early life—as measured during pregnancy and/or childhood—has a detrimental impact on the health outcomes of the child. Previous works indicate that polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) bioaccumulate in human placenta tissue, possibly contributing to prenatal exposures to environmental contaminants.[16-18] PBDEs—largely used as flame retardants in electronic equipment, carpets, and in polyurethane foam used in furniture—have been detected in umbilical cord blood.[19-20] Because the human placenta is the interface between the maternal/external environment and the embryo, it can be used as an environmental monitoring system. In fact, placental examination is a useful tool for estimating both maternal and fetal exposures.[18, 21, 22] Moreover, the placenta plays an active role in the homeostasis of the intrauterine environment and also mediates signal transmission from the fetus to the mother and vice versa. Nutrition supply, endocrine/immune regulation, and gas exchange are orchestrated by the placenta. All of this evidence makes the placenta a highly informative organ for the study of pregnancy.[23]

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The presence of toxicants in the placenta can cause alterations of its structure and function as well as interfere with fetal development. An example is provided by exposure to cigarette smoking during pregnancy: a modification of the gene expression of placental and fetal cells has been demonstrated in relation to both direct and indirect tobacco smoke exposure.[24-26] High concentrations of mercury in fetal tissues are associated with the reduction of hormone synthesis and oxygen consumption by the placenta.[27,28]

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Persistent organic compounds have been measured in fetal tissues, in particular in the placenta. Exposure to PCBs and PBDEs has been shown to interfere with fetal development, resulting in significant weight reduction at birth.[29]

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Umbilical cord blood and the placenta are also noninvasive indicators for exposure to heavy metals, and may be easily collected along with maternal blood.[30] The cadmium level in the placenta is also a valuable biomarker of dietary metal exposure related to specific dietary habits and soil

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2 characteristics. Lead and mercury have been shown to be easily transferred through the placental and
3
4 blood barriers.[28, 31]
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7 Monitoring pollutant concentrations in human tissues, along with the extensive characterization of
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9 the entire environmental matrix proposed in the International Centre of Advanced Study in
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11 Environment, Ecosystem and Human Health (CISAS) project will provide new insights into toxicant
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13 transfer routes from the environment to the human fetus. Our analysis will be focused on the
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15 investigation of toxicants that are transferred from the mother's blood to the developing fetus and on
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17 the influence of toxicants on fetal development, pregnancy outcomes, and late-onset health
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19 consequences. Particular attention will also be paid to the possible interaction between environmental
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21 exposures and the low socioeconomic status which often characterizes the investigated population.
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30 **METHODS AND ANALYSIS**

31 **Experimental context**

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33 The CISAS project, funded by the Italian Ministry of Education, Universities and Research, aims at
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35 understanding the chemical-physical processes that regulate the distribution of contaminants in
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37 various environmental matrices and their transfer to the ecosystem and humans. The CISAS Project
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39 has been developed for three selected National Priority Contaminated Sites (NPCSs) in southern Italy:
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41 in these contexts, the influence of environmental pollution on pregnant women is of special interest.
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43 The three selected NPCSs are: Milazzo-Valle del Mela, Augusta-Priolo, and Crotona. Those in
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45 Milazzo and Augusta are widely industrialized coastal areas located in eastern Sicily in which large
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47 production sites are present, including refineries, petrochemical and cement plants, power plants,
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49 numerous hazardous waste dumps, and the former Eternit plant in Siracusa where asbestos was
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51 processed.[32] In the Crotona area, located in the region of Calabria, the most relevant environmental
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53 impact is due to three disused industrial areas (the former Pertusola, Fosfotec, and Agricoltura sites)
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2 which operated between the 1920s and the 1990s, mainly in the fields of zinc, phosphoric acid, and
3 complex fertilizer production.[33]
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6 During the course of the (ongoing) CISAS project, the Neonatal Environment and Health Outcomes
7 birth cohort (NEHO) birth cohort will enroll pregnant women living in the three NPCSSs, along with
8 pregnant women living in surrounding areas, outside them. The NEHO cohort is aimed at
9 understanding the processes and mechanisms involved in the transfer of heavy metals and Persistent
10 Organic Pollutants (POPs, e.g., PBDEs, PCBs, chlorinated pesticides) from the environment to the
11 ecosystem and humans. In the context of the CISAS project, the same toxicants will be evaluated in
12 all environmental matrices (atmosphere, soil, sediment, inland waters and sea) as well as the food
13 chain (fish, meat, eggs, milk and dairy products, sampled from local producers of each studied area).
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28 **Study population and recruitment**

29 Starting in January 2018, the NEHO cohort began recruiting pregnant women living in the three
30 NPCSSs of Crotona, Milazzo-Valle del Mela and Augusta-Priolo, in southern Italy (Figure 1), along
31 with pregnant women living in surrounding areas presenting similar geographic and socio-
32 demographic characteristics.
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39 The NEHO study involves twenty-six cities subdivided into study areas and local reference areas.
40 Table 1 shows the selected cities in Sicily and Calabria, as well as their respective distance from
41 industrial areas.
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46 Local reference areas were identified as “local controls” by ISTISAN reports[32, 34] and by the
47 SENTIERI Project, a study of the epidemiological evidence of associations between causes of death
48 and environmental exposures[35-36] coordinated by the Italian National Institute of Health and
49 supported by the Ministry of Health. To maximize recruitment efficiency, maternity units were
50 selected in each NPCSS on the basis of both the deliveries/population ratio and available resources.
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57 The NEHO cohort recruits pregnant women in four selected maternity units located in the public
58 hospitals of four cities: the “G. Fogliani” Hospital in Milazzo (for the Milazzo-Valle del Mela NPCSS),
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the General Hospital of Lentini and the “Umberto I” Hospital in Siracusa (for the Augusta-Priolo NPC), and the “San Giovanni di Dio” Hospital in Crotone.

Table 1 - The communities selected for the project in both study and local reference areas, with their respective distance from industrial areas.

National Priority Contaminated Sites	Study areas		Local reference areas	
	Name	km	Name	km
Milazzo - Valle del Mela	Milazzo	-	Barcellona P.d.G.	8
	Pace del Mela	6	Spadafora	13
	San Filippo del Mela	5	Terme Vigliatore	12
Augusta - Priolo			Villafranca Tirrena	22
	Augusta	9	Avola	27
	Floridia	8	Canicattini Bagni	19
	Melilli	5	Carlentini	22
	Priolo Gargallo	-	Lentini	22
Crotone	Solarino	8		
	Crotone	-	Botricello	28
			Cariati	49
			Crucoli and local districts	39
				32
			Cirò Marina	14
			Isola di C. Rizzuto	26
			Melissa and local districts	28
				20
S. Leonardo di Cutro			23	
Strongoli				
Marina di Strongoli				

All pregnant women treated at one of the maternity units during the last two months of pregnancy are asked to participate—on a voluntary basis—in the study, which means receiving follow-up visits until delivery. Thereafter, the children are followed from birth to 24 months of age to assess their exposure to toxicants through their diet, and physical environment, as well as their cognitive and behavioral

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3 development. In order to limit possible bias due to the self-selection of enrolled women and exclusive
4
5 recruitment in public hospitals, we have organized periodic meetings in birthing classes and with
6
7 general practitioners.
8

9
10 Figure 2 shows the selected cities and the distribution of the hospitals where the pregnant women are
11
12 recruited in each NPCCS.
13

14
15 Because the recruitment of the NEHO cohort is carried out in a heavily polluted area, detailed
16
17 information is also collected on daily commuting to work, use of electronic devices at home, dietary
18
19 habits (validated food frequency questionnaires are used), including data on the type of water
20
21 consumed and the place of fish, meat, and vegetable purchases, food packaging, use of plastic dishes,
22
23 detergents, etc.
24

25
26 After recruitment, mothers are asked to fill out the second part of the baseline questionnaire by means
27
28 of a web-based interface. The second part includes questions on health status, smoking habits, diet,
29
30 occupational exposures, and any other possible chemical exposures in the periconceptual period.
31
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35 **Inclusion criteria**

36
37 The general criteria for combined residential and hospital-based recruitment of healthy pregnant
38
39 women are:
40

- 41 • residence in a study area or a local reference area for at least one year;
- 42
- 43 • ability to speak and understand the Italian language;
- 44
- 45 • being 18-40 years old at the time of delivery;
- 46
- 47 • not following any program of assisted reproduction;
- 48
- 49 • absence of serious chronic diseases, such as diabetes, hypertension, etc.;
- 50
- 51 • absence of any evident complications during pregnancy diagnosed previous to signing informed
52
53 consent.
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60 **Questionnaire**

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2
3 As in previous studies, the NEHO questionnaire collects comprehensive information on pre-
4 pregnancy health status, such as physical activity, lifestyle, stress factors, socio-demographic
5 characteristics, use of medication, and information about previous births (including stillbirths). The
6
7 same data are also collected for the gestational period, along with smoking habits (including e-
8
9 cigarette use), possible chemical exposures, and maternal health characteristics.
10
11

12
13 Subsequently, after delivery, information is collected on newborns over a two-year period regarding:
14
15 use of medicine, nutritional outcomes (including growth and breastfeeding), neurocognitive
16
17 development, infections and injuries, hospitalizations, and characteristics of home environments.
18
19

20
21 In addition to the above-listed variables, the NEHO cohort collects detailed information about diet:
22
23 the origin of the food (whether local or unknown) and place of purchase (local markets or large
24
25 supermarket chains), as well as information about drinking water. Information on domestic and/or
26
27 working environment exposures is also collected.
28
29

30 31 **Collection of biological samples**

32
33 Biological material is collected, managed, and briefly cryopreserved in the recruiting centers and
34
35 periodically transferred to the NEHO biobank, located at the Institute of Biomedicine and Molecular
36
37 Immunology (Palermo, Italy), where it is stored at -80°C. All four maternity units follow the same
38
39 protocol for sample collection, processing, and storage. All personnel involved are periodically
40
41 trained and all protocols are updated at regular intervals.
42
43

44
45 Blood samples (15ml) are drawn from a mother's cubital vein at enrollment and after delivery and
46
47 from the child's umbilical cord immediately after delivery (5ml in K2-EDTA and 10ml in serum
48
49 separator tubes). Blood tubes are stored at 4°C and centrifuged within 24 hours for 10 minutes at
50
51 2,000 x g. Serum is divided into 8 aliquots of approximately 0.5ml. Fractionated K2-EDTA blood is
52
53 dispensed in 4 aliquots of plasma (0.5ml each). One aliquot of at least 0.1ml of white blood cells
54
55 (buffy coat) is stored in cryotubes.
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3 In addition, 12 placenta samples are systematically collected from central and peripheral region by
4 means of a biopsy punch, stored in cryotubes with RNAlater, and frozen at -20°C within 2 hours of
5 delivery. Cryotubes with maternal and cord blood samples are periodically transported in dry ice to
6 the NEHO biobank where they are stored in -80°C freezers. Placenta samples are also transferred to
7 the central biobank for long-term storage at -80°C. Incomplete sample collection is not considered as
8 exclusion criteria for follow-up.
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16 Figure 3 provides a schematic overview of the project's time course.
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21 **Biobank**

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23 The long-term perspective of the project and the possibly delayed evidence of children's impairment
24 in physical, psychological, social and cognitive health necessitate the creation of a biobank for the
25 storage of maternal and fetal tissues.
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30 The preservation of high-quality placental tissue specimens will enable the search for new biomarkers
31 of prenatal exposure to pollutants as well as promote a better understanding of the mechanisms
32 through which potential disruptors are transmitted from mother to fetus. This may suggest possible
33 interventions to be made during pregnancy for the prevention of some adult diseases.
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42 **Transcriptomics**

43
44 The placenta has an active role in fetal development. The impairment of placental formation,
45 differentiation, and/or function severely affects fetal development and is associated with a wide range
46 of pregnancy complications, including pregnancy loss.[37] Other complications linked to placental
47 dysfunction, including gestational diabetes, hypertension, pre-eclampsia, and intrauterine growth
48 restriction can irreversibly result in greater susceptibility to multifactorial disorders during the entire
49 lifespan.[38-40] It is known that toxic substances may interfere with placental signaling cascades
50 involved in metabolism, nutrient and waste product transport, and steroid hormone and enzyme
51 production and release.[41] Therefore, in the context of the NEHO cohort, we will investigate the
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1
2 relationship between exposure to environmental toxic compounds (both heavy metals and POPs) and
3
4 shifts in gene expression by means of a whole transcriptome analysis. RNA microarray analysis will
5
6 be performed by SurePrint G3 Human Gene Expression v3 8x60k Microarray, acquired by the Agilent
7
8 G2565CA Microarray scanner. The raw data will be analyzed using R biostatistical computing
9
10 platform RStudio GUI. Gene set enrichment analysis will be performed to visualize the regulated
11
12 biological processes.
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18 **Outcome assessment**

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20 At the time of delivery, a clinical evaluation is performed: delivery details, birth outcomes, infant
21
22 anthropometry, including birth weight, head circumference and Apgar score at 5 and 10 minutes are
23
24 recorded. The presence of any possible congenital defects is noted for each newborn.
25
26

27 Children are followed-up on during the first two years of life through web-administered
28
29 questionnaires at 6, 12, and 24 months: breastfeeding, nutritional supplements, and vaccination data
30
31 are recorded by regular parental surveys conducted via web.
32
33

34 Similarly to the second part of the baseline questionnaire, the follow-up questionnaires (6 and 12
35
36 months) are self-administered. If requested, telephone helpline service is provided for those
37
38 experiencing difficulties in using the on-line self-administered questionnaires.
39
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41 Women are regularly contacted by phone or email and are encouraged to follow the timeline for
42
43 questionnaire submission.
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45

46 Finally, at 24 months after birth, all participant will be invited to an infant clinical evaluation. A
47
48 trained psychologist will administer the Bayley-III test, including cognitive, language, and motor
49
50 scales. Physiological and behavioral development will be evaluated by means of the CAT/CLAMS
51
52 test[42].
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55 Long-term outcomes will be evaluated by means of regional health records providing information on
56
57 causes of hospitalization and death.
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60 All the collected data are organized in a database and undergo a pseudonymization procedure.

Table 2 shows the major outcomes that will be evaluated in the NEHO cohort.

Table 2 – Outcomes of the study, separately for age, with their description.

Project stage	Outcome description	
At the birth	<ul style="list-style-type: none"> • Gestational age, • Weight, • Height, • Head circumference • Apgar score (5', 10'), • Type of delivery • Congenital birth defects 	➤ Clinical evaluation
6 months	<ul style="list-style-type: none"> • Anthropometric child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • CAT/CLAMS part 1 	➤ Self-reported
12 months	<ul style="list-style-type: none"> • Anthropometric child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Accidents, • Sleep quality, • CAT/CLAMS part 2 	➤ Self-reported
24 months	<ul style="list-style-type: none"> • Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • Barkley's scale of infant development 	➤ Clinical evaluation

Statistics

Primary analyses will concern the evaluation of possible differences in toxicant concentrations in maternal blood, placental tissue, and cord blood by exploring correlations between toxicant concentrations in different biological matrices. Moreover, the possible effects of confounders/effect modifiers will be evaluated for each toxicant by means of generalized linear models. Clinical

1
2 outcomes will be evaluated by means of logistic models (for dichotomous variables) or generalized
3 linear models (for continuous variables). Finally, subsamples of placental specimens will be evaluated
4 for transcriptome analysis.
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9 The NEHO cohort will enroll an estimated total of 800 mother-child pairs from the three highly
10 polluted areas, by January 2021. Sorkun et al (2007) found increased (0.048 ± 0.014 vs 0.038 ± 0.012)
11 cadmium concentration in placentas of Turkish women living in a highly polluted area with respect
12 to women living in a rural control area.[43] Based on these data, we estimate that a sample of 38
13 subjects for each study and control area (76 subjects as total sample) will be adequate for detecting
14 such differences in placental heavy metals, with an alpha level of 0.05 and a power ($1-\beta$) of 0.95.
15
16 Moreover, García-Esquinas et al (2013) found significantly reduced 5-minute Apgar scores (9.13 ± 0.6
17 vs 9.40 ± 0.5) in newborns with umbilical cadmium levels $>0.30 \mu\text{g/l}$.[44] From these data, we
18 estimate that a sample of 129 subjects for each study and control area (258 subjects as total sample)
19 will be adequate for detecting such differences in 5-minute Apgar scores, with an alpha level of 0.05
20 and a power ($1-\beta$) of 0.95.
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38 **Aims**

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40 NEHO is the first cohort in Italy to study and analyze pollutant blood levels in pregnant women living
41 near highly polluted areas along with fetal exposure. The project will attempt to identify the influence
42 of environmental risk factors for placental function, pregnancy outcomes, and newborns' health
43 outcomes.
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49 Finally, NEHO will enroll mother-child pairs residing in disadvantaged areas with low socio-
50 economic status, thus representing a particular condition in the context of Italian and European birth
51 cohorts.
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55 Thus, on the basis of the collected data, the NEHO cohort is aimed at:
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- evaluating the risk of heavy metals and emerging contaminants during pregnancy in a cohort of mother-child pairs residing in highly polluted areas characterized by different levels of environmental pollution;
- evaluating the bioaccumulation features and patterns of toxicants by examining their distribution in maternal, placental, and fetal tissues;
- determining whether the bioaccumulation of toxicants might impact placental mRNA expression;
- understanding whether prenatal exposure to contaminants may cause negative pregnancy outcomes and/or long-term effects on children's health and disease susceptibility;
- defining the associations of placental contamination and gene expression patterns with long-term infant health outcomes, to evaluate the validity of placental analyses in predicting future infant health outcomes.

Patient and public involvement

The recruitment is based on healthy pregnant volunteers. Patients and the public were neither involved in the study design nor in the establishment of questions and/or outcome definition. No individual results will be provided to participants unless there is a possible impact on their health.

Ethics and dissemination

The collection of human tissues and the creation of a biobank for medical research involve important ethical and legal issues:[45] consequently, a complete explanation of the details and the aims of the research project to participants is a prerequisite for enrollment. To promote and guarantee respect for pregnant women's free choice, during a routine visit pregnant women receive a great deal of information about the collection and storage of biological samples until the child turns eighteen. The participants are required to sign a consent form confirming their complete understanding of the project's scope and indicating their agreement to take part in the project. This includes knowing how to withdraw from the project at any time if they change their mind.

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2
3 Qualified project staff (biologists, midwives, nurses, gynecologists) provide complete information
4 about the project and manage the completion of the baseline questionnaire and the collection of blood
5 samples. Aimed at making data totally comparable with previous large Italian birth cohorts, the
6 questionnaire was developed from the *Piccolipiù* questionnaire.[14]
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11 After completing the questionnaire, a unique identification code is automatically assigned to each
12 woman to identify the questionnaires and the biological samples collected during the study,
13 preserving complete pseudonymization. The study is being conducted following the Declaration of
14 Helsinki. All the adopted procedures comply with the General Data Protection Regulation (UE
15 2016/679) and Italian laws concerning data protection.
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20 The results of the study will be communicated to participants, the local Regional Health Authorities
21 and clinical professionals only on an aggregated basis through *ad hoc* meetings. The Researchers will
22 also disseminate results by means of peer-reviewed journals and scientific conferences.
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34 **DECLARATIONS**

35 **Ethics approval and consent to participate**

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38 The NEHO study protocol has been approved by the Ethics Committees responsible for the three
39 involved NPCSS: the Ethics Committee of the University Hospitals of Messina for the NPCSS of
40 Milazzo-Valle del Mela (September 18, 2017, n. 9/2017); the Ethics Committee “Catania 2” for the
41 NPCSS of Augusta-Priolo (July 11, 2017, n. 38/2017/CECT2); the Ethics Committee of the Region of
42 Calabria for the NPCSS of Crotone (July 20, 2017, n. 173). Each participant read the information sheet
43 and signed the informed consent. Copies of participants’ information sheets are available at the
44 website: www.neho.it.
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57 **Availability of data and materials**

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59 Documentation for the study, information sheet, and informed consent is available at www.neho.it.
60

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SR, GD, PC, and FC made substantial contributions to the conceptualization and design of the study and are involved in study monitoring. They drafted and critically revised the manuscript for its intellectual content, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

AA, PA, ABi, ABu, PD, RDS, VLR, AL, LLP, BM, FP, GP, and AZC made substantial contributions to data acquisition and were involved in drafting the manuscript. Each of the authors read and approved the final version of the manuscript.

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1
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3 behavioral development.
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FIGURE LEGENDS

Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=2ca75003ef9d477fb22db19832c9554f>. Last accessed March 29, 2019.

Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. The National Priority Contaminated Sites are in red, the surrounding areas are in green. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=61145ee86375431f9c54762de4ccd9e7>

Figure 3 - Schematic overview of the project's time course.

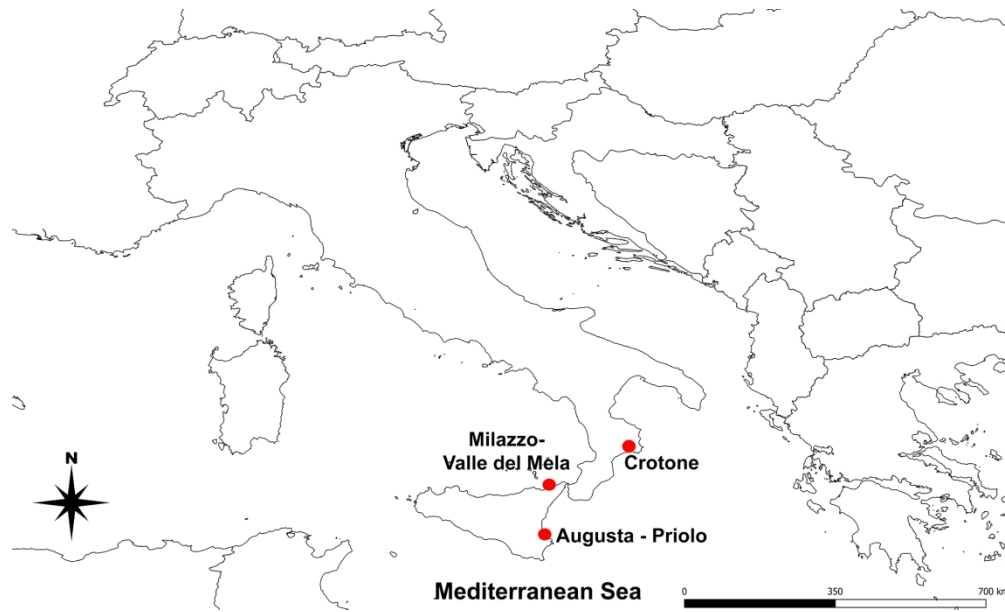


Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=2ca75003ef9d477fb22db19832c9554f>. Last accessed March 29, 2019

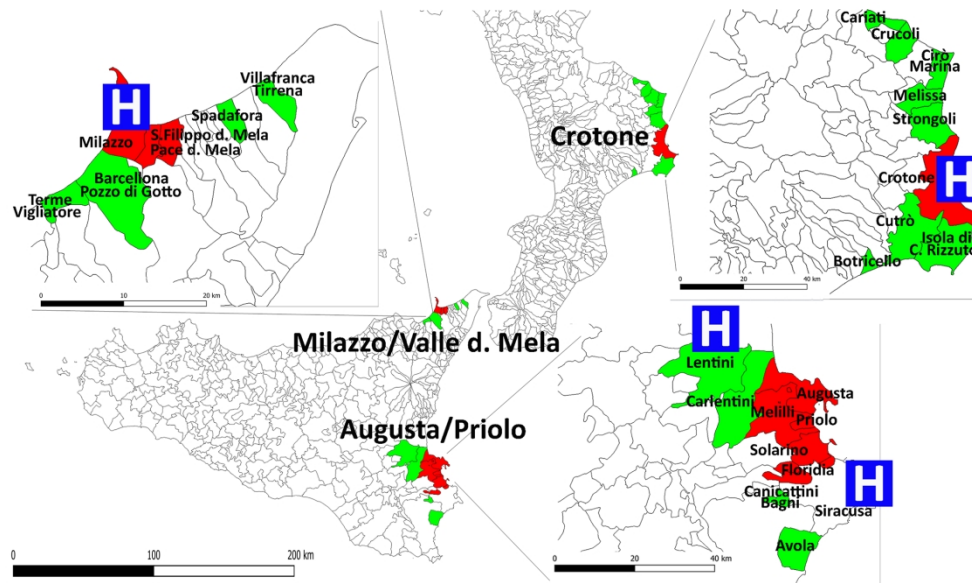


Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. In red the National Priority Contaminated Sites, in green the surrounding areas. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL <http://qgis.org>; shapefile from <https://www.arcgis.com/home/item.html?id=61145ee86375431f9c54762de4ccd9e7>

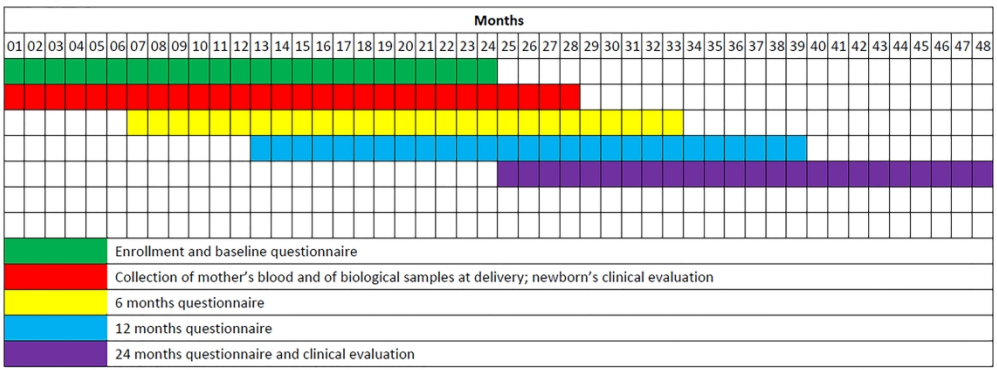


Figure 3 - Schematic overview of the project's time course

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	7-9
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	9
		(b) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	12-13
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	10-11
Bias	9	Describe any efforts to address potential sources of bias	8
Study size	10	Explain how the study size was arrived at	14
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	13
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	13
		(b) Describe any methods used to examine subgroups and interactions	13
		(c) Explain how missing data were addressed	
		(d) If applicable, explain how loss to follow-up was addressed	
		(e) Describe any sensitivity analyses	
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	16

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.