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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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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

Strenghts 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

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

The three selected NPCS are: Milazzo-Valle del Mela, Augusta-Priolo, and Crotone. Those of Milazzo and Augusta are wide industrialized coastal areas located in eastern Sicily, in which large production sites are present, mainly refineries, petrochemical complexes, power plants, and cement plants, numerous hazardous waste dumps and the former Eternit plant in Syracuse where asbestos was processed.[16] In Crotone area, located in the region of Calabria, the most relevant environmental impact is due to three disused industrial areas (ex Pertusola, ex Fosfotec, and ex Agricoltura) which operated between the 1920s and the 1990s, mainly in the field of production of zinc, phosphoric acid, and complex fertilizers.[17]

During the course of CISAS project, the birth cohort NEHO (Neonatal Environment and Health Outcomes) will enroll pregnant women living in the three NPCS, along with pregnant women living in surrounding areas, outside the NPCS. NEHO cohort is aimed at understanding processes and mechanisms for the transfer of heavy metals and POPs (e.g., PBDEs, PCBs, chlorinated pesticides) from the environment to the ecosystem and to humans. In the context of CISAS Project, the same toxicants will be evaluated in all the environmental matrices (atmosphere, soil, sediment, inland waters and sea) and in the food chain (fish, meat, eggs, milk and dairy products, sampled from local producers of each studied area).

Epidemiologic evidences support the concept that early life exposure to pollutants – as measured during pregnancy and/or during childhood – is detrimental to health outcomes of the child. Previous works indicate that polybrominated diphenyl ethers (PBDE) and polychlorinated biphenyls (PCB) bioaccumulate in human placenta tissue possibly contributing to prenatal exposures to the environmental contaminants.[18-20] PBDEs – largely used as flame retardants in electronic equipment, carpet, and in the polyurethane foam used in furniture – have been detected in umbilical

cord blood.[21-22] Attention will be focused on the investigation of toxicants that are transferred from the mother's blood to the developing fetus and on the influence of toxicants on both pregnancy outcome and fetal development and late onset consequences. Because human placenta represents the interface between maternal/external environment and embryo, it can be taken as an environmental monitoring system. In fact, placental examination constitutes an useful tool for estimating both maternal and fetal exposures.[20, 23, 24] Moreover, placenta has an active role in the homeostasis of the intrauterine environment and also mediates signal transmission form the fetus to the mother and vice versa. Nutrition supply, endocrine/immune regulation, and gas exchange are orchestrated by the placenta. All these evidences pone the placenta as highly informative organ in the study of pregnancy.[25]

The presence of toxicants in the placenta can cause alterations of its structure and function along with fetal development interference. 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.[26-28] High concentrations of mercury in fetal tissues are associated with the reduction of hormone synthesis and the consumption of oxygen by the placenta.[29,30]

Persistent organic compounds have been measured in fetal tissues and 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.[31]

Umbilical cord blood and placenta are also noninvasive indicators for exposure to heavy metals, and may be easily collected along with maternal blood.[32] Cadmium level in placenta is also a valuable biomarker of metal dietary exposure related to specific dietary habits and soil characteristics. Lead and mercury have been shown to be easily transferred through the placental and blood barriers.[30, 33]

Monitoring pollutants concentration in human tissues along with the extensive characterization of all the environmental matrix proposed in CISAS project will provide new insights on the toxicants' transfer routes from environment to human fetus.

Particular attention will be paid to the possible interaction between the environmental exposures and the low socioeconomic status which often characterizes the investigated population.

METHODS AND ANALYSIS

Study population and recruitment

From January 2018, NEHO cohort started to recruit pregnant women living in the three selected NPCS of Crotone, Milazzo-Valle del Mela and Augusta-Priolo, in southern Italy (Figure 1), along with pregnant women living in surrounding areas presenting similar geographic and socio-economic characteristics.

The NEHO study involves twenty-six cities subdivided in study areas and local reference areas. Table 1 shows the selected cities in Sicily and Calabria and their respective distance from industrial areas. Local reference areas were identified as "local control" by ISTISAN reports[16, 34] and by SENTIERI Project, an epidemiological study on the epidemiological evidence on the association between causes of death and environmental exposures,[35-36] coordinated by the Italian National Institute of Health and supported by the Ministry of Health. Aimed at maximizing the recruitment efficiency, in each NPCS, maternal units were selected on the basis of both deliveries/population ratio and available resources.

NEHO study recruits pregnant women in four selected maternal units located in the public hospitals of four cities: "G. Fogliani" Hospital in Milazzo (for Milazzo-Valle del Mela NCPS), General Hospital in Lentini and "Umberto I" Hospital in Siracusa (for Augusta-Priolo NCPS), and "San Giovanni di Dio" Hospital in Crotone.

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

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 periconceptional 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 periods, along with smoking habits (including e-cigarette use), possible chemical exposures, and maternal health characteristics.

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

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

Collection of biologic samples

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

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

In addition, 12 sections of placenta are systematically collected from central and peripheral region by means of a biopsy punch, stored in cryotubes with RNAlater and frozen at -20°C, within 2 hours from delivery. Cryotubes with blood and cord samples are periodically transported in dry ice to the NEHO biobank where they are stored in -80°C freezers. Placenta samples are also transferred to the central

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

Figure 3 provides a schematic overview of the project's time course.

Biobank

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

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

Transcriptomics

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

computing platform RStudio GUI. Gene set enrichment analysis will be performed to visualise regulated biological processes.

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 in the first two years of life through questionnaires via web 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.

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 [43].

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

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

Table 2 shows the major outcome 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	• Gestational age, • Weight, • Height, • Head Circumference • Apgar score (5', 10'), • Type of Delivery • Congenital Birth defects	➢ Clinical evaluation
6 months	• Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • CAT/CLAMS part 1	➢ Self-reported
12 months	• Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • CAT/CLAMS part 2	➢ Self-reported
24 months	• 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.

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

Aims

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

Finally, 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.

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

- evaluating the risk of heavy metals and emerging contaminants during pregnancy in a cohort of mother-child pairs resident in highly polluted areas characterized by different levels of environmental pollution;
- evaluating the bioaccumulation features and patterns of toxicants by examining their distribution among maternal, placental and fetal tissues;
- determining whether the bio-accumulation 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 health and disease predisposition;
- 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:[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 Helsinky. 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 Crotone (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

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 make substantial contributions to acquisition of data and were involved in drafting the manuscript. Each of the authors read and approved the final version of the manuscript.

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REFERENCES

1. Fleming TP, Watkins AJ, Velazquez MA, et al. Origins of lifetime health around the time of conception: causes and consequences. Lancet. 2018.5.391. 1842–1852. doi:10.1016/S0140-6736(18)30312-X.
2. World Health Organization. The European health report 2005: public health action for healthier children and populations. WHO Regional Office for Europe; 2005.
3. Barker DJ, Osmond C, Winter PD et al. Weight in infancy and death from ischaemic heart disease. Lancet. 1989.2. 577–580.
4. Barker DJP. Fetal and infant origins of adult disease. BMJ. 1990.301.1111
5. Barker DJ. Maternal nutrition, fetal nutrition and disease in later life. Nutr.1997.13. 807–813.
6. Barker DJP. The Developmental Origins of Adult Disease. J Am Coll Nutr 2004.23.Suppl 6. 588S–595S.

7. Barker DJP. The origins of the developmental origins theory. J Intern Med. 2007.261. 412–417. doi:10.1111/j.1365-2796.2007.01809.x

8. Gluckman P, Hanson M. Developmental Origins of Health and Disease. Cambridge University Press.2006.

9. Kordas K. European birth cohorts offer insights on environmental factors affecting human development and health. Int J Epidemiol. 2016.55. 731–734. doi:10.1093/eurpub/ckw068

10. Richiardi L, Baussano I, Vizzini L, et al. NINFEA cohort: Feasibility of recruiting a birth cohort through the Internet: the experience of the NINFEA cohort. Eur J Epidemiol.2007.22. 831–837. doi:10.1007/s10654-007-9194-2

11. Vecchi Brumatti L, Montico M, Russian S, et al. Analysis of motivations that lead women to participate (or not) in a newborn cohort study. BMC Pediatr. 2013.11. 13–53. doi:10.1186/1471-2431-13-53

12. Brescianini S, Fagnani C, Toccaceli V, et al. An update on the Italian twin register: advances, in cohort recruitment, project building and network development. Twin Res Hum Genet. 2013.16. 190–196. doi:10.1017/thg.2012.85

13. Farchi S, Forastiere F, Vecchi Brumatti L, et al. Piccolipiù, a multicenter birth cohort in Italy: protocol of the study. BMC Pediatr 2014.14.36 doi:10.1186/1471-2431-14.36.

14. Porta D, Fantini MP, on behalf of the GASPII and Co.N.ER Study Groups. Prospective cohort studies of newborns in Italy to evaluate the role of environmental and genetic characteristics on common childhood disorders. Ital J Pediatr.2006.32. 350–357.

15. Barchitta M, Maugeri A, La Rosa MC, et al. Single Nucleotide Polymorphisms in Vitamin D Receptor Gene Affect Birth Weight and the Risk of Preterm Birth: Results From the "Mamma & Bambino" Cohort and A Meta-Analysis. Nutr.2018.10.1172. doi:10.3390/nu10091172

16. Carere M, Musmeci L, Bianchi F, et al. Study for the environmental health characterization of the contaminated sites of Gela and Priolo. Istituto Superiore di Sanità. Rapporti ISTISAN. 2016;16/35,67p.

17. Barone V, Calenda C, Motta F, Oranges T. Inquinamento e recupero nel crotonese. Ecoscienza 2010.3.

18. Vizcaino E, Grimalt JO, Fernández-Somoano A, et al. Transport of persistent organic pollutants across the human placenta. Environ Int. 2014.65. 107–115. doi:10.1016/j.envint.2014.01.004

19. Leonetti C, Butt CM, Hoffman K, et al. Concentrations of Polybrominated Diphenyl Ethers (PBDEs) and 2,4,6-Tribromophenol in Human Placental Tissues. Environ Int. 2016. 23–29 doi:10.1016/j.envint.12.002.

20. Jeong Y, Lee S, Kim S, et al. Placental transfer of persistent organic pollutants and feasibility using the placenta as a non-invasive biomonitoring matrix. Sci Total Environ. 2018.612. 1498–1505. doi: 10.1016/j.scitotenv.2017.07.054.

21. Foster WG, Gregorovich S, Morrison KM, et al. Human maternal and umbilical cord blood concentrations of polybrominated diphenyl ethers. Chemosphere. 2011.84. 1301–1309. doi:10.1016/j.chemosphere.2011.05.028

22. Arbuckle TE, Kubwabo C, Walker M, et al. Umbilical cord blood levels of perfluoroalkyl acids and polybrominated flame retardants. Int J Hyg Environ Health. 2013.216. 184–194 doi:10.1016/j.ijheh.2012.03.004.

23. Jones R & Golding J. Choosing the types of biological sample to collect in longitudinal birth cohort studies. Paediatr Perinat Epidemiol. 2009.23S.1. 103–113. doi:10.1111/j.1365-3016.2008.01000.x.

24. Leino O, Kiviranta H, Karjalainen AK, et al. Pollutant concentrations in placenta. Food Chem Toxicol. 2013.54. 59–69. doi:10.1016/j.fct.2011.10.058.

25. Soares MJ, Varberg KM, Iqbal K. Hemochorial placentation: development, function, and adaptations. Biol Reprod. 2018.99(1). 196–211. doi:10.1093/biolre/ioy049.

26. Bruin JE, Gerstein HC, Holloway AC: Long-term consequences of fetal and neonatal nicotine exposure: a critical review. Toxicol Sci. 2010.116Suppl.2. 364–374. doi:10.1093/toxsci/kfq103

27. Votavova H, Dostalova Merkerova M, Fejglova K, et al. Transcriptome alterations in maternal

and fetal cells induced by tobacco smoke. Placenta. 2011.32Suppl.10. 763–770. doi:10.1016/j.placenta.2011.06.022

28. Piasek M, Mikolić A, Sekovanić A, et al. Cadmium in Placenta – A Valuable Biomarker of Exposure During Pregnancy in Biomedical Research. J Toxicol and Env Health. 2014.77. 1071–1074 doi:10.1080/15287394.2014.915779.

29. Gundacker C, Fröhlich S, Graf-Rohrmeister K, et al. Perinatal lead and mercury exposure in Austria. Sci Total Environ. 2010.408.5744–5749. doi:10.1016/j.scitotenv.2010.07.079

30. Gundacker C, Hengstschlager M. The role of the placenta in fetal exposure to heavy metals. Wien Med Wochenschr. 2012.162. 201–206. doi:10.1007/s10354-012-0074-3.

31. Rylander L, Stromberg U, Hagmar L. Lowered birth weight among infants born to women with a high intake of fish contaminated with persistent organochlorine compounds. Chemosphere. 2000.40. 1255–1262.

32. Esteban-Vasallo MD, Aragonés N, Pollan M, et al. Mercury, Cadmium, and Lead Levels in Human Placenta: A Systematic Review. Environ Health Persp. 2012.120. 1369–1377. doi:10.1289/ehp.1204952

33. Caserta D, Graziano A, Lo Monte G, et al. Heavy metals and placental fetal-maternal barrier: A mini-review on the major concerns. Eur. Rev. Med. Pharmacol. Sci. 2013.17. 2198–2206.

34. Comba P, Pitimada M. Epidemiological study of Calabria Region contaminated sites: aims, methods and feasibility. Istituto Superiore di Sanità. Rapporti ISTISAN. 2016;16/19.ii.135p.

35. Pirastu R, Ancona C, Iavarone I, et al. Mortality study of residents in Italian polluted sites: evaluation of the epidemiological evidence. Epidemiol Prev. 2010.34.(5-6 Suppl 3). 1–2.

36. Pirastu R, Iavarone I, Pasetto R, et al. SENTIERI Project - Mortality study of residents in Italian polluted sites: Results.Epidemiol Prev. 2001.35.(5-6 Suppl 4): 1–204.

37. Gardosi J, Kady SM., McGeown P, et al. Classification of stillbirth by relevant condition at death (ReCoDe): population based cohort study. Brit Med J. 2005.331. 1113–1117. doi:10.1136

38. Salafia CM, Pezzullo JC, López-Zeno JA, et al. Placental pathologic features of preterm preeclampsia. Am J Obstet Gynecol. 1995.173(4). 1097–1105.

39. Junaid TO, Brownbill P, Chalmers N, et al. Fetoplacental vascular alterations associated with fetal growth restriction. Placenta. 2004. 35. 808–815, doi:10.1016

40. Sobrevia L, Abarzúa F, Nien JK, et al. Review: Differential placental macrovascular and microvascular endothelial dysfunction in gestational diabetes, Placenta, 2011. 32 Sup2. S159–S164. doi:10.1016/j.placenta.2010.12.011.

41. Maltepe E, Fisher SJ. Placenta: the forgotten organ. Annu Rev Cell Dev Biol. 2015.31. 523–552. doi:10.1146/annurev-cellbio-100814-125620

42. Sorkun HC, Bir F, Akbulut M, et al. The effects of air pollution and smoking on placental cadmium, zinc concentration and metallothionein expression. Toxicology. 2007.238. 15–22.

43. Capute AJ, Accardo PJ. The Infant Neurodevelopmental Assessment: A clinical interpretive manual for CAT-CLAMS in the first two years of life. Part 1. Curr Probl Pediatr. 1996.26.7. 238–257. doi:10.1016/S0045-9380(06)80061-7

44. Toccaceli V, Serino L, Stazi MA. Informed consent, and an ethico-legal framework for paediatric observational research and biobanking: the experience of an Italian birth cohort study. Cell Tissue Bank. 2014.15. 579–590. doi:10.1007/s10561-014-9431-3.

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.

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

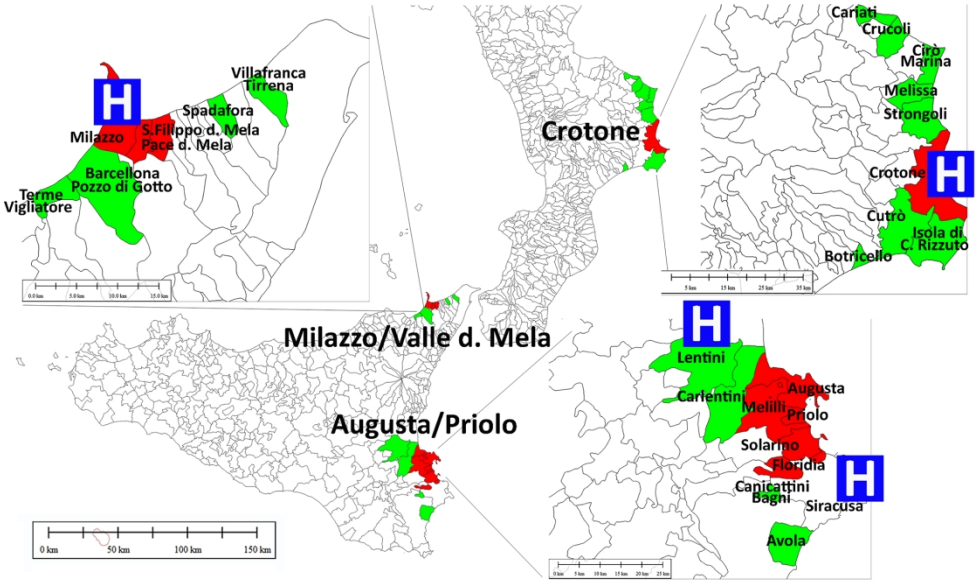

Figure 2 - The selected communities and the distribution of the maternal units (H) where the pregnant women are recruited. In red the National Priority Contaminated Sites, in green the surrounding areas

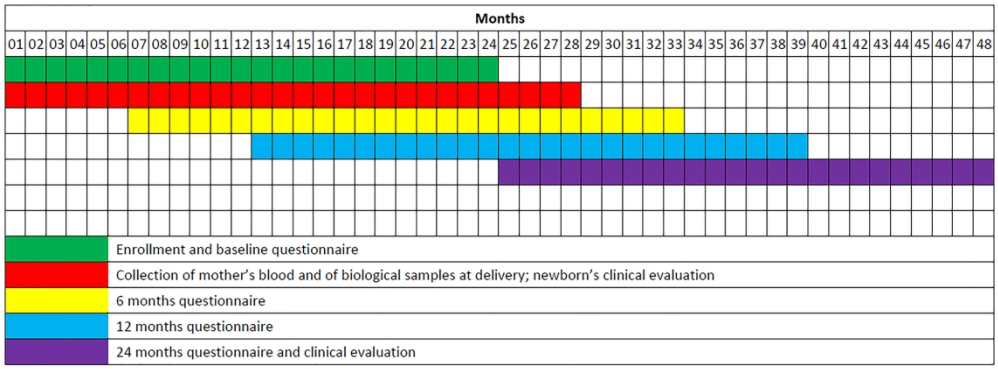

Figure 3 - Schematic overview of the project's time course

189x70mm (300 x 300 DPI)

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.

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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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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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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

Strenghts 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

polychlorinated biphenyls (PCB) bioaccumulate in human placenta tissue possibly contributing to prenatal exposures to the environmental contaminants. [16-18] PBDEs – largely used as flame retardants in electronic equipment, carpet, and in the polyurethane foam used in furniture – have been detected in umbilical cord blood. [19-20] Because human placenta represents the interface between maternal/external environment and embryo, it can be used as an environmental monitoring system. In fact, placental examination constitutes an useful tool for estimating both maternal and fetal exposures. [18, 21, 22] Moreover, placenta has 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 these evidences pone the placenta as a highly informative organ in the study of pregnancy.[23]

The presence of toxicants in the placenta can cause alterations of its structure and function along with fetal development interference. 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 the oxygen consumption ~~of~~ by the placenta.[27,28]

Persistent organic compounds have been measured in fetal tissues and 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]

Umbilical cord blood and placenta are also noninvasive indicators for exposure to heavy metals, and may be easily collected along with maternal blood.[30] Cadmium level in placenta is also a valuable biomarker of metal dietary exposure related to specific dietary habits and soil characteristics. Lead and mercury have been shown to be easily transferred through the placental and blood barriers.[28, 31]

Monitoring pollutants concentration in human tissues along with the extensive characterization of all the environmental matrix proposed in CISAS project will provide new insights on the toxicants'

transfer routes from environment to human fetus. Attention will be focused on the investigation of toxicants that are transferred from the mother's blood to the developing fetus and on the influence of toxicants on fetal development, pregnancy outcomes, and late onset health consequences.

Particular attention will be paid to the possible interaction between the environmental exposures and the low socioeconomic status which often characterizes the investigated population.

METHODS AND ANALYSIS

Experimental context

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 various environmental matrices and their transfer to the ecosystem and the humans. CISAS Project is developed in three selected National Priority Contaminated Sites (NPCS) of southern Italy: in its context the influence of environmental pollution on pregnant women is of special interest.

The three selected NPCS are: Milazzo-Valle del Mela, Augusta-Priolo, and Crotone. Those of Milazzo and Augusta are wide industrialized coastal areas located in eastern Sicily, in which large production sites are present: mainly refineries, petrochemical and cement plants, power plants, numerous hazardous waste dumps and the former Eternit plant in Syracuse where asbestos was processed.[32] In Crotone area, located in the region of Calabria, the most relevant environmental impact is due to three disused industrial areas (former Pertusola, former Fosfotec, and former Agricoltura) which operated between the 1920s and the 1990s, mainly in the field of production of zinc, phosphoric acid, and complex fertilizers.[33]

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

mechanisms for the transfer of heavy metals and POPs (e.g., PBDEs, PCBs, chlorinated pesticides) from the environment to the ecosystem and to humans. In the context of CISAS Project, the same toxicants will be evaluated in all the environmental matrices (atmosphere, soil, sediment, inland waters and sea) and in the food chain (fish, meat, eggs, milk and dairy products, sampled from local producers of each studied area).

Study population and recruitment

From January 2018, NEHO cohort started to recruit pregnant women living in the three selected NPCS of Crotone, Milazzo-Valle del Mela and Augusta-Priolo, in southern Italy (Figure 1), along with pregnant women living in surrounding areas presenting similar geographic and socio-demographic characteristics.

The NEHO study involves twenty-six cities subdivided in study areas and local reference areas. Table 1 shows the selected cities in Sicily and Calabria and their respective distance from industrial areas.

Local reference areas were identified as "local control" by ISTISAN reports[32, 34] and by SENTIERI Project, a study on the epidemiological evidence on the association between causes of death and environmental exposures,[35-36] coordinated by the Italian National Institute of Health and supported by the Ministry of Health. Aimed at maximizing the recruitment efficiency, in each NPCS, maternal units were selected on the basis of both deliveries/population ratio and available resources.

NEHO study recruits pregnant women in four selected maternal units located in the public hospitals of four cities: "G. Fogliani" Hospital in Milazzo (for Milazzo-Valle del Mela NCPS), General Hospital in Lentini and "Umberto I" Hospital in Siracusa (for Augusta-Priolo NCPS), and "San Giovanni di Dio" Hospital in Crotone.

All the pregnant women attending 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

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 periconceptional 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.

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

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

Collection of biological samples

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

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

In addition, 12 samples of placenta are systematically collected from central and peripheral region by means of a biopsy punch, stored in cryotubes with RNAlater and frozen at -20°C, within 2 hours from delivery. Cryotubes with maternal and cord blood samples are periodically transported in dry ice to the NEHO biobank where they are stored in -80°C freezers. Placenta samples are also transferred to the central biobank for long-term storage at -80°C. Incomplete sample collection is not considered as exclusion criteria for follow up.

Figure 3 provides a schematic overview of the project's time course.

Biobank

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

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

Transcriptomics

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

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	• Gestational age, • Weight, • Height, • Head Circumference • Apgar score (5', 10'), • Type of Delivery • Congenital Birth defects	➢ Clinical evaluation
6 months	• Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • CAT/CLAMS part 1	➢ Self-reported
12 months	• Anthropometrics child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Incidents, • Sleep quality, • CAT/CLAMS part 2	➢ Self-reported
24 months	• 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.

NEHO estimates to enroll a total of 800 mother-child pairs from the three highly polluted areas, within January 2021. Sorkun et al (2007) found increased (0.048±0.014 vs 0.038±0.012) cadmium concentration in placenta of Turkish women living in a highly polluted area with respect to women living in a control rural area.[43] Founded on these data, we estimated that a sample of 38 subjects for each study and control areas (76 subjects as total sample) will be adequate for detecting such differences in placental heavy metals, with an alpha level of 0.05 and a power (1-β) of 0.95. Moreover, García-Esquinas et al (2013) found significantly reduced 5-minutes Apgar score (9.13±0.6 vs 9.40±0.5) in newborns with umbilical cadmium levels >0.30 µg/l.[44] From these data, we estimated that a sample of 129 subjects for each study and control areas (258 subjects as total sample) will be adequate for detecting such differences in 5-minutes Apgar score, with an alpha level of 0.05 and a power (1-β) of 0.95.

Aims

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

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

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

- evaluating the risk of heavy metals and emerging contaminants during pregnancy in a cohort of mother-child pairs resident in highly polluted areas characterized by different levels of environmental pollution;
- evaluating the bioaccumulation features and patterns of toxicants by examining their distribution among maternal, placental and fetal tissues;
- determining whether the bio-accumulation 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 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

preserving complete pseudonymization. The study is conducted following the Declaration of Helsinky. 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 for 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 Crotone (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, information sheet, and informed consent are available at www.neho.it.

Competing interest

The authors declare that they have no competing interests.

Funding

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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 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 make substantial contributions to acquisition of data and were involved in drafting the manuscript. Each of the authors read and approved the final version of the manuscript.

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REFERENCES

1. Fleming TP, Watkins AJ, Velazquez MA, et al. Origins of lifetime health around the time of conception: causes and consequences. Lancet. 2018.5.391. 1842–1852. doi:10.1016/S0140-6736(18)30312-X.
2. World Health Organization. The European health report 2005: public health action for healthier

children and populations. WHO Regional Office for Europe; 2005.

3. Barker DJ, Osmond C, Winter PD et al. Weight in infancy and death from ischaemic heart disease. Lancet. 1989.2. 577–580.

4. Barker DJP. Fetal and infant origins of adult disease. BMJ. 1990.301.1111

5. Barker DJ. Maternal nutrition, fetal nutrition and disease in later life. Nutr.1997.13. 807–813.

6. Barker DJP. The Developmental Origins of Adult Disease. J Am Coll Nutr 2004.23.Suppl 6. 588S–595S.

7. Barker DJP. The origins of the developmental origins theory. J Intern Med. 2007.261. 412–417. doi:10.1111/j.1365-2796.2007.01809.x

8. Gluckman P, Hanson M. Developmental Origins of Health and Disease. Cambridge University Press.2006.

9. Kordas K. European birth cohorts offer insights on environmental factors affecting human development and health. Int J Epidemiol. 2016.55. 731–734. doi:10.1093/eurpub/ckw068

10. Richiardi L, Baussano I, Vizzini L, et al. NINFEA cohort: Feasibility of recruiting a birth cohort through the Internet: the experience of the NINFEA cohort. Eur J Epidemiol.2007.22. 831–837. doi:10.1007/s10654-007-9194-2

11. Vecchi Brumatti L, Montico M, Russian S, et al. Analysis of motivations that lead women to participate (or not) in a newborn cohort study. BMC Pediatr. 2013.11. 13–53. doi:10.1186/1471-2431-13-53

12. Brescianini S, Fagnani C, Toccaceli V, et al. An update on the Italian twin register: advances, in cohort recruitment, project building and network development. Twin Res Hum Genet. 2013.16. 190–196. doi:10.1017/thg.2012.85

13. Farchi S, Forastiere F, Vecchi Brumatti L, et al. Piccolipiù, a multicenter birth cohort in Italy: protocol of the study. BMC Pediatr 2014.14.36 doi:10.1186/1471-2431-14.36.

14. Porta D, Fantini MP, on behalf of the GASPII and Co.N.ER Study Groups. Prospective cohort studies of newborns in Italy to evaluate the role of environmental and genetic characteristics on common childhood disorders. Ital J Pediatr.2006.32. 350–357.

15. Barchitta M, Maugeri A, La Rosa MC, et al. Single Nucleotide Polymorphisms in Vitamin D Receptor Gene Affect Birth Weight and the Risk of Preterm Birth: Results From the "Mamma & Bambino" Cohort and A Meta-Analysis. Nutr.2018.10.1172. doi:10.3390/nu10091172

16. Vizcaino E, Grimalt JO, Fernández-Somoano A, et al. Transport of persistent organic pollutants across the human placenta. Environ Int. 2014.65. 107–115. doi:10.1016/j.envint.2014.01.004

17. Leonetti C, Butt CM, Hoffman K, et al. Concentrations of Polybrominated Diphenyl Ethers (PBDEs) and 2,4,6-Tribromophenol in Human Placental Tissues. Environ Int. 2016. 23–29 doi:10.1016/j.envint.12.002.

18. Jeong Y, Lee S, Kim S, et al. Placental transfer of persistent organic pollutants and feasibility using the placenta as a non-invasive biomonitoring matrix. Sci Total Environ. 2018.612. 1498–1505. doi: 10.1016/j.scitotenv.2017.07.054.

19. Foster WG, Gregorovich S, Morrison KM, et al. Human maternal and umbilical cord blood concentrations of polybrominated diphenyl ethers. Chemosphere. 2011.84. 1301–1309. doi:10.1016/j.chemosphere.2011.05.028

20. Arbuckle TE, Kubwabo C, Walker M, et al. Umbilical cord blood levels of perfluoroalkyl acids and polybrominated flame retardants. Int J Hyg Environ Health. 2013.216. 184–194 doi:10.1016/j.ijheh.2012.03.004.

21. Jones R & Golding J. Choosing the types of biological sample to collect in longitudinal birth cohort studies. Paediatr Perinat Epidemiol. 2009.23S.1. 103–113. doi:10.1111/j.1365-3016.2008.01000.x.

22. Leino O, Kiviranta H, Karjalainen AK, et al. Pollutant concentrations in placenta. Food Chem Toxicol. 2013.54. 59–69. doi:10.1016/j.fct.2011.10.058.

23. Soares MJ, Varberg KM, Iqbal K. Hemochorial placentation: development, function, and

adaptations. Biol Reprod. 2018.99(1). 196–211. doi:10.1093/biolre/ioy049.

24. Bruin JE, Gerstein HC, Holloway AC: Long-term consequences of fetal and neonatal nicotine exposure: a critical review. Toxicol Sci. 2010.116Suppl.2. 364–374. doi:10.1093/toxsci/kfq103

25. Votavova H, Dostalova Merkerova M, Fejglova K, et al. Transcriptome alterations in maternal and fetal cells induced by tobacco smoke. Placenta. 2011.32Suppl.10. 763–770. doi:10.1016/j.placenta.2011.06.022

26. Piasek M, Mikolić A, Sekovanić A, et al. Cadmium in Placenta – A Valuable Biomarker of Exposure During Pregnancy in Biomedical Research. J Toxicol and Env Health. 2014.77. 1071–1074 doi:10.1080/15287394.2014.915779.

27. Gundacker C, Fröhlich S, Graf-Rohrmeister K, et al. Perinatal lead and mercury exposure in Austria. Sci Total Environ. 2010.408.5744–5749. doi:10.1016/j.scitotenv.2010.07.079

28. Gundacker C, Hengstschlager M. The role of the placenta in fetal exposure to heavy metals. Wien Med Wochenschr. 2012.162. 201–206. doi:10.1007/s10354-012-0074-3.

29. Rylander L, Stromberg U, Hagmar L. Lowered birth weight among infants born to women with a high intake of fish contaminated with persistent organochlorine compounds. Chemosphere. 2000.40. 1255–1262.

30. Esteban-Vasallo MD, Aragonés N, Pollan M, et al. Mercury, Cadmium, and Lead Levels in Human Placenta: A Systematic Review. Environ Health Persp. 2012.120. 1369–1377. doi:10.1289/ehp.1204952

31. Caserta D, Graziano A, Lo Monte G, et al. Heavy metals and placental fetal-maternal barrier: A mini-review on the major concerns. Eur. Rev. Med. Pharmacol. Sci. 2013.17. 2198–2206.

32. Carere M, Musmeci L, Bianchi F, et al. Study for the environmental health characterization of the contaminated sites of Gela and Priolo. Istituto Superiore di Sanità. Rapporti ISTISAN. 2016;16/35,67p.

33. Barone V, Calenda C, Motta F, Oranges T. Inquinamento e recupero nel crotonese. Ecoscienza 2010.3.

34. Comba P, Pitimada M. Epidemiological study of Calabria Region contaminated sites: aims, methods and feasibility. Istituto Superiore di Sanità. Rapporti ISTISAN. 2016;16/19.ii.135p.

35. Pirastu R, Ancona C, Iavarone I, et al. Mortality study of residents in Italian polluted sites: evaluation of the epidemiological evidence. Epidemiol Prev. 2010.34.(5-6 Suppl 3). 1–2.

36. Pirastu R, Iavarone I, Pasetto R, et al. SENTIERI Project - Mortality study of residents in Italian polluted sites: Results.Epidemiol Prev. 2001.35.(5-6 Suppl 4): 1–204.

37. Gardosi J, Kady SM., McGeown P, et al. Classification of stillbirth by relevant condition at death (ReCoDe): population based cohort study. Brit Med J. 2005.331. 1113–1117. doi:10.1136

38. Salafia CM, Pezzullo JC, López-Zeno JA, et al. Placental pathologic features of preterm preeclampsia. Am J Obstet Gynecol. 1995.173(4). 1097–1105.

39. Junaid TO, Brownbill P, Chalmers N, et al. Fetoplacental vascular alterations associated with fetal growth restriction. Placenta. 2004. 35. 808–815, doi:10.1016

40. Sobrevia L, Abarzúa F, Nien JK, et al. Review: Differential placental macrovascular and microvascular endothelial dysfunction in gestational diabetes, Placenta, 2011. 32 Sup2. S159–S164. doi:10.1016/j.placenta.2010.12.011.

41. Maltepe E, Fisher SJ. Placenta: the forgotten organ. Annu Rev Cell Dev Biol. 2015.31. 523–552. doi:10.1146/annurev-cellbio-100814-125620

42. Capute AJ, Accardo PJ. The Infant Neurodevelopmental Assessment: A clinical interpretive manual for CAT-CLAMS in the first two years of life. Part 1. Curr Probl Pediatr. 1996.26.7. 238–257. doi:10.1016/S0045-9380(06)80061-7

43. Sorkun HC, Bir F, Akbulut M, et al. The effects of air pollution and smoking on placental cadmium, zinc concentration and metallothionein expression. Toxicology. 2007.238. 15–22.

44. García-Esquinas E, Pérez-Gómez B, Fernández-Navarro P, et al. Lead, mercury and cadmium in umbilical cord blood and its association with parental epidemiological variables and birth factors. BMC Public Health. 2013;13:841. doi: 10.1186/1471-2458-13-841.

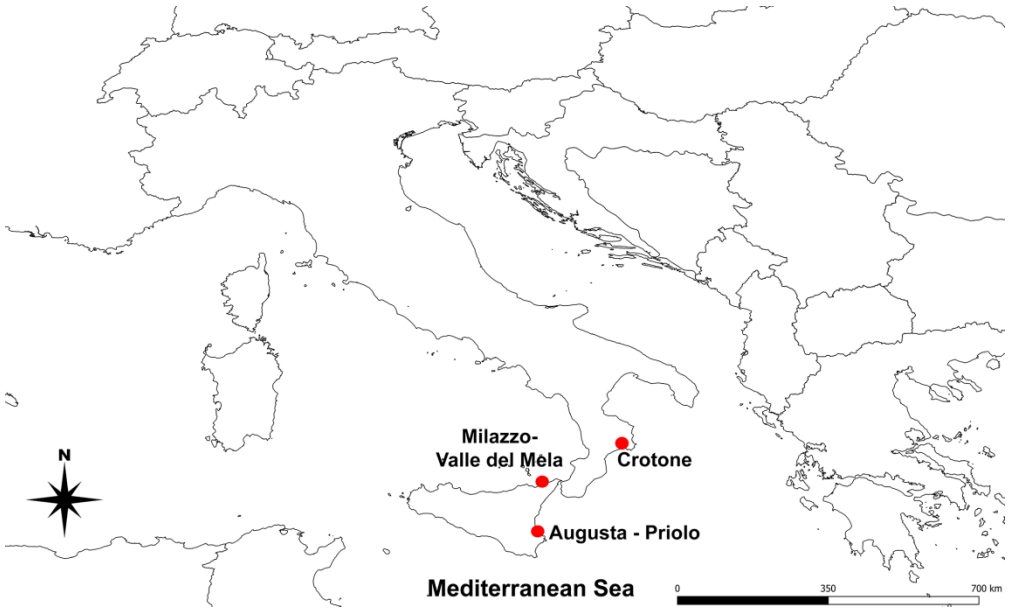
45. Toccaceli V, Serino L, Stazi MA. Informed consent, and an ethico-legal framework for paediatric observational research and biobanking: the experience of an Italian birth cohort study. Cell Tissue Bank. 2014.15. 579–590. doi:10.1007/s10561-014-9431-3.

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.



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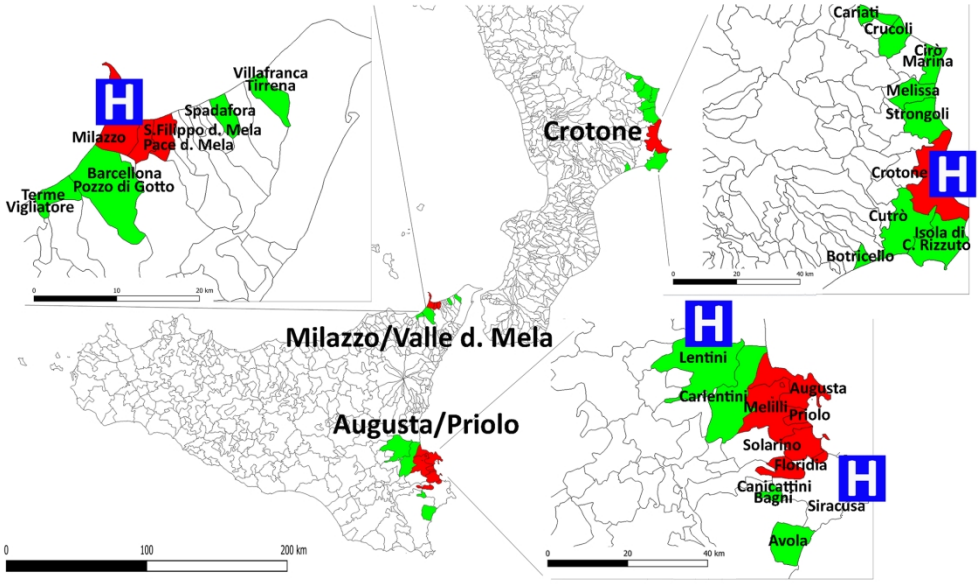

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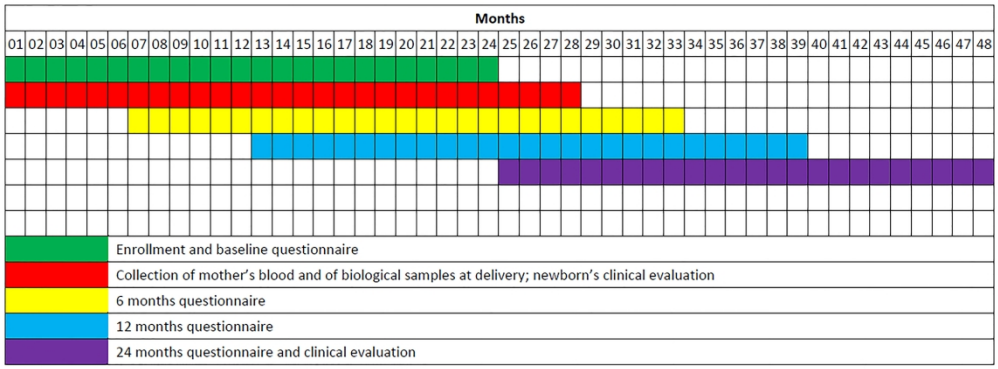

Figure 3 - Schematic overview of the project's time course

189x70mm (300 x 300 DPI)

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

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

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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Word count: 3,835

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 enrolls 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 NPCSs 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]

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]

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]

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]

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

characteristics. Lead and mercury have been shown to be easily transferred through the placental and blood barriers.[28, 31]

Monitoring pollutant concentrations in human tissues, along with the extensive characterization of the entire environmental matrix proposed in the International Centre of Advanced Study in Environment, Ecosystem and Human Health (CISAS) project will provide new insights into toxicant transfer routes from the environment to the human fetus. Our analysis will be focused on the investigation of toxicants that are transferred from the mother's blood to the developing fetus and on the influence of toxicants on fetal development, pregnancy outcomes, and late-onset health consequences. Particular attention will also be paid to the possible interaction between environmental exposures and the low socioeconomic status which often characterizes the investigated population.

METHODS AND ANALYSIS

Experimental context

The CISAS project, funded by the Italian Ministry of Education, Universities and Research, aims at understanding the chemical-physical processes that regulate the distribution of contaminants in various environmental matrices and their transfer to the ecosystem and humans. The CISAS Project has been developed for three selected National Priority Contaminated Sites (NPCSs) in southern Italy: in these contexts, the influence of environmental pollution on pregnant women is of special interest. The three selected NPCSs are: Milazzo-Valle del Mela, Augusta-Priolo, and Crotone. Those in Milazzo and Augusta are widely industrialized coastal areas located in eastern Sicily in which large production sites are present, including refineries, petrochemical and cement plants, power plants, numerous hazardous waste dumps, and the former Eternit plant in Siracusa where asbestos was processed.[32] In the Crotone area, located in the region of Calabria, the most relevant environmental impact is due to three disused industrial areas (the former Pertusola, Fosfotec, and Agricoltura sites)

which operated between the 1920s and the 1990s, mainly in the fields of zinc, phosphoric acid, and complex fertilizer production.[33]

During the course of the (ongoing) CISAS project, the Neonatal Environment and Health Outcomes birth cohort (NEHO) birth cohort will enroll pregnant women living in the three NPCSs, along with pregnant women living in surrounding areas, outside them. The NEHO cohort is aimed at understanding the processes and mechanisms involved in the transfer of heavy metals and Persistent Organic Pollutants (POPs, e.g., PBDEs, PCBs, chlorinated pesticides) from the environment to the ecosystem and humans. In the context of the CISAS project, the same toxicants will be evaluated in all environmental matrices (atmosphere, soil, sediment, inland waters and sea) as well as the food chain (fish, meat, eggs, milk and dairy products, sampled from local producers of each studied area).

Study population and recruitment

Starting in January 2018, the NEHO cohort began recruiting pregnant women living in the three NPCSs of Crotone, Milazzo-Valle del Mela and Augusta-Priolo, in southern Italy (Figure 1), along with pregnant women living in surrounding areas presenting similar geographic and socio-demographic characteristics.

The NEHO study involves twenty-six cities subdivided into study areas and local reference areas. Table 1 shows the selected cities in Sicily and Calabria, as well as their respective distance from industrial areas.

Local reference areas were identified as “local controls” by ISTISAN reports[32, 34] and by the SENTIERI Project, a study of the epidemiological evidence of associations between causes of death and environmental exposures[35-36] coordinated by the Italian National Institute of Health and supported by the Ministry of Health. To maximize recruitment efficiency, maternity units were selected in each NPCS on the basis of both the deliveries/population ratio and available resources.

The NEHO cohort recruits pregnant women in four selected maternity units located in the public hospitals of four cities: the “G. Fogliani” Hospital in Milazzo (for the Milazzo-Valle del Mela NPCS),

the General Hospital of Lentini and the "Umberto I" Hospital in Siracusa (for the Augusta-Priolo NPCS), 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
			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 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

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

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 the 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), including data on the type of water consumed and the place of fish, meat, and vegetable purchases, food packaging, use of plastic dishes, detergents, etc.

After recruitment, mothers are asked to fill out the second part of the baseline questionnaire by means of 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 periconceptional period.

Inclusion criteria

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

- residence in a study area or a local reference area for at least one year;
- ability to speak and understand the Italian language;
- being 18-40 years old at the time of delivery;
- not following any program of assisted reproduction;
- absence of serious chronic diseases, such as diabetes, hypertension, etc.;
- absence of any evident complications during pregnancy diagnosed previous to signing informed consent.

Questionnaire

As in previous studies, the 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 for the gestational period, along with smoking habits (including e-cigarette use), possible chemical exposures, and maternal health characteristics.

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

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

Collection of biological samples

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

Blood samples (15ml) are drawn from a mother's cubital vein at enrollment and after delivery and from the child's umbilical cord immediately after delivery (5ml in K2-EDTA and 10ml in serum separator tubes). Blood tubes are stored at 4°C and centrifuged within 24 hours for 10 minutes at 2,000 x g. Serum is divided into 8 aliquots of approximately 0.5ml. Fractionated K2-EDTA blood is dispensed in 4 aliquots of plasma (0.5ml each). One aliquot of at least 0.1ml of white blood cells (buffy coat) is stored in cryotubes.

In addition, 12 placenta samples are systematically collected from central and peripheral region by means of a biopsy punch, stored in cryotubes with RNAlater, and frozen at -20°C within 2 hours of delivery. Cryotubes with maternal and cord blood samples are periodically transported in dry ice to the NEHO biobank where they are stored in -80°C freezers. Placenta samples are also transferred to the central biobank for long-term storage at -80°C. Incomplete sample collection is not considered as exclusion criteria for follow-up.

Figure 3 provides a schematic overview of the project's time course.

Biobank

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

The preservation of high-quality placental tissue specimens will enable the search for new biomarkers of prenatal exposure to pollutants as well as promote a better understanding of the mechanisms through which potential disruptors are transmitted from mother to fetus. This may suggest possible interventions to be made during pregnancy for the prevention of some adult diseases.

Transcriptomics

The placenta has an active role in fetal development. The impairment of placental formation, differentiation, and/or function severely affects fetal development and is associated with a wide range of pregnancy complications, including pregnancy loss.[37] Other complications linked to placental dysfunction, including gestational diabetes, hypertension, pre-eclampsia, and intrauterine growth restriction can irreversibly result in greater susceptibility to multifactorial disorders during the entire lifespam.[38-40] It is known that toxic substances may interfere with placental signaling cascades involved in metabolism, nutrient and waste product transport, and steroid hormone and enzyme production and release.[41] Therefore, in the context of the NEHO cohort, we will investigate the

relationship between exposure to environmental toxic compounds (both heavy metals and POPs) and shifts in gene expression by means of a whole transcriptome analysis. RNA microarray analysis will be performed by SurePrint G3 Human Gene Expression v3 8x60k Microarray, acquired by the Agilent G2565CA Microarray scanner. The raw data will be analyzed using R biostatistical computing platform RStudio GUI. Gene set enrichment analysis will be performed to visualize the regulated biological processes.

Outcome assessment

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

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

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

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

Finally, at 24 months after 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 the 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 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	• Gestational age, • Weight, • Height, • Head circumference • Apgar score (5', 10'), • Type of delivery • Congenital birth defects	➢ Clinical evaluation
6 months	• Anthropometric child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • CAT/CLAMS part 1	➢ Self-reported
12 months	• Anthropometric child data • Respiratory diseases, • Allergic diseases, • Viral infections, • Hospitalization, • Accidents, • Sleep quality, • CAT/CLAMS part 2	➢ Self-reported
24 months	• 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

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.

The NEHO cohort will enroll an estimated total of 800 mother-child pairs from the three highly polluted areas, by January 2021. Sorkun et al (2007) found increased (0.048±0.014 vs 0.038±0.012) cadmium concentration in placentas of Turkish women living in a highly polluted area with respect to women living in a rural control area.[43] Based on these data, we estimate that a sample of 38 subjects for each study and control area (76 subjects as total sample) will be adequate for detecting such differences in placental heavy metals, with an alpha level of 0.05 and a power (1-β) of 0.95. Moreover, García-Esquinas et al (2013) found significantly reduced 5-minute Apgar scores (9.13±0.6 vs 9.40±0.5) in newborns with umbilical cadmium levels >0.30 µg/l.[44] From these data, we estimate that a sample of 129 subjects for each study and control area (258 subjects as total sample) will be adequate for detecting such differences in 5-minute Apgar scores, with an alpha level of 0.05 and a power (1-β) of 0.95.

Aims

NEHO is the first cohort in Italy to study and analyze pollutant blood levels in pregnant women living near highly polluted areas along with fetal exposure. The project will attempt to identify the influence of environmental risk factors for placental function, pregnancy outcomes, and newborns' health outcomes.

Finally, NEHO will enroll mother-child pairs residing in disadvantaged areas with low socio-economic status, thus representing a particular condition in the context of Italian and European birth cohorts.

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

- 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.

Qualified project staff (biologists, midwives, nurses, gynecologists) provide complete information about the project and manage the completion of the 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 the *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, preserving complete pseudonymization. The study is being conducted following the Declaration of Helsinki. All the adopted procedures comply with the General Data Protection Regulation (UE 2016/679) and Italian laws concerning data protection.

The results of the study will be communicated to participants, the local Regional Health Authorities and clinical professionals only on an aggregated basis through *ad hoc* meetings. The Researchers will also disseminate 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 responsible for the three involved NPCSs: the Ethics Committee of the University Hospitals of Messina for the NPCS of Milazzo-Valle del Mela (September 18, 2017, n. 9/2017); the Ethics Committee "Catania 2" for the NPCS of Augusta-Priolo (July 11, 2017, n. 38/2017/CECT2); the Ethics Committee of the Region of Calabria for the NPCS of Crotone (July 20, 2017, n. 173). Each participant read the information sheet and signed the informed consent. Copies of participants' information sheets are available at the website: www.neho.it.

Availability of data and materials

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

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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REFERENCES

1. Fleming TP, Watkins AJ, Velazquez MA, et al. Origins of lifetime health around the time of conception: causes and consequences. Lancet. 2018.5.391. 1842–1852. doi:10.1016/S0140-6736(18)30312-X.
2. World Health Organization. The European health report 2005: public health action for healthier children and populations. WHO Regional Office for Europe; 2005.
3. Barker DJ, Osmond C, Winter PD et al. Weight in infancy and death from ischaemic heart disease. Lancet. 1989.2. 577–580.
4. Barker DJP. Fetal and infant origins of adult disease. BMJ. 1990.301.1111
5. Barker DJ. Maternal nutrition, fetal nutrition and disease in later life. Nutr.1997.13. 807–813.
6. Barker DJP. The Developmental Origins of Adult Disease. J Am Coll Nutr 2004.23.Suppl 6. 588S–595S.
7. Barker DJP. The origins of the developmental origins theory. J Intern Med. 2007.261. 412–417. doi:10.1111/j.1365-2796.2007.01809.x
8. Gluckman P, Hanson M. Developmental Origins of Health and Disease. Cambridge University Press.2006.
9. Kordas K. European birth cohorts offer insights on environmental factors affecting human development and health. Int J Epidemiol. 2016.55. 731–734. doi:10.1093/eurpub/ckw068
10. Richiardi L, Baussano I, Vizzini L, et al. NINFEA cohort: Feasibility of recruiting a birth cohort through the Internet: the experience of the NINFEA cohort. Eur J Epidemiol.2007.22. 831–837. doi:10.1007/s10654-007-9194-2
11. Vecchi Brumatti L, Montico M, Russian S, et al. Analysis of motivations that lead women to

participate (or not) in a newborn cohort study. BMC Pediatr. 2013.11. 13–53. doi:10.1186/1471-2431-13-53

12. Brescianini S, Fagnani C, Toccaceli V, et al. An update on the Italian twin register: advances, in cohort recruitment, project building and network development. Twin Res Hum Genet. 2013.16. 190–196. doi:10.1017/thg.2012.85

13. Farchi S, Forastiere F, Vecchi Brumatti L, et al. Piccolipiù, a multicenter birth cohort in Italy: protocol of the study. BMC Pediatr 2014.14.36 doi:10.1186/1471-2431-14.36.

14. Porta D, Fantini MP, on behalf of the GASPII and Co.N.ER Study Groups. Prospective cohort studies of newborns in Italy to evaluate the role of environmental and genetic characteristics on common childhood disorders. Ital J Pediatr.2006.32. 350–357.

15. Barchitta M, Maugeri A, La Rosa MC, et al. Single Nucleotide Polymorphisms in Vitamin D Receptor Gene Affect Birth Weight and the Risk of Preterm Birth: Results From the "Mamma & Bambino" Cohort and A Meta-Analysis. Nutr.2018.10.1172. doi:10.3390/nu10091172

16. Vizcaino E, Grimalt JO, Fernández-Somoano A, et al. Transport of persistent organic pollutants across the human placenta. Environ Int. 2014.65. 107–115. doi:10.1016/j.envint.2014.01.004

17. Leonetti C, Butt CM, Hoffman K, et al. Concentrations of Polybrominated Diphenyl Ethers (PBDEs) and 2,4,6-Tribromophenol in Human Placental Tissues. Environ Int. 2016. 23–29 doi:10.1016/j.envint.12.002.

18. Jeong Y, Lee S, Kim S, et al. Placental transfer of persistent organic pollutants and feasibility using the placenta as a non-invasive biomonitoring matrix. Sci Total Environ. 2018.612. 1498–1505. doi: 10.1016/j.scitotenv.2017.07.054.

19. Foster WG, Gregorovich S, Morrison KM, et al. Human maternal and umbilical cord blood concentrations of polybrominated diphenyl ethers. Chemosphere. 2011.84. 1301–1309. doi:10.1016/j.chemosphere.2011.05.028

20. Arbuckle TE, Kubwabo C, Walker M, et al. Umbilical cord blood levels of perfluoroalkyl acids and polybrominated flame retardants. Int J Hyg Environ Health. 2013.216. 184–194

doi:10.1016/j.ijheh.2012.03.004.

21. Jones R & Golding J. Choosing the types of biological sample to collect in longitudinal birth cohort studies. Paediatr Perinat Epidemiol. 2009.23S.1. 103–113. doi:10.1111/j.1365-3016.2008.01000.x.

22. Leino O, Kiviranta H, Karjalainen AK, et al. Pollutant concentrations in placenta. Food Chem Toxicol. 2013.54. 59–69. doi:10.1016/j.fct.2011.10.058.

23. Soares MJ, Varberg KM, Iqbal K. Hemochorial placentation: development, function, and adaptations. Biol Reprod. 2018.99(1). 196–211. doi:10.1093/biolre/ioy049.

24. Bruin JE, Gerstein HC, Holloway AC: Long-term consequences of fetal and neonatal nicotine exposure: a critical review. Toxicol Sci. 2010.116Suppl.2. 364–374. doi:10.1093/toxsci/kfq103

25. Votavova H, Dostalova Merkerova M, Fejglova K, et al. Transcriptome alterations in maternal and fetal cells induced by tobacco smoke. Placenta. 2011.32Suppl.10. 763–770. doi:10.1016/j.placenta.2011.06.022

26. Piasek M, Mikolić A, Sekovanić A, et al. Cadmium in Placenta – A Valuable Biomarker of Exposure During Pregnancy in Biomedical Research. J Toxicol and Env Health. 2014.77. 1071–1074 doi:10.1080/15287394.2014.915779.

27. Gundacker C, Fröhlich S, Graf-Rohrmeister K, et al. Perinatal lead and mercury exposure in Austria. Sci Total Environ. 2010.408.5744–5749. doi:10.1016/j.scitotenv.2010.07.079

28. Gundacker C, Hengstschlager M. The role of the placenta in fetal exposure to heavy metals. Wien Med Wochenschr. 2012.162. 201–206. doi:10.1007/s10354-012-0074-3.

29. Rylander L, Stromberg U, Hagmar L. Lowered birth weight among infants born to women with a high intake of fish contaminated with persistent organochlorine compounds. Chemosphere. 2000.40. 1255–1262.

30. Esteban-Vasallo MD, Aragonés N, Pollan M, et al. Mercury, Cadmium, and Lead Levels in Human Placenta: A Systematic Review. Environ Health Persp. 2012.120. 1369–1377. doi:10.1289/ehp.1204952

31. Caserta D, Graziano A, Lo Monte G, et al. Heavy metals and placental fetal-maternal barrier: A mini-review on the major concerns. Eur. Rev. Med. Pharmacol. Sci. 2013.17. 2198–2206.

32. Carere M, Musmeci L, Bianchi F, et al. Study for the environmental health characterization of the contaminated sites of Gela and Priolo. Istituto Superiore di Sanità. Rapporti ISTISAN. 2016;16/35,67p.

33. Barone V, Calenda C, Motta F, Oranges T. Inquinamento e recupero nel crotonese. Ecoscienza 2010.3.

34. Comba P, Pitimada M. Epidemiological study of Calabria Region contaminated sites: aims, methods and feasibility. Istituto Superiore di Sanità. Rapporti ISTISAN. 2016;16/19.ii.135p.

35. Pirastu R, Ancona C, Iavarone I, et al. Mortality study of residents in Italian polluted sites: evaluation of the epidemiological evidence. Epidemiol Prev. 2010.34.(5-6 Suppl 3). 1–2.

36. Pirastu R, Iavarone I, Pasetto R, et al. SENTIERI Project - Mortality study of residents in Italian polluted sites: Results.Epidemiol Prev. 2001.35.(5-6 Suppl 4): 1–204.

37. Gardosi J, Kady SM., McGeown P, et al. Classification of stillbirth by relevant condition at death (ReCoDe): population based cohort study. Brit Med J. 2005.331. 1113–1117. doi:10.1136

38. Salafia CM, Pezzullo JC, López-Zeno JA, et al. Placental pathologic features of preterm preeclampsia. Am J Obstet Gynecol. 1995.173(4). 1097–1105.

39. Junaid TO, Brownbill P, Chalmers N, et al. Fetoplacental vascular alterations associated with fetal growth restriction. Placenta. 2004. 35. 808–815, doi:10.1016

40. Sobrevia L, Abarzúa F, Nien JK, et al. Review: Differential placental macrovascular and microvascular endothelial dysfunction in gestational diabetes, Placenta, 2011. 32 Sup2. S159–S164. doi:10.1016/j.placenta.2010.12.011.

41. Maltepe E, Fisher SJ. Placenta: the forgotten organ. Annu Rev Cell Dev Biol. 2015.31. 523–552. doi:10.1146/annurev-cellbio-100814-125620

42. Capute AJ, Accardo PJ. The Infant Neurodevelopmental Assessment: A clinical interpretive manual for CAT-CLAMS in the first two years of life. Part 1. Curr Probl Pediatr. 1996.26.7. 238–257. doi:10.1016/S0045-9380(06)80061-7

43. Sorkun HC, Bir F, Akbulut M, et al. The effects of air pollution and smoking on placental cadmium, zinc concentration and metallothionein expression. Toxicology. 2007.238. 15–22.

44. García-Esquinas E, Pérez-Gómez B, Fernández-Navarro P, et al. Lead, mercury and cadmium in umbilical cord blood and its association with parental epidemiological variables and birth factors. BMC Public Health. 2013;13:841. doi: 10.1186/1471-2458-13-841.

45. Toccaceli V, Serino L, Stazi MA. Informed consent, and an ethico-legal framework for paediatric observational research and biobanking: the experience of an Italian birth cohort study. Cell Tissue Bank. 2014.15. 579–590. doi:10.1007/s10561-014-9431-3.

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.

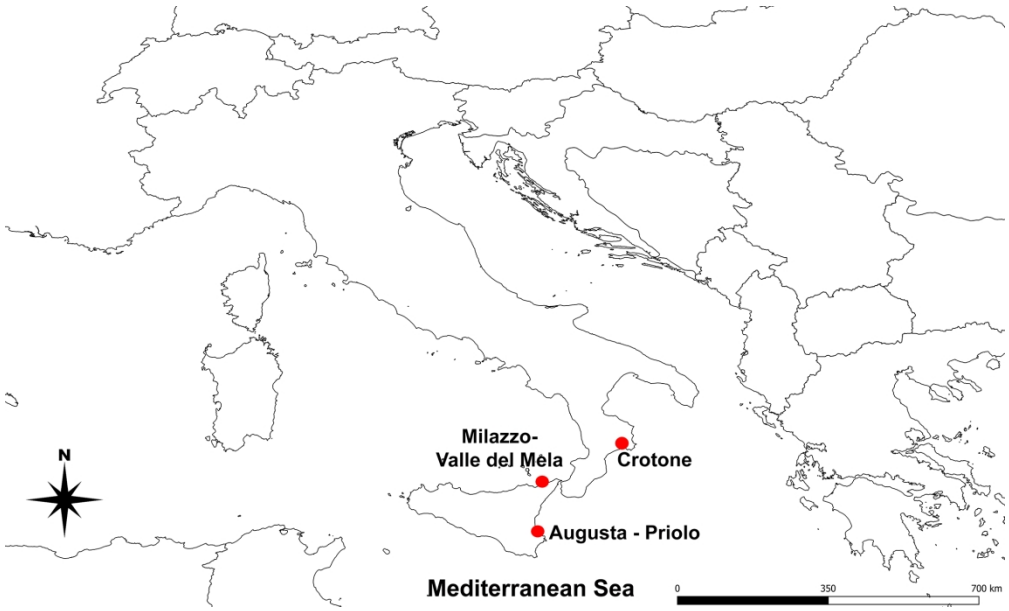
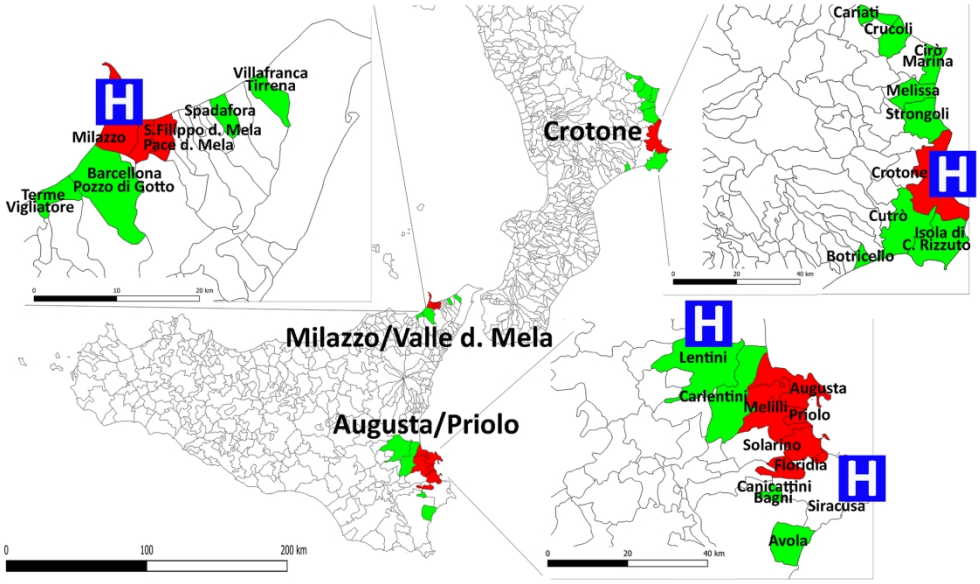


Figure 1 - The three selected National Priority Contaminated Sites involved in the study in the south Mediterranean area of Italy. Created through QGIS Development Team, 2009. QGIS Geographic Information System. Open Source Geospatial Foundation. URL http://qgis.org; shapefile from https://www.arcgis.com/home/item.html?id=2ca75003ef9d477fb22db19832c9554f.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



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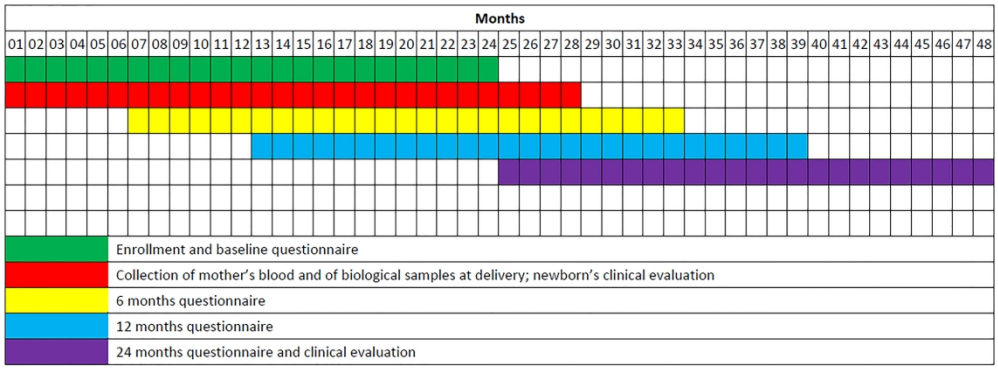

Figure 3 - Schematic overview of the project's time course

189x70mm (300 x 300 DPI)

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.