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Etiological epidemiological studies using biomarkers of exposure and early effect in three contaminated sites.

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1
2
3
4
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7
8
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11
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Etiological epidemiological studies using biomarkers of exposure and early effect in three contaminated sites.

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ABSTRACT / 300 words

Introduction

Environmental pollution has been progressively becoming one the main risk factors to human diseases. In particular, populations living in high-contaminated sites are particularly exposed to environmental toxicants, with consequent increased risks to human health. In Italy, there are currently ongoing three epidemiological etiological studies aimed at evaluating the association between exposure to inorganic and organic chemicals and presence of biological markers of early effects in population living in three National Priority Contamined Sites (NCPSs). Specifically, the correlations concern pre-clinical indicators of liver disease in Priolo NCPS, thyroid diseases in Milazzo-Valle del Mela NCPS, and cardiovascular risk and kidney damage in Crotone NCPS.

Methods and analysis

Overall, approximately 1,300 subjects of both sexes will be enrolled in the three NCPSs according to specific inclusion criteria. For each subject, serum and urine specimens are collected, on which the determination of biological markers of exposure and early effects for the selected outcomes are performed. Individual information on environmental and occupational exposure, medical history, diet, and life habits are obtained through questionnaires provided by web platform. In Milazzo-Valle del Mela and Crotone NCPSs, not invasive instrumental and imaging examinations are performed in order to evaluate further risk factors of thyroid carcinoma and cardiovascular disease, respectively.

Ethics and dissemination

The protocol studies have been approved by the Ethics Committees responsible for the three involved NPCSs: the Ethics Committee 'Catania 2' for the NPCS of Priolo (21 July 2017, n. 500/2017/CECT2), the Ethics Committee of the University Hospitals of Messina for the NPCS of Milazzo-Valle del Mela (19 February 2018, n.2/2018); the Ethics Committee of the Region of Calabria for the NPCS of Crotone (20 July 2017, n. 174). Results will be disseminated among policy makers, citizens, stakeholders and scientific community through the organization of conferences and events, and the publication on international peer/reviewed journals.

1

Strengths and limitations of this study

- In the framework of the CISAS project epidemiological studies based on the evaluation of biological markers of exposure and early effects in populations living in high-contaminated sites of southern Italy are proposed.
- The levels of exposure and the presence of risk factors and early-effects indicators of selected outcomes are evaluated by using biohumoral tests, instrumental and imaging examinations, and information by individual questionnaire.
- The sample size in the three studies is representative of the population distributions by sex, age class, and municipality, and it is established to evaluate statistically significant differences in biological markers between the study and the reference areas.
- The cross-sectional study design is the weakest of the etiological studies, it is informative but inadequate to infer causation in the environment-health relationship.
- The biological markers of health risk, although predictive of important health outcomes, are affected by limits of precision and accuracy proper of biomarkers commonly used in clinical practices.

2

Introduction

In the last decades, research on environment and health has progressively gained importance for contemporary society, given the faster and faster environmental changes and the consequent threats to human health. In 2012, it has been estimated that 12.6 million deaths globally, representing 23% of all deaths and 26% of deaths among children under five years, were attributable to environmental exposures.[1]

The Sixth Ministerial Conference on Environment and Health of 53 Countries of the World Health Organization (WHO) European Region, included for the first time contaminated sites as leading environmental risks and consequently among the priority actions for public health.[2] In the all EU Member States, the total number of potentially contaminated sites is estimated in 2.8 million, of which 65,500 have been already remediated or are under aftercare measures, representing an increment of more than 8,500 new remediated sites in the last 5 years.[3] Nonetheless, since 2011, a reduction in the number of sites under remediation has been reported by Belgium, Estonia, Italy, Latvia, Norway and Slovakia.[3] The contamination of these areas particularly concerns soils and water for which industrial and commercial activities as well as waste disposal and treatment represent the main sources of pollution.[4] As regards air pollution, over the period 2008-2012, considering a total of 14,325 single facilities in the EU-27, plus Norway and Switzerland, the cost of damage to health and environment produced by their emissions ranged between 329 and 1,065 billion euros.[5] It is important to note that fifty per cent of the total damage cost occurs as a result of emissions from 147 plants, and countries such as Germany, Poland, the United Kingdom, France and Italy, having a high number of large facilities, contribute the most to total estimated damage costs.[5] Air pollutants (carbon dioxide, nitrogen oxides, sulfur dioxide, particulate matter), heavy metals (e.g., cadmium (Cd), lead (Pb), arsenic (As), mercury), organic compounds (benzene, dioxins and furans, polycyclic aromatic hydrocarbons) represent the main environmental determinants used to quantify the impacts and associated damage costs.[5]

In Italy there are currently 41 National Priority Contaminated Sites (NPCSs), with a total surface area of 1712 km^2 and representing 0.57% of the national surface area. NCPSs are identified as highly contaminated areas by the Ministry of Environment, in agreement with the regions concerned.[6]

The Italian SENTIERI project, initiated in 2006, provided an ecological epidemiological observation of the populations residing in the aggregation of municipalities included in each NCPS.[7] The current update of SENTIERI covers 45 sites, which include 319 municipalities, with a total population of approximately 5,900,000 inhabitants (9.8% of national population).[4] An overall excess of 5,267 and 6,725 deaths due to all natural causes was detected, respectively, in the male and female population, while the excess due to oncological causes was equal to 3,375 men and 1,910 women.[4] The study is based on the a priori identification of the health effects expected to be associated to the main source of pollution (i.e., industrial plants, harbors, landfills, incinerators), and provided the first solid basis for a global evaluation of the impact of industrial areas on the health of exposed communities. Nonetheless, its ecological design and the consequent use of aggregated data both for exposure and health indicators does not allow to infer any causal relationship between exposures and health outcomes.[8]

With the aim to of studying in depth the relationships between environment and health in selected NCPSs, the Italian Ministry of Education, Universities and Research has funded the ongoing "International Centre of Advanced Study in Environment, Ecosystem and Human Health" (CISAS) project, which involves four institutes of the National Research Council, in collaboration with scientific and regulatory bodies (http://www.cisas.cnr.it/). CISAS is a multidisciplinary study is aimed at understanding environmental pollution phenomena and their impact to ecosystem and human health in highly contaminated sites, organized in six working packages (WPs). The research activity, to be developed by 2021, is focused on the three NCPSs of Priolo and Milazzo-Valle del Mela (Sicily, Italy), and Crotone (Calabria, Italy), characterized by severe environmental contamination and relating health impacts.

The environmental and health studies currently in progress have the objective to assess the levels of contamination of air, water and soil, the routes of chemicals in the environmental matrices, the exposure profiles of local communities through the main route of exposures, including the consumption of food of local production, in addition to investigate the effects of toxicants on pregnancy outcomes in a newborn cohort study.[9]

The Institute of Clinical Physiology of the National Research Council (IFC-CNR) is coordinating a working package developing epidemiological studies with etiological design aimed at evaluating the relationship of

3

exposure to specific pollutants with selected early markers exposure or health risk. Such indicators were identified on the basis of the existing scientific evidence of association with environmental pollutants and the relevance for research and innovation. These studies, in order to assess environmental exposure, consider residence in exposed or unexposed area and the measurement of individual exposure markers. Hereinafter, we describe rationale, objectives, and results expected for each planned study.

Study aims

The CISAS project embraces multidisciplinary activities with the purpose to deepen knowledge on environmental pollution phenomena and their impact on the ecosystem and human health, in order to plan actions mitigating harmful environmental effects and for preventing risks to human health. Within CISAS, three epidemiological studies, started in January 2019, are currently in progress with the following objectives:

- evaluating the association between exposure to organic pollutants and presence of biomarkers and risk factors for hepatic disease and hepatocellular carcinoma (HCC) in Priolo NCPS;
- exploring the relationship between exposure to heavy metals and increased risk of clinical and subclinical hypothyroidism and early indicators of thyroid carcinoma in Milazzo-Valle del Mela NCPS;
- defining the association of exposure to heavy metals and pre-clinical alterations of cardiovascular risk and renal damage in Crotone NCPS;
- enhancing the participation of the general practitioners, environmental and health operators, public administrators, citizens associations and committees;
- supporting a decision-making participated process.

Background

The NCPS of Priolo

Priolo NCPS, other than the Gela and Milazzo NCPSs, is defined by the Sicilian Region as a "high-crisis environmental area", being characterized by the presence of major industrial settlements, mainly petrochemical plants, which in the last decades have caused a progressive contaminations of environmental matrices. In this area high concentrations of persistent and bioaccumulative toxic compounds such as heavy metals, polychlorinated biphenyls (PCBs), dioxins, polycyclic aromatic hydrocarbons, and volatile organic compounds, have been detected.[10] Meanwhile, there has been increased incidence of several types of tumors, in particular mesothelioma, liver and pancreatic neoplasms, in the population of both sexes living in the NCPS, if compared to the rate recorded in Cancer Registries of Central-Southern Italy.[11] Hepatocellular carcinoma accounts for >90% of primary liver cancer cases[12] and it is currently the third leading cause of cancer deaths worldwide (https://emedicine.medscape.com/article/197319-overview). In addition to alcoholism, hepatitis B virus, hepatitis C virus, and illness conditions including Nonalcoholic Fatty Acid Liver Disease (NAFLD), obesity, type 2 diabetes, cirrhosis,[13] the exposure to endocrine disrupting chemicals able to induce metabolic alterations, particularly on lipids, and consequent lipotoxicity and oxidative stress,[14] represent a further risk factor for HCC.[13,15]

The NCPS of Milazzo-Valle del Mela

The high-level contamination of the area is primarily attributable to the presence of plants for the electricity production, a refinery, a steel plant and fuel oil power plant. At global level, the last three decades have been characterized by a significant increase of thyroid carcinoma, the most frequent endocrine malignancy, and, among the acknowledged risk factors including age, sex, radiation, a familiar history of thyroid cancer,[16] much evidence supports the likely etiological role of organic compounds (i.e., PCBs, polybrominated diphenyl ethers, phthalates, bisphenols) and heavy metals (i.e., Cd, Pb) that may act as thyroid disruptors through

4

multiple mechanisms such as interference with thyroid hormone (TH) synthesis, TH metabolism and excretion, and TH action,[17] which, however, have not been completely elucidated.[18,19] In the early 2000s a higher incidence and hospitalization for thyroid cancer was observed in the population living in the NCPS of Milazzo when compared with the regional rate.[11] Recently, a biomonitoring study reported that adolescents living in the contaminated area exhibit a significantly more elevated concentration of urinary Cd than peer controls.[20] Exposure to Pb and Cd, in particular, was reported to be related to thyroid dysfunction in adult women[21] while Cd levels were higher in the blood and thyroid tissue of patients with thyroid cancer.[22,23]

The NCPS of Crotone

The past presence of industrial activities, namely for production of zinc, phosphates, nitrogen fertilizers, resulted in a widespread contamination by heavy metals and hydrocarbons in this site.[7] In this area an excess of mortality for kidney cancer in both sexes, and for urinary tract infections and kidney failure in women were observed. In addition, increased hospitalization rate was found in men and women for ischemic heart disease, nephritis, and nephrotic syndrome, while a higher rate of renal tumors emerged among males.[24] There are increasing suggestions that exposure to As and other toxic metals may be an independent risk factor for cardiovascular disease.[25,26] A recent systematic review and meta-analysis reported that exposure to As, Cd, Pb, and copper is significantly and positively associated with cardiovascular disease, coronary heart disease, and stroke, and for As, Cd, and Pb a linear dose-response relationship was observed. The mechanisms involved comprise induced oxidative stress, inflammation, and endothelial cell damage, which can lead to atherosclerosis.[27] Furthermore, the kidney is a target organ of heavy metal toxicity for its capability to reabsorb and concentrate divalent ions and metals[28] Pb and Cd are established nephrotoxins at high exposure levels[29] and even at environmental exposure may contribute to the risk of chronic kidney disease, a progressive and irreversible deterioration in the renal function, especially in adults with hypertension or diabetes.[30,31]

Study design

The three epidemiological studies have a cross-sectional design and are based on the measurement of exposure and early-effect biological markers. For each subject recruited, the studies provide for:

- the administration of a questionnaire aimed at gathering anthropometric data, information on residential history, socioeconomic and health conditions, environmental and occupational exposure, lifestyle, diet, risk perception;
- the collection of biological samples (serum and urine);
- the measurement of systolic and diastolic blood pressure and heart rate, and neck ultrasound in Milazzo-Valle del Mela NCPS;
- Carotid Ultrasound, Brachial Artery Flow-Mediated Dilation, Arterial Tonometry, Electrocardiogram in Crotone NCPS.

Definition of study and reference areas

The NCPS of Priolo (defined by the Decree 426/1998 of the Italian Ministry of the Environment) comprises the municipalities of Augusta, Melilli, Priolo Gargallo and Syracuse (184.308 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html). The latter has been excluded from the study area as its territorial and demographic characteristics are very different from the other municipalities included in the NCPS, namely it has a much larger population (122,291 total inhabitants) and only a small part of the resident population is resident close to the boundaries of the perimeter of the NCPS. Furthermore, differently from the other municipalities that are homogeneous in both demographic and environmental characteristics, inhabitants of Syracuse are exposed to multiple sources of pollution with a significant component dependent on heating and vehicular traffic. Two other municipalities, Noto and Avola (55,621 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html) were included in the reference area because of the similar demographic

5

and territorial characteristics to those of the study area, i.e., located along the coast and not interested by environmental issues of industrial origin (Figure 1).

The NCPS of Milazzo-Valle del Mela (defined by the Decree 266/2005 of the Italian Ministry of the Environment) includes the municipalities of Milazzo, San Filippo del Mela, Pace del Mela (about 45,000 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html). Other four municipalities (Barcellona Pozzo di Gotto, Terme Vigliatore, Villafranca Tirrena and Spadafora) were considered as reference area (62,583 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html) (Figure 1).

As regards the NCPS of Crotone, defined by the Decree 468/2001 of the Italian Ministry of the Environment (total inhabitants 62,178 on 1 January 2016, http://www.demo.istat.it/index.html), two municipalities, Cutro and Isola di Capo Rizzuto (total inhabitants 28.184 on 1 January 2016, http://www.demo.istat.it/index.html), were selected as reference area (Figure 1).

Inclusion criteria for recruitment

For the three epidemiological studies inclusion criteria have been established as follows:

For the NCPS of Priolo:

- Men and women aged 40-70 years (the age class most at risk for the development of liver disease);[32]
- residence in the study area for at least 10 years before the enrollment: since liver disease is a long-term disease, this time of residence is considered sufficient for the evaluation of early-effects biological markers;[33]
- absence of known liver diseases;
- signed informed consent to participate in the study.

Subjects with an acknowledged diagnosis of liver disease, positivity for hepatitis B and C virus infections, with abuse of alcohol or consumption of other drugs are excluded from the study.

For the NCPS of Milazzo-Valle del Mela:

- Men and women aged 18-44 years (thus excluding women in menopausal status, which could lead to a misclassification of early effects biological markers);
- residence in the study area for at least 10 years before the enrollment[34,35];
- absence of thyroid diseases;
- signed informed consent to participate in the study.

Individuals with an acknowledged diagnosis of thyroid disease or subjected to pharmacological therapy for thyroid disease are excluded from the study.

For the NCPS of Crotone:

- Men and women aged 40-70 years (only subjects in cardiovascular primary prevention were selected due to the high sensibility and vulnerability of this population);[36]
- residence in the study area for at least 5 years before the enrollment, as this period of time is optimal to link the measured CV biomarkers to the CV outcomes in terms of both mortality and morbidity;[37,38]
- subjects in primary prevention for cardiovascular risk;
- signed informed consent to participate in the study.

The exclusion criteria include the presence of systemic diseases, past cardiovascular events, and/or history of nephropathy.

Questionnaires

6

In addition to some common sections (i.e., personal data, sports, smoking and alcohol consumption, medical history and clinical conditions, environmental and socioeconomic conditions, occupational activity, exposure to physical and chemical agents, diet, reproductive history, hazard and risk perception, access to information on environment and health), the questionnaire administered to the participants includes other sections aimed at providing insights about potential sources of exposure, and previous and current diseases of subjects and their own families specific for each NCPS:

- Priolo NCPS: physical and/or sporting activity practiced and at what level, vaccinations, dental fillings, tattoos and /or piercings, family medical history, previous and current cardiovascular diseases, previous and current non-cardiovascular diseases, domestic heating/cooling modality, owning of televisions/tablets/computers/mobile phones.
- Milazzo-Valle del Mela NCPS: symptomatology associated to thyroid diseases, use of supplements, history of adverse reproductive outcomes and information on menstrual cycle for women, information on sterility/infertility for both sexes.
- Crotone NCPS: sleep disorders, adverse life events, family medical history, primary factors of cardiovascular risk, previous and current cardiovascular diseases, previous and current non-cardiovascular diseases

The questionnaires have been tested for comprehension and readability by three researchers independently. In order to avoid both paper administration and human errors, an online platform has been created for entering answers to the questionnaires. The questionnaires are administered by one or more operators who reported the answers on the platform. To adequately train each operator, a guide for the correct administration of the questionnaires have been produced and delivered to the contact person of each area. The questionnaires may be requested to these authors.

Biohumoral tests

In the three NCPSs and corresponding reference areas a sample of venous blood and a sample of urine is collected for all participants (Table 1). Blood tubes are stored at 4°C and centrifuged within 24 hours for analyses performed locally, whereas aliquots of plasma and urine are stored at -20°C until their transfer to the biobank of IFC-CNR for supplementary tests.

NCPS "Priolo"

In this study the results deriving from blood clinical tests are used with the anthropometric data to determine the Fatty Liver Index (FLI), the Fibrotic Liver Index (FIB-4) and the NAFLD Fibrosis Score (NFS), in order to evaluate the presence of hepatic steatosis and fibrosis, respectively.[39,40,41] According to the European Association for the Study of the Liver, European Association for the Study of Diabetes and European Association for the Study of Obesity, FLI, FIB-4 and NFS are non-invasive markers, which can be used with liver enzymes to identify subjects at risk of liver disease.[39]

-The FLI is calculated by the following formula:[40]

$$(e^{0.953*\log_e (triglycerides) + 0.139*BMI + 0.718*\log_e (ggt) + 0.053*waist\ circumference - 15.745}) / (1 + e^{0.953*\log_e (triglycerides) + 0.139*BMI + 0.718*\log_e (ggt) + 0.053*waist\ circumference - 15.745}) *100$$

with the cut off FLI<30 for presence of NAFLD

-The FIB-4 computation is based on the following formula:[43]

$$age\ (years) \times AST\ [U/L]/(platelets\ [10^9/L] \times (ALT\ [U/L])1/2)$$

with the cut off FIB-4 >1.30 for presence of fibrosis

-The NAFLD fibrosis score (NFS)[44] is calculated by the following formula (http://gihep.com/calculators/hepatology/nafld-fibrosis-score/):

7

$$-1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m2)} + 1.13 \times \text{IFG/diabetes (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet } (\times 109/l) - 0.66 \times \text{albumin (g/dl)}$$

with the cut off NFS >-1.455 for presence of fibrosis

Furthermore, serological lipidomics markers and urinary phthalates and bisphenols are evaluated by Quadrupole Time-of-Flight Liquid Chromatography/Mass Spectrometry and Gas Chromatography/Mass Spectrometry, while serum inflammatory markers are measured by Luminex technology (Table 1).

NCPS "Milazzo – Valle del Mela"

In addition to the determination of serum thyroid hormones, namely thyroxine (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH), also the evaluation of the gene expression of nuclear receptors of thyroid hormone (THR-alpha, -beta) and retinoic acid (RAR-alpha, -gamma), and of 9-cis-retinoic acid receptor (RXR-alpha) levels in whole blood are performed as previously done (Table 1).[45] The analysis of gene expression crossed with the related hormone levels allows to assess the possible association between exposure and clinical or sub-clinical hypothyroidism or other subclinical conditions of alterations of thyroid hormones as well. Furthermore, urinary Cd and Pb, considered as markers of individual environmental exposure, are determined with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) by an accredited laboratory.

NCPS "Crotone"

Markers of inflammation, oxidative stress, cardiac biomarkers, markers of blood vulnerability to thrombosis, and bone health are measured in plasma specimens of each participant (Table 1). The potential association between exposure to heavy metals and cardiovascular disease (CVD) has several clinical implications, mainly due to an increase in oxidative stress and inflammatory status.[45,46] In addition, chronic exposure to Cd can lead to increased arterial stiffness and altered endothelial function.[47,48] It is interesting to note that different heavy metals are pollutants able of critically influencing bone mass[49] that is associated, according to recent studies, to a higher risk of CVD.[50,51]

The impact of environmental exposure to heavy metals on renal function are assessed through the determination of biomarkers of tubular damage in urine samples, indexed by urinary creatinine excretion levels. Moreover, kidney damage are further estimated by the glomerular filtration rate (GFR) using serum creatinine-based formulas (Cockcroft–Gault and Modification of Diet in Renal Disease)[52] and serum cystatin-based formula[53] for predicting GFR.

These biochemical tests are performed using standard available automated methods, or manual colorimetric and ELISA commercially available kits, according to the manufacturer's instructions.

Finally, individual exposure to Cd and Pb are determined both in blood and urine specimens with ICP-MS by an accredited laboratory.

Table 1. Summary of clinical chemistry tests on blood and urine specimens by study area.

Area	Test	Amount (ml)
	Blood	
	Hemochrome, FBG, triglycerides, total and HDL cholesterol, SGOT, SGPt, GGT, HbA1c, bilirubin, ferritin, transferrin, albumin	10
Priolo NCPS	Lipidomic profile (including free fatty acid profile), leptin, insulin, inflammatory biomarkers (MCP-1, TNFα)	10
	Total	20
	Urine	
	Measurement of phthalates and bisphenols	10
	Total	10

8

	Blood	
Milazzo - Valle del Mela NCPS	Hemochrome, ESR, fibrinogen, CRP, glycemia, creatinine, triglycerides, total, HDL and LDL cholesterol	12
	TSH, FT3, FT4, thyroglobulin, antithyroglobulin Ab	6
	Calcitonin	6
	Cd, Pb	6
	THR-alpha, THR-beta, RAR-alpha, RAR-gamma, RXR-alpha	4
	Total	34
	Urine	
	Early morning urine: Iodine, creatinine	10
	24 h urine: Cd, Pb	10
	Total	20
Crotone NCPS Cardiovascular system	**Blood**	
	Hemochrome, Triglycerides, total, HDL and LDL cholesterol, urea, creatinine, ALT, AST, GGT, CRP, glycemia, fibrinogen, TSH	15
	Inflammatory markers (IL-6)	1
	Oxidative stress markers (hydroperoxides, total antioxidant capacity)	2
	Markers of blood vulnerability to thrombosis: (circulating microparticles, tPA, and CD40 ligand)	6
	Fibrosis (galectin-3) and cardiac damage markers (BNP)	3
	Bone health markers (vitamin D, osteocalcin)	3
	Cd, Pb	6
	Total	36
Crotone NCPS Kidney	**Blood**	
	Cystatin C	10
	Total	10
	Urine	
	Early morning urine: Markers of tubular damage (NAG, β2-MG, KIM-1, NGAL, L-FABP)	10
	24-h urine: Albumin, protein	10
	24-h urine: Cd, Pb	10
	Total	30

Abbreviations: β2-MG: β2-microglobulin; BNP: N-terminal pro B-type natriuretic peptide; Cd: cadmium; CRP: C reactive protein; FBG: fasting blood glucose; FT3: free triiodothyronine; FT4: thyroxine; GGT: gamma glutamyl transferase; IL-6: interleukine 6; HDL: high density lipoprotein; KIM-1: Kidney injury molecule-1; LDL: low density lipoprotein; L-FABP: Liver-type fatty acid binding protein; NAG: N-acetyl-β-D-glucosaminidase; NGAL: neutrophil gelatinase-associated lipocalin; Pb: lead; RAR: retinoid acid receptor; RXR: retinoid X receptor; SGOT: alanine aminotransferase; SGPT: aspartate aminotransferase; THR: thyroid hormone receptor; tPA: tissue plasminogen activator; TSH: thyroid stimulating hormone.

Instrumental and imaging examinations

NCPS "Milazzo - Valle del Mela"

In addition to biohumoral tests, a neck ultrasonography is performed in all subjects by a commercially available system focusing on the examination of the following parameters: general characteristics of the thyroid parenchyma (glandular volume, echogenicity, echo structure, vascularization and presence of parenchymal calcifications), morpho-structural evaluation of possible nodular lesions (location, size, echogenicity, echo structure, calcifications, content and vascular pattern), possible presence of lymphadenopathy. In the case of indications emerging from the ultrasound scan for the opportunity to proceed with fine-needle aspiration biopsy (i.e., presence of thyroid nodules with diameter > 10 mm or nodules < 10 mm in diameter that require further evaluation because of clinical symptoms or associated lymphadenopathy)[54], this information is transmitted to the subject and to the attending physician.

NCPS "Crotone"

9

Brachial artery flow mediated dilation is used to assess endothelial function by processing of ultrasound images of the brachial artery following International consensus guidelines.[55] In particular, maximal percentage increase of the brachial artery diameter following reactive hyperaemia is measured using an automatic edge detection system (FMD Studio, Quipu, Pisa Italy). Furthermore, carotid-femoral pulse wave velocity (PWV) is assessed as a surrogate marker of regional arterial stiffness. For this purpose, an applanation tonometer is employed to record pressure waveforms at the carotid and femoral artery levels, thus obtaining the PWV value. The carotid pressure curve is also rescaled in order to estimate central blood pressure and augmentation index. Ultrasound scans of the common carotid artery are acquired and processed in order to assess anatomical and functional parameters. In particular, intimal-media thickness, plaque presence and carotid stiffness is estimated using an automatic software (Carotid Studio, Quipu, Pisa, Italy).[56] Finally, heart rate variability is performed automatically both in time and frequency domain by processing 12-lead ECG traces.

Statistics

Distribution of people residing in each study and reference has allowed to extract a sample that reflects distribution by sex, age class and municipality of residence of inhabitants in NCPSs and corresponding reference areas. Thus, from the list of patients of Local Health Companies of Siracusa, Messina and Crotone the sample, sized in proportion to the population of every municipality included in the study, was randomly selected with stratification by sex and age class. To calculate the statistical power of each study, the following indicators were considered:

- Priolo NCPS: NAFLD Fibrosis Score NFS and FLI In order to obtain a difference of -1 between the value of NFS - 2.4 obtained in a general population [39] and the cutoff of NFS =- 1.45[44] to distinguish the subjects with fibrosis from those without fibrosis; and a difference of -15 between the reference value of FLI = 35 obtained in a general population[39] and the value of NFS = 50 for the subjects with NAFLD[44] a sample of 500 subjects was defined, 250 in the NCPS and 250 in the reference area, with a statistical power $(1-\beta)$ of 90% and a significance level (α) of 0.05 using a two-tailed t-test.

- Milazzo-Valle del Mela NCPS: Thyroid nodules with > 10 mm diameter. Palpable thyroid nodules > 10 mm diameter are detected in approximately 5% of the population[54]: most of them are benign but 5% develops to malignant nodules.[57] With a sample of 480 subjects, 240 for each area, α=0.05, 1-β=0.8 through bilateral test, and a difference of risk between the two areas corresponding to 0.07, was obtained.

- Crotone NCPS: Arterial stiffness. This is the indicator that, among those of imaging, has the characteristics of the lowest accuracy and precision. With regard to this biomarker, hypothesizing a difference between the exposed and unexposed population of 8% and a standard deviation of 20%, considering a sample of 150 individuals in each exposure group, and assuming a first type error of 5% for bilateral testing, statistical power of 90% was achieved.

  As the pilot study on the evaluation of kidney damage is based on a sample halved compared to the main study assessing the cardiovascular risk in the same population, the enrollment ends when for 75 subjects living in each area who have joined the first study, the possibility and the willingness to participate in the second survey is verified.

The comparison between the levels of exposure to the selected pollutants and the levels of biological markers will be performed through univariate and multivariate statistical analyses (adjusting for confounding or modification covariates), using parametric and non-parametric statistical methods, chosen after checking the normality of the distribution of the dependent variables considered.

Patient and public involvement

The recruitment is based on healthy volunteers adhering inclusion criteria. Patients and the public were not involved in the study design but they are partially involved in the conduction (questionnaires and relationship with the general practitioners) and in the dissemination, as the protocol plans a public presentation of aggregated results to all stakeholders including the patients.

10

Ethics approval

The protocol studies have been approved by the Ethics Committees responsible for the three involved NPCSs: the Ethics Committee 'Catania 2' for the NPCS of Priolo (21 July 2017, n. 500/2017/CECT2), the Ethics Committee of the University Hospitals of Messina for the NPCS of Milazzo-Valle del Mela (19 February 2018, n.2/2018); the Ethics Committee of the Region of Calabria for the NPCS of Crotone (20 July 2017, n. 174). Results will be disseminated among policy makers, citizens, stakeholders and scientific community through the organization of conferences and events, and the publication on international peer/reviewed journals.

Ethics and dissemination

The subjects enrolled may participate to the studies after receiving an extensive explanation about project details and research aims, and signing the informed consent. Each participant is assigned a unique identification code for the chemical tests, instrumental examinations and questionnaires in order to link all the data preserving complete pseudonymisation. All the images acquired are stored on digital support in pseudonymizated form and data are re-evaluated by an operator "blind" and with quantitative "operator-independent" systems, in order to reduce inter- and intra-observer variability. The procedures adopted comply the General Data Protection Regulation (EU) 2016/67 regulating data protection and privacy for all individual citizens of the European Union and the European Economic Area.

In the case of studies based on individual markers of exposure of early effect specific ethical requirements regulate not only the protection of volunteers' privacy, but also the dissemination and communication of results.[58] The content of the questionnaire is developed in accordance with ethical requirements, and is included in the approval procedures of local Ethics Committees. The studies are being conducted in compliance with the principles of Declaration of Helsinki.

Among the requirements, there are the respect of the right to know as the basis of self-determination in the use of research results, the distribution of benefits to different groups, and the choice to deliver the results to the research subjects if they want. At enrollment, an information sheet and the informed consent to be signed provide a complete explanation of project details and research aims. Moreover, all the subjects are made aware that they may withdraw from participating at any time.[59]

CISAS has been giving particular importance to the communication of information about the project activities to policy makers, citizens, and stakeholders.[60] Public conferences have been organized in the previous months and others will be held at the end of the studies to make available the overall results to the local Regional Health Authorities, the authorities of the municipalities involved, and the scientific community.[60] Findings will also be published on international peer-reviewed journals.

On the main topics of the project, educational and training activities in the schools located in the three contaminated areas have been scheduled from before the start to after the end of the studies.

Results expected and impacts on research

The described epidemiological study designs in CISAS project represent the first example of analytical investigations of human biomonitoring based on the evaluation of exposure and pre-clinical effects in populations residing in NCPSs of Southern Italy. The results obtained will allow to develop epidemiological knowledge in the examined areas, and in particular will provide insights in the relationships between priority pollutants of each contaminated site and selected health outcomes, in the view of improving the evidence on and in the molecular mechanisms involved as well. In particular, it will be possible:

- to identify whether subjects exposed to endocrine disrupting chemicals exhibit increased hepatic biomarkers, and alterations of lipid metabolism that may promote the development of liver disease and, with time, possibly HCC;
- to evaluate the association between exposure to heavy metals and increased risk of indicators of thyroid disease and thyroid cancer;
- to assess the effect of exposure to heavy metals on cardiovascular risk and on glomerular and tubular function in cardiovascular primary prevention subjects.

11

The innovative feature of these studies is represented by the assessment of non-invasive, exposure and early-effects biomarkers that would be helpful to implement integrated health-environment surveillance systems in NCPSs and seek novel strategies aimed at decreasing the adverse effects of environmental pollution, including specific remediation programs of the environmental matrices.

Such an approach could be applied in other contaminated sites having similar environment and health characteristics, as it represents a valuable method to monitor exposed communities that could be followed over time for a reliable exposure assessment. Information on early health effects can be particularly useful for planning specific prevention actions for polluted areas.

It is hoped that the participatory process can contribute both to decision-making processes based on scientific evidence and to grow scientific citizenship.

Contributors

AG, CV, DB, FB, FF, FM, FS, GT, MI conceptualized the design and methodology of epidemiological studies; FG drafted the manuscript; FG and FB critically reviewed the manuscript and gave final approval of the version to be published. CV, DB, EB, FB, FG, FM, FS, GQ, GT, LC, MI contributed in drafting the manuscript. All the authors read and approved the final version of the manuscript.

Competing interests

None to declare.

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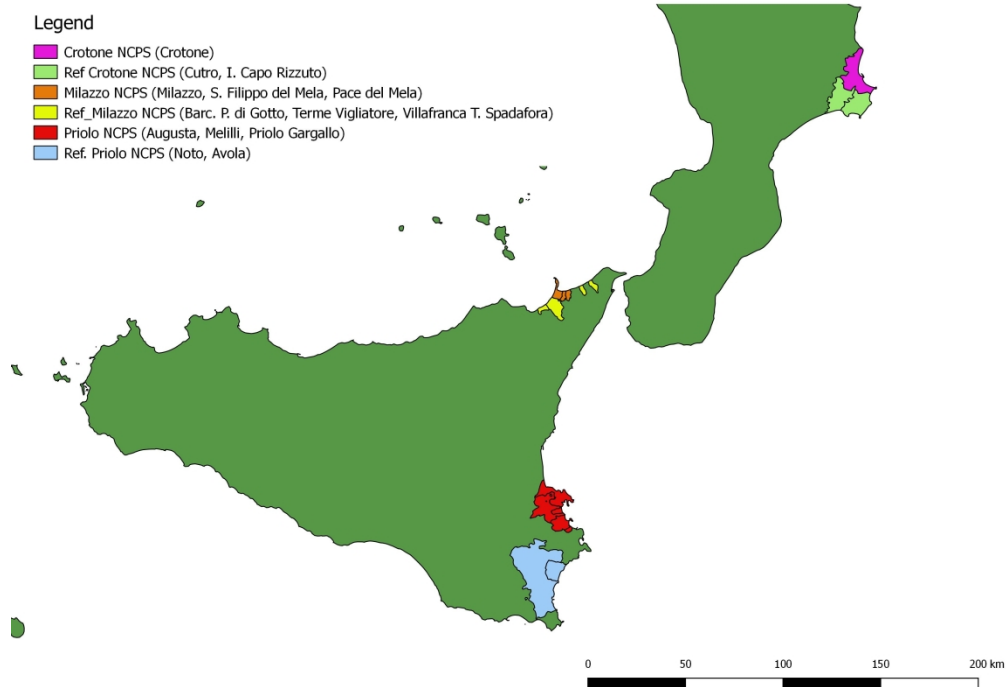Figure 1. Map of National Priority Contaminated Sites and reference areas in CISAS project

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Biomarkers of exposure and early effect in three contaminated sites of southern Italy: protocols for etiological epidemiological studies.

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Biomarkers of exposure and early effect in three contaminated sites of southern Italy: protocols for etiological epidemiological studies

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ABSTRACT

Introduction

Environmental pollution has been progressively becoming one the main risk factors to human diseases. In particular, populations living in high-contaminated sites are particularly exposed to environmental toxicants, with consequent increased risks to human health. In Italy, there are currently ongoing three epidemiological etiological studies aimed at evaluating the association between exposure to inorganic and organic chemicals and presence of biological markers of early effects in population living in three National Priority Contaminated Sites (NCPSs). Specifically, the correlations concern pre-clinical indicators of liver disease in Priolo NCPS, thyroid diseases in Milazzo-Valle del Mela NCPS, and cardiovascular risk and kidney damage in Crotone NCPS.

Methods and analysis

Overall, approximately 1,300 subjects of both sexes will be enrolled in the three NCPSs according to specific inclusion criteria. For each subject, serum and urine specimens are collected, on which the determination of biological markers of exposure and early effects for the selected outcomes are performed. Individual information on environmental and occupational exposure, medical history, diet, and life habits is obtained through questionnaires provided by web platform. In Milazzo-Valle del Mela and Crotone NCPSs, not invasive instrumental and imaging examinations are performed in order to evaluate further risk factors of thyroid carcinoma and cardiovascular disease, respectively.

Ethics and dissemination

The protocol studies have been approved by the Ethics Committees responsible for the three involved NPCSs: the Ethics Committee 'Catania 2' for the NPCS of Priolo (21 July 2017, n. 500/2017/CECT2), the Ethics Committee of the University Hospitals of Messina for the NPCS of Milazzo-Valle del Mela (19 February 2018, n.2/2018); the Ethics Committee of the Region of Calabria for the NPCS of Crotone (20 July 2017, n. 174). Results will be disseminated among policy makers, citizens, stakeholders and scientific community through the organization of conferences and events, and the publication on international peer/reviewed journals.

1

Strengths and limitations of this study

- In the framework of the CISAS project epidemiological studies based on the evaluation of biological markers of exposure and early effects in populations living in high-contaminated sites of southern Italy are proposed.
- The levels of exposure and the presence of risk factors and early-effects indicators of selected outcomes are evaluated by using biohumoral tests, instrumental and imaging examinations, and information by individual questionnaire.
- The sample size in the three studies is representative of the subpopulation with the distribution by sex, age class, and municipality, also considering inclusion/exclusion criteria as established by the study protocols, and it has been defined to evaluate statistically significant differences in biological markers between the study and the reference areas.
- The cross-sectional study design is the weakest of the etiological studies, but it is sufficiently informative to investigate the relationship between environmental exposure and early indicators of disease.
- The biological markers of health risk, although predictive of important health outcomes, are affected by limits of precision and accuracy proper of biomarkers commonly used in clinical practices.

2

INTRODUCTION

In the last decades, research on environment and health has progressively gained importance for contemporary society, given the faster and faster environmental changes and the consequent threats to human health. In 2012, it has been estimated that 12.6 million deaths globally, representing 23% of all deaths and 26% of deaths among children under five years, were attributable to environmental exposures.[1]

The Sixth Ministerial Conference on Environment and Health of 53 Countries of the World Health Organization (WHO) European Region, included for the first time contaminated sites as leading environmental risks and consequently among the priority actions for public health.[2] In the all EU Member States, the total number of potentially contaminated sites is estimated in 2.8 million, of which 65,500 have been already remediated or are under aftercare measures, representing an increment of more than 8,500 new remediated sites in the last 5 years.[3] Nonetheless, since 2011, a reduction in the number of sites under remediation has been reported by Belgium, Estonia, Italy, Latvia, Norway and Slovakia.[3] The contamination of these areas particularly concerns soils and water for which industrial and commercial activities as well as waste disposal and treatment represent the main sources of pollution.[4] As regards air pollution, over the period 2008-2012, considering a total of 14,325 single facilities in the EU-27, plus Norway and Switzerland, the cost of damage to health and environment produced by their emissions ranged between 329 and 1,065 billion euros.[5] It is important to note that fifty per cent of the total damage cost occurs as a result of emissions from 147 plants, and countries such as Germany, Poland, the United Kingdom, France and Italy, having a high number of large facilities, contribute the most to total estimated damage costs.[5] Air pollutants (carbon dioxide, nitrogen oxides, sulfur dioxide, particulate matter), heavy metals (e.g., cadmium (Cd), lead (Pb), arsenic (As), mercury), organic compounds (benzene, dioxins and furans, polycyclic aromatic hydrocarbons) represent the main environmental determinants used to quantify the impacts and associated damage costs.[5]

In Italy there are currently 41 National Priority Contaminated Sites (NPCSs), with a total surface area of 1712 km^2 and representing 0.57% of the national surface area. NCPSs are identified as highly contaminated areas by the Ministry of Environment, in agreement with the regions concerned.[6]

The Italian SENTIERI project, initiated in 2006, provided an ecological epidemiological observation of the populations residing in the aggregation of municipalities included in each NCPS.[7] The current update of SENTIERI covers 45 sites, which include 319 municipalities, with a total population of approximately 5,900,000 inhabitants (9.8% of national population).[4] An overall excess of 5,267 and 6,725 deaths due to all natural causes was detected, respectively, in the male and female population, while the excess due to oncological causes was equal to 3,375 men and 1,910 women.[4] The study is based on the a priori identification of the health effects expected to be associated to the main source of pollution (i.e., industrial plants, harbors, landfills, incinerators), and provided the first solid basis for a global evaluation of the impact of industrial areas on the health of exposed communities. Nonetheless, its ecological design and the consequent use of aggregated data both for exposure and health indicators does not allow to infer any causal relationship between exposures and health outcomes.[8]

With the aim to of studying in depth the relationships between environment and health in selected NCPSs, the Italian Ministry of Education, Universities and Research has funded the ongoing "International Centre of Advanced Study in Environment, Ecosystem and Human Health" (CISAS) project, which involves four institutes of the National Research Council, in collaboration with scientific and regulatory bodies (http://www.cisas.cnr.it/). CISAS is a multidisciplinary study is aimed at understanding environmental pollution phenomena and their impact to ecosystem and human health in highly contaminated sites, organized in six working packages (WPs). The research activity, to be developed by 2021, is focused on the three NCPSs of Priolo and Milazzo-Valle del Mela (Sicily, Italy), and Crotone (Calabria, Italy), characterized by severe environmental contamination and relating health impacts.

The environmental and health studies currently in progress have the objective to assess the levels of contamination of air, water and soil, the routes of chemicals in the environmental matrices, the exposure profiles of local communities through the main route of exposures, including the consumption of food of local production, in addition to investigate the effects of toxicants on pregnancy outcomes in a newborn cohort study.[9]

3

The Institute of Clinical Physiology of the National Research Council (IFC-CNR) is coordinating a working package developing epidemiological studies with etiological design aimed at evaluating the relationship of exposure to specific pollutants with selected early markers exposure or health risk. Such indicators were identified on the basis of the existing scientific evidence of association with environmental pollutants and the relevance for research and innovation. These studies, in order to assess environmental exposure, consider residence in exposed or unexposed area and the measurement of individual exposure markers. Hereinafter, we describe rationale, objectives, and results expected for each planned study.

METHODS AND ANALYSIS
Experimental context
The NCPS of Priolo

Priolo NCPS, other than the Gela and Milazzo NCPSs, is defined by the Sicilian Region as a "high-crisis environmental area", being characterized by the presence of major industrial settlements, mainly petrochemical plants, which in the last decades have caused a progressive contaminations of environmental matrices. In this area high concentrations of persistent and bioaccumulative toxic compounds such as heavy metals, polychlorinated biphenyls (PCBs), dioxins, polycyclic aromatic hydrocarbons, and volatile organic compounds, have been detected.[10] Meanwhile, there has been increased incidence of several types of tumors, in particular mesothelioma, liver and pancreatic neoplasms, in the population of both sexes living in the NCPS, if compared to the rate recorded in Cancer Registries of Central-Southern Italy.[11] Hepatocellular carcinoma accounts for >90% of primary liver cancer cases[12] and it is currently the third leading cause of cancer deaths worldwide (https://emedicine.medscape.com/article/197319-overview). In addition to alcoholism, hepatitis B virus, hepatitis C virus, and illness conditions including Nonalcoholic Fatty Acid Liver Disease (NAFLD), obesity, type 2 diabetes, cirrhosis,[13] the exposure to endocrine disrupting chemicals able to induce metabolic alterations, particularly on lipids, and consequent lipotoxicity and oxidative stress,[14] represent a further risk factor for HCC.[13,15]

The NCPS of Milazzo-Valle del Mela

The high-level contamination of the area is primarily attributable to the presence of plants for the electricity production, a refinery, a steel plant and fuel oil power plant. At global level, the last three decades have been characterized by a significant increase of thyroid carcinoma, the most frequent endocrine malignancy, and, among the acknowledged risk factors including age, sex, radiation, a familiar history of thyroid cancer,[16] much evidence supports the likely etiological role of organic compounds (i.e., PCBs, polybrominated diphenyl ethers, phthalates, bisphenols) and heavy metals (i.e., Cd, Pb) that may act as thyroid disruptors through multiple mechanisms such as interference with thyroid hormone (TH) synthesis, TH metabolism and excretion, and TH action,[17] which, however, have not been completely elucidated.[18,19] In the early 2000s a higher incidence and hospitalization for thyroid cancer was observed in the population living in the NCPS of Milazzo when compared with the regional rate.[11] Recently, a biomonitoring study reported that adolescents living in the contaminated area exhibit a significantly more elevated concentration of urinary Cd than peer controls.[20] Exposure to Pb and Cd, in particular, was reported to be related to thyroid dysfunction in adult women[21] while Cd levels were higher in the blood and thyroid tissue of patients with thyroid cancer.[22,23]

The NCPS of Crotone

The past presence of industrial activities, namely for production of zinc, phosphates, nitrogen fertilizers, resulted in a widespread contamination by heavy metals and hydrocarbons in this site.[7] In this area an excess of mortality for kidney cancer in both sexes, and for urinary tract infections and kidney failure in women were observed. In addition, increased hospitalization rate was found in men and women for ischemic heart disease,

4

nephritis, and nephrotic syndrome, while a higher rate of renal tumors emerged among males.[24] There are increasing suggestions that exposure to As and other toxic metals may be an independent risk factor for cardiovascular disease.[25,26] A recent systematic review and meta-analysis reported that exposure to As, Cd, Pb, and copper is significantly and positively associated with cardiovascular disease, coronary heart disease, and stroke, and for As, Cd, and Pb a linear dose-response relationship was observed. The mechanisms involved comprise induced oxidative stress, inflammation, and endothelial cell damage, which can lead to atherosclerosis.[27] Furthermore, the kidney is a target organ of heavy metal toxicity for its capability to reabsorb and concentrate divalent ions and metals[28] Pb and Cd are established nephrotoxins at high exposure levels[29] and even at environmental exposure may contribute to the risk of chronic kidney disease, a progressive and irreversible deterioration in the renal function, especially in adults with hypertension or diabetes.[30,31]

Study design

The three epidemiological studies have a cross-sectional design and are based on the measurement of exposure and early-effect biological markers. For each subject recruited, the studies provide for:

- the administration of a questionnaire aimed at gathering anthropometric data, information on residential history, socioeconomic and health conditions, environmental and occupational exposure, lifestyle, diet, risk perception;
- the collection of biological samples (serum and urine);
- the measurement of systolic and diastolic blood pressure and heart rate, and neck ultrasound in Milazzo-Valle del Mela NCPS;
- Carotid Ultrasound, Brachial Artery Flow-Mediated Dilation, Arterial Tonometry, Electrocardiogram in Crotone NCPS.

Definition of study and reference areas

The NCPS of Priolo (defined by the Decree 426/1998 of the Italian Ministry of the Environment) comprises the municipalities of Augusta, Melilli, Priolo Gargallo and Syracuse (184.308 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html). The latter has been excluded from the study area as its territorial and demographic characteristics are very different from the other municipalities included in the NCPS, namely it has a much larger population (122,291 total inhabitants) and only a small part of the resident population is resident close to the boundaries of the perimeter of the NCPS. Furthermore, differently from the other municipalities that are homogeneous in both demographic and environmental characteristics, inhabitants of Syracuse are exposed to multiple sources of pollution with a significant component dependent on heating and vehicular traffic. Two other municipalities, Noto and Avola (55,621 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html) were included in the reference area because of the similar demographic and territorial characteristics to those of the study area, i.e., located along the coast and not interested by environmental issues of industrial origin (Figure 1).

The NCPS of Milazzo-Valle del Mela (defined by the Decree 266/2005 of the Italian Ministry of the Environment) includes the municipalities of Milazzo, San Filippo del Mela, Pace del Mela (about 45,000 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html). Other four municipalities (Barcellona Pozzo di Gotto, Terme Vigliatore, Villafranca Tirrena and Spadafora) were considered as reference area (62,583 total inhabitants on 1 January 2016, http://www.demo.istat.it/index.html) (Figure 1).

As regards the NCPS of Crotone, defined by the Decree 468/2001 of the Italian Ministry of the Environment (total inhabitants 62,178 on 1 January 2016, http://www.demo.istat.it/index.html), two municipalities, Cutro and Isola di Capo Rizzuto (total inhabitants 28.184 on 1 January 2016, http://www.demo.istat.it/index.html), were selected as reference area (Figure 1).

5

Inclusion criteria for recruitment

For the three epidemiological studies inclusion criteria have been established as follows:

For the NCPS of Priolo:

- Men and women aged 40-70 years (the age class most at risk for the development of liver disease);[32]
- residence in the study area for at least 10 years before the enrollment: since liver disease is a long-term disease, this time of residence is considered sufficient for the evaluation of early-effects biological markers;[33]
- absence of known liver diseases;
- signed informed consent to participate in the study.

Subjects with an acknowledged diagnosis of liver disease, positivity for hepatitis B and C virus infections, with abuse of alcohol or consumption of other drugs are excluded from the study.

For the NCPS of Milazzo-Valle del Mela:

- Men and women aged 18-44 years (thus excluding women in menopausal status, which could lead to a misclassification of early effects biological markers);
- residence in the study area for at least 10 years before the enrollment[34,35];
- absence of thyroid diseases;
- signed informed consent to participate in the study.

Individuals with an acknowledged diagnosis of thyroid disease or subjected to pharmacological therapy for thyroid disease are excluded from the study.

For the NCPS of Crotone:

- Men and women aged 40-70 years (only subjects in cardiovascular primary prevention were selected due to the high sensibility and vulnerability of this population);[36]
- residence in the study area for at least 5 years before the enrollment, as this period of time is optimal to link the measured CV biomarkers to the CV outcomes in terms of both mortality and morbidity;[37,38]
- subjects in primary prevention for cardiovascular risk;
- signed informed consent to participate in the study.

The exclusion criteria include the presence of systemic diseases, past cardiovascular events, and/or history of nephropathy.

Questionnaires

In addition to some common sections (i.e., personal data, sports, smoking and alcohol consumption, medical history and clinical conditions, environmental and socioeconomic conditions, occupational activity, exposure to physical and chemical agents, diet, reproductive history, hazard and risk perception, access to information on environment and health), the questionnaire administered to the participants includes other sections aimed at providing insights about potential sources of exposure, and previous and current diseases of subjects and their own families specific for each NCPS:

- Priolo NCPS: physical and/or sporting activity practiced and at what level, vaccinations, dental fillings, tattoos and /or piercings, family medical history, previous and current cardiovascular diseases, previous and current non-cardiovascular diseases, domestic heating/cooling modality, owning of televisions/tablets/computers/mobile phones.
- Milazzo-Valle del Mela NCPS: symptomatology associated to thyroid diseases, use of supplements, history of adverse reproductive outcomes and information on menstrual cycle for women, information on sterility/infertility for both sexes.

6

- Crotone NCPS: sleep disorders, adverse life events, family medical history, primary factors of cardiovascular risk, previous and current cardiovascular diseases, previous and current non-cardiovascular diseases

The questionnaires have been tested for comprehension and readability by three researchers independently. In order to avoid both paper administration and human errors, an online platform has been created for entering answers to the questionnaires. The questionnaires are administered by one or more operators who reported the answers on the platform. To adequately train each operator, a guide for the correct administration of the questionnaires have been produced and delivered to the contact person of each area. The questionnaires may be requested to these authors.

Biohumoral tests

In the three NCPSs and corresponding reference areas a sample of venous blood and a sample of urine is collected for all participants (Table 1). Blood tubes are stored at 4°C and centrifuged within 24 hours for analyses performed locally, whereas aliquots of plasma and urine are stored at -20°C until their transfer to the biobank of IFC-CNR for supplementary tests.

NCPS "Priolo"

In this study the results deriving from blood clinical tests are used with the anthropometric data to determine the Fatty Liver Index (FLI), the Fibrotic Liver Index (FIB-4) and the NAFLD Fibrosis Score (NFS), in order to evaluate the presence of hepatic steatosis and fibrosis, respectively.[39,40,41] According to the European Association for the Study of the Liver, European Association for the Study of Diabetes and European Association for the Study of Obesity, FLI, FIB-4 and NFS are non-invasive markers, which can be used with liver enzymes to identify subjects at risk of liver disease.[42]

-The FLI is calculated by the following formula:[40]

$$(e^{0.953*log_e \text{ (triglycerides)} + 0.139*BMI + 0.718*log_e \text{ (ggt)} + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*log_e \text{ (triglycerides)} + 0.139*BMI + 0.718*log_e \text{ (ggt)} + 0.053*\text{waist circumference} - 15.745}) * 100$$

with the cut off FLI<30 for presence of NAFLD

-The FIB-4 computation is based on the following formula:[43]

$$\text{age (years)} \times AST [U/L]/(\text{platelets} [109/L] \times (ALT [U/L])1/2)$$

with the cut off FIB-4 >1.30 for presence of fibrosis

-The NAFLD fibrosis score (NFS)[44] is calculated by the following formula (http://gihep.com/calculators/hepatology/nafld-fibrosis-score/):

$$-1.675 + 0.037 \times \text{age (years)} + 0.094 \times BMI (kg/m2) + 1.13 \times IFG/\text{diabetes (yes = 1, no = 0)} + 0.99 \times AST/ALT \text{ ratio} - 0.013 \times \text{platelet} (\times 109/l) - 0.66 \times \text{albumin (g/dl)}$$

with the cut off NFS >-1.455 for presence of fibrosis

Furthermore, serological lipidomics markers and urinary phthalates and bisphenols are evaluated by Quadrupole Time-of-Flight Liquid Chromatography/Mass Spectrometry and Gas Chromatography/Mass Spectrometry, while serum inflammatory markers are measured by Luminex technology (Table 1).

NCPS "Milazzo – Valle del Mela"

In addition to the determination of serum thyroid hormones, namely thyroxine (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH), also the evaluation of the gene expression of nuclear receptors of thyroid

7

hormone (THR-alpha, -beta) and retinoic acid (RAR-alpha, -gamma), and of 9-cis-retinoic acid receptor (RXR-alpha) levels in whole blood are performed as previously done (Table 1).[45] The analysis of gene expression crossed with the related hormone levels allows to assess the possible association between exposure and clinical or sub-clinical hypothyroidism or other subclinical conditions of alterations of thyroid hormones as well. Furthermore, urinary Cd and Pb, considered as markers of individual environmental exposure, are determined with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) by an accredited laboratory.

NCPS "Crotone"

Markers of inflammation, oxidative stress, cardiac biomarkers, markers of blood vulnerability to thrombosis, and bone health are measured in plasma specimens of each participant (Table 1). The potential association between exposure to heavy metals and cardiovascular disease (CVD) has several clinical implications, mainly due to an increase in oxidative stress and inflammatory status.[46,47] In addition, chronic exposure to Cd can lead to increased arterial stiffness and altered endothelial function.[48,49] It is interesting to note that different heavy metals are pollutants able of critically influencing bone mass[50] that is associated, according to recent studies, to a higher risk of CVD.[51,52]

The impact of environmental exposure to heavy metals on renal function are assessed through the determination of biomarkers of tubular damage in urine samples, indexed by urinary creatinine excretion levels. Moreover, kidney damage is further estimated by the glomerular filtration rate (GFR) using serum creatinine-based formulas (Cockcroft–Gault and Modification of Diet in Renal Disease)[53] and serum cystatin-based formula[54] for predicting GFR.

These biochemical tests are performed using standard available automated methods, or manual colorimetric and ELISA commercially available kits, according to the manufacturer's instructions.

Finally, individual exposure to Cd and Pb are determined both in blood and urine specimens with ICP-MS by an accredited laboratory.

Table 1. Summary of clinical chemistry tests on blood and urine specimens by study area.

Area	Test	Amount (ml)
Priolo NCPS	**Blood**	
	Hemochrome, FBG, triglycerides, total and HDL cholesterol, SGOT, SGPt, GGT, HbA1c, bilirubin, ferritin, transferrin, albumin	10
	Lipidomic profile (including free fatty acid profile), leptin, insulin, inflammatory biomarkers (MCP-1, TNFα)	10
	Total	20
	Urine	
	Measurement of phthalates and bisphenols	10
	Total	10
Milazzo - Valle del Mela NCPS	**Blood**	
	Hemochrome, ESR, fibrinogen, CRP, glycemia, creatinine, triglycerides, total, HDL and LDL cholesterol	12
	TSH, FT3, FT4, thyroglobulin, antithyroglobulin Ab	6
	Calcitonin	6
	Cd, Pb	6
	THR-alpha, THR-beta, RAR-alpha, RAR-gamma, RXR-alpha	4
	Total	34
	Urine	
	Early morning urine: Iodine, creatinine	10
	24 h urine: Cd, Pb	10
	Total	20
	Blood	
	Hemochrome, Triglycerides, total, HDL and LDL cholesterol, urea,	15

8

Crotone NCPS Cardiovascular system	creatinine, ALT, AST, GGT, CRP, glycemia, fibrinogen, TSH	
	Inflammatory markers (IL-6)	1
	Oxidative stress markers (hydroperoxides, total antioxidant capacity)	2
	Markers of blood vulnerability to thrombosis: (circulating microparticles, tPA, and CD40 ligand)	6
	Fibrosis (galectin-3) and cardiac damage markers (BNP)	3
	Bone health markers (vitamin D, osteocalcin)	3
	Cd, Pb	6
	Total	36
Crotone NCPS Kidney	**Blood**	
	Cystatin C	10
	Total	10
	Urine	
	Early morning urine: Markers of tubular damage (NAG, β2-MG, KIM-1, NGAL, L-FABP)	10
	24-h urine: Albumin, protein	10
	24-h urine: Cd, Pb	10
	Total	30

Abbreviations: β2-MG: β2-microglobulin; BNP: N-terminal pro B-type natriuretic peptide; Cd: cadmium; CRP: C reactive protein; FBG: fasting blood glucose; FT3: free triiodothyronine; FT4: thyroxine; GGT: gamma glutamyl transferase; IL-6: interleukine 6: HDL: high density lipoprotein; KIM-1: Kidney injury molecule-1; LDL: low density lipoprotein; L-FABP: Liver-type fatty acid binding protein; NAG: N-acetyl-β-D-glucosaminidase; NGAL: neutrophil gelatinase-associated lipocalin; Pb: lead; RAR: retinoid acid receptor; RXR: retinoid X receptor; SGOT: alanine aminotransferase; SGPT: aspartate aminotransferase; THR: thyroid hormone receptor; tPA: tissue plasminogen activator; TSH: thyroid stimulating hormone.

Instrumental and imaging examinations

NCPS "Milazzo - Valle del Mela"

In addition to biohumoral tests, a neck ultrasonography is performed in all subjects by a commercially available system focusing on the examination of the following parameters: general characteristics of the thyroid parenchyma (glandular volume, echogenicity, echo structure, vascularization and presence of parenchymal calcifications), morpho-structural evaluation of possible nodular lesions (location, size, echogenicity, echo structure, calcifications, content and vascular pattern), possible presence of lymphadenopathy. In the case of indications emerging from the ultrasound scan for the opportunity to proceed with fine-needle aspiration biopsy (i.e., presence of thyroid nodules with diameter > 10 mm or nodules < 10 mm in diameter that require further evaluation because of clinical symptoms or associated lymphadenopathy)[55], this information is transmitted to the subject and to the attending physician.

NCPS "Crotone"

Brachial artery flow mediated dilation is used to assess endothelial function by processing of ultrasound images of the brachial artery following International consensus guidelines.[56] In particular, maximal percentage increase of the brachial artery diameter following reactive hyperaemia is measured using an automatic edge detection system (FMD Studio, Quipu, Pisa Italy). Furthermore, carotid-femoral pulse wave velocity (PWV) is assessed as a surrogate marker of regional arterial stiffness. For this purpose, an applanation tonometer is employed to record pressure waveforms at the carotid and femoral artery levels, thus obtaining the PWV value. The carotid pressure curve is also rescaled in order to estimate central blood pressure and augmentation index. Ultrasound scans of the common carotid artery are acquired and processed in order to assess anatomical and functional parameters. In particular, intimal-media thickness, plaque presence and carotid stiffness is estimated using an automatic software (Carotid Studio, Quipu, Pisa, Italy).[57] Finally, heart rate variability is performed automatically both in time and frequency domain by processing 12-lead ECG traces.

9

Statistics

Distribution of people residing in each study and reference has allowed to extract a sample that reflects distribution by sex, age class and municipality of residence of inhabitants in NCPSs and corresponding reference areas. Thus, from the list of patients of Local Health Companies of Siracusa, Messina and Crotone the sample, sized in proportion to the population of every municipality included in the study, was randomly selected with stratification by sex and age class. To calculate the statistical power of each study, the following indicators were considered:

- Priolo NCPS: NAFLD Fibrosis Score NFS and FLI In order to obtain a difference of -1 between the value of NFS - 2.4 obtained in a general population [39] and the cutoff of NFS =- 1.45[44] to distinguish the subjects with fibrosis from those without fibrosis; and a difference of -15 between the reference value of FLI = 35 obtained in a general population[39] and the value of NFS = 50 for the subjects with NAFLD[44] a sample of 500 subjects was defined, 250 in the NCPS and 250 in the reference area, with a statistical power (1-β) of 90% and a significance level (α) of 0.05 using a two-tailed t-test.
- Milazzo-Valle del Mela NCPS: Thyroid nodules with > 10 mm diameter. Palpable thyroid nodules > 10 mm diameter are detected in approximately 5% of the population[55]: most of them are benign but 5% develops to malignant nodules.[58] With a sample of 480 subjects, 240 for each area, α=0.05, 1-β=0.8 through bilateral test, and a difference of risk between the two areas corresponding to 0.07, was obtained. A statistical power of 80%, and not of 90% as for the other two studies, was considered acceptable given the available funds, which limit the recruitment of a higher number of cases than estimated using a power of 80%.
- Crotone NCPS: Arterial stiffness. This is the indicator that, among those of imaging, has the characteristics of the lowest accuracy and precision. With regard to this biomarker, hypothesizing a difference between the exposed and unexposed population of 8% and a standard deviation of 20%, considering a sample of 150 individuals in each exposure group, and assuming a first type error of 5% for bilateral testing, statistical power of 90% was achieved.
  As the pilot study on the evaluation of kidney damage is based on a sample halved compared to the main study assessing the cardiovascular risk in the same population, the enrollment ends when for 75 subjects living in each area who have joined the first study, the possibility and the willingness to participate in the second survey is verified.

The comparison between the levels of exposure to the selected pollutants and the levels of biological markers will be performed through univariate and multivariate statistical analyses (adjusting for confounding or modification covariates), using parametric and non-parametric statistical methods, chosen after checking the normality of the distribution of the dependent variables considered.

Aims

The CISAS project embraces multidisciplinary activities with the purpose to deepen knowledge on environmental pollution phenomena and their impact on the ecosystem and human health, in order to plan actions mitigating harmful environmental effects and for preventing risks to human health.

The three described epidemiological studies in CISAS project, started in January 2019 and currently ongoing, represent the first example of analytical investigations of human biomonitoring based on the evaluation of exposure and pre-clinical effects in populations residing in NCPSs of southern Italy. The results obtained will allow to develop epidemiological knowledge in the examined areas, and in particular will provide insights in the relationships between priority pollutants of each contaminated site and selected health outcomes, in the view of improving the evidence on and in the molecular mechanisms involved as well. In particular, it will be possible:

10

- to identify whether subjects exposed to endocrine disrupting chemicals exhibit increased hepatic biomarkers, and alterations of lipid metabolism that may promote the development of liver disease and, with time, possibly HCC;

- to evaluate the association between exposure to heavy metals and increased risk of indicators of thyroid disease and thyroid cancer;

- to assess the effect of exposure to heavy metals on cardiovascular risk and on glomerular and tubular function in cardiovascular primary prevention subjects.

The innovative feature of these studies is represented by the assessment of non-invasive, exposure and early-effects biomarkers that would be helpful to implement integrated health-environment surveillance systems in NCPSs and seek novel strategies aimed at decreasing the adverse effects of environmental pollution, including specific remediation programs of the environmental matrices. Furthermore, all data collected within CISAS project will allow the evaluation of codified cardiovascular risk scores, such as the ACC/AHA atherosclerotic cardiovascular disease (ASCVD) risk score. These scores could be very useful in order to estimate the impact of environmental exposure on future risk of developing cardiovascular diseases.

Such an approach could be applied in other contaminated sites having similar environment and health characteristics, as it represents a valuable method to monitor exposed communities that could be followed over time for a reliable exposure assessment. Information on early health effects can be particularly useful for planning specific prevention actions for polluted areas.

Patient and public involvement

The recruitment is based on healthy volunteers adhering inclusion criteria. Patients and the public were not involved in the study design but they are partially involved in the conduction (questionnaires and relationship with the general practitioners) and in the dissemination, as the protocol plans a public presentation of aggregated results to all stakeholders including the patients.

Ethics approval

The protocol studies have been approved by the Ethics Committees responsible for the three involved NPCSs: the Ethics Committee 'Catania 2' for the NPCS of Priolo (21 July 2017, n. 500/2017/CECT2), the Ethics Committee of the University Hospitals of Messina for the NPCS of Milazzo-Valle del Mela (19 February 2018, n.2/2018); the Ethics Committee of the Region of Calabria for the NPCS of Crotone (20 July 2017, n. 174). Results will be disseminated among policy makers, citizens, stakeholders and scientific community through the organization of conferences and events, and the publication on international peer/reviewed journals.

Ethics and dissemination

The subjects enrolled may participate to the studies after receiving an extensive explanation about project details and research aims, and signing the informed consent. Each participant is assigned a unique identification code for the chemical tests, instrumental examinations and questionnaires in order to link all the data preserving complete pseudonymisation. All the images acquired are stored on digital support in pseudonymizated form and data are re-evaluated by an operator "blind" and with quantitative "operator-independent" systems, in order to reduce inter- and intra-observer variability. The procedures adopted comply the General Data Protection Regulation (EU) 2016/67 regulating data protection and privacy for all individual citizens of the European Union and the European Economic Area.

In the case of studies based on individual markers of exposure of early effect specific ethical requirements regulate not only the protection of volunteers' privacy, but also the dissemination and communication of results.[59] The content of the questionnaire is developed in accordance with ethical requirements, and is included in the approval procedures of local Ethics Committees. The studies are being conducted in compliance with the principles of Declaration of Helsinki.

11

Among the requirements, there are the respect of the right to know as the basis of self-determination in the use of research results, the distribution of benefits to different groups, and the choice to deliver the results to the research subjects if they want. At enrollment, an information sheet and the informed consent to be signed provide a complete explanation of project details and research aims. Moreover, all the subjects are made aware that they may withdraw from participating at any time.[60]

CISAS has been giving particular importance to the communication of information about the project activities to policy makers, citizens, and stakeholders.[61] Public conferences have been organized in the previous months and others will be held at the end of the studies to make available the overall results to the local Regional Health Authorities, the authorities of the municipalities involved, and the scientific community.[61] Findings will also be published on international peer-reviewed journals.

On the main topics of the project, educational and training activities in the schools located in the three contaminated areas have been scheduled from before the start to after the end of the studies.

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Contributors

AG, CV, DB, FB, FF, FM, FS, GT, MI conceptualized the design and methodology of epidemiological studies; FG drafted the manuscript; FG and FB critically reviewed the manuscript and gave final approval of the version to be published. CV, DB, EB, FB, FG, FM, FS, GQ, GT, LC, MI contributed in drafting the manuscript. All the authors read and approved the final version of the manuscript.

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Competing interests

None to declare.

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Figure 1. Map of National Priority Contaminated Sites and reference areas in CISAS project. Created through QGIS 2.18 Development Team. QGIS Geographic Information System. Open Source Geospatial Foundation. URL: https://www.qgis.org/it/site/forusers/visualchangelog218/index.html.
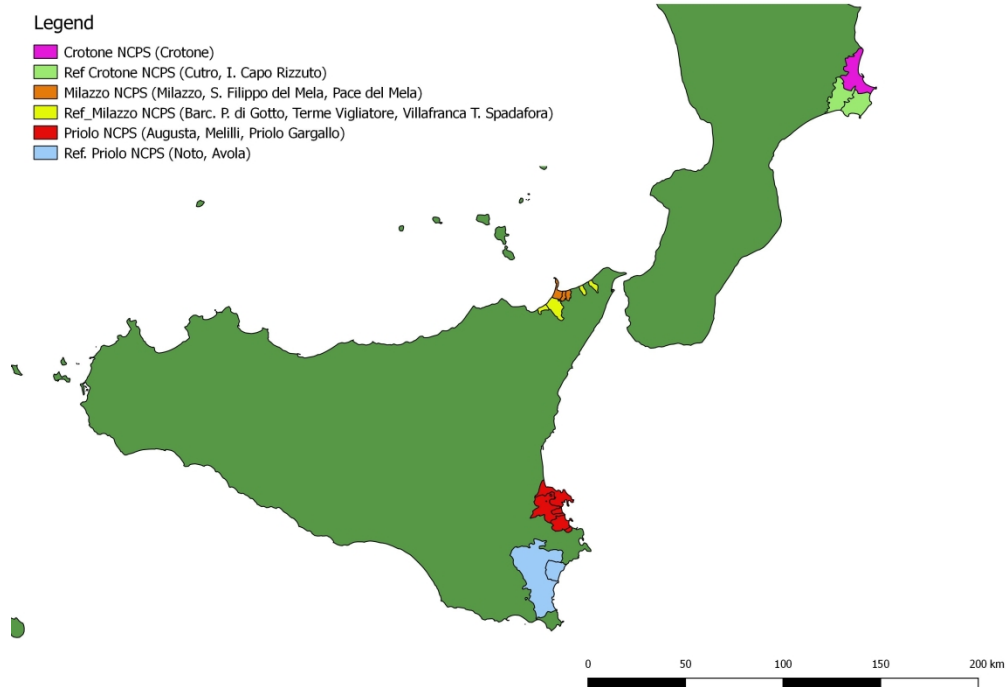
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Figure 1. Map of National Priority Contaminated Sites and reference areas in CISAS project

296x209mm (300 x 300 DPI)