UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Prenatal Exposure to Residential Traffic-Related Air Pollution and Immune System at Birth: Results from the NELA Cohort

Exposición Residencial a Contaminación Atmosférica Relacionada con el Tráfico durante la Etapa Prenatal y Sistema Inmunitario al Nacimiento: Resultados de la Cohorte NELA

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Exposición residencial a contaminación atmosférica relacionada con el tráfico durante la etapa prenatal y sistema inmunitario al nacimiento: resultados de la cohorte NELA

Memoria presentada para optar al grado de Doctor con Mención internacional por:

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“The balance of nature is not a status quo;
it is fluid, ever shifting, in a constant state of adjustment.
Man, too, is part of this balance.”

- Rachel Carson, “Silent Spring”

“"The materials of science are the materials of life itself.
Science is the way, the how and the why
for everything in our experience.”

- Rachel Carson

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DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS/PREFACE

The current doctoral thesis is presented as a compendium of three articles that have been previously published, in accordance with the authorization of the director and the academic tutor of the doctoral thesis (PhD) candidate; and the Academic Commission responsible for the Doctoral Program in Health Sciences of the University of Murcia. Thus, this PhD is built upon the following articles:

1. **García-Serna AM**, Martín-Orozco E, Hernández-Caselles T, Morales E. Prenatal and Perinatal Environmental Influences Shaping the Neonatal Immune System: A Focus on Asthma and Allergy Origins. Int J Environ Res Public Health. 2021; 18(8):3962. doi: 10.3390/ijerph18083962
2. **García-Serna AM**, Hernández-Caselles T, Jiménez-Guerrero P, Martín-Orozco E, Pérez-Fernández V, Cantero-Cano E, Muñoz-García M, Ballesteros-Meseguer C, Pérez de Los Cobos I, García-Marcos L, Morales E; NELA Study group. Air pollution from traffic during pregnancy impairs newborn's cord blood immune cells: The NELA cohort. Environ Res. 2021;198:110468. doi: 10.1016/j.envres.2020.110468
3. **García-Serna AM**, Martín-Orozco E, Jiménez-Guerrero P, Hernández-Caselles T, Pérez-Fernández V, Cantero-Cano E, Muñoz-García M, Molina-Ruano MD, Rojo-Atenza E, García-Marcos L, Morales E; NELA Study Group. Cytokine profiles in cord blood in relation to prenatal traffic-related air pollution: The NELA cohort. Pediatr Allergy Immunol. 2022;33(2):e13732. doi: 10.1111/pai.13732.

Additionally, three manuscripts related to the research work carried out during the doctoral period are included in the appendix. These manuscripts will be submitted for publication.

1. **García-Serna AM,** Morales M, Cantero-Cano E, Norte-Muñoz M, Gil-Buendía MA, Velazquez-Marin J, Hernández-Caselles T, Pérez-Fernández V, Martínez-Torres AE, García-Marcos L, Martin-Orozco E. Cytokine production by newborns. Influence of sex and season of birth. Pediatric Research. Forthcoming 2022.
2. **García-Serna AM**, Martin-Orozco E, Jimenez-Guerrero P, Hernández-Caselles T, Perez-Fernandez V, Cantero-Cano E, Muñoz-García M, Elena MC, Soler-Sánchez J, García-Marcos L, Morales E. Traffic-related air pollution *in utero* modifies cytokine responses to stimuli of umbilical cord blood cells: the NELA cohort.
3. **García-Serna AM**, Salas LA, Morales E. Pregnancy exposure to outdoor air pollution impacts miRNA expression in cord blood at birth: the NELA cohort study.

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ABBREVIATIONS

APC	Allophycocyanin
BKMR	Bayesian kernel machine regression
BMI	Body Mass Index
CCR4	C–C Chemokine Receptor Type 4
CCR6	C–C Chemokine Receptor Type 6
CD	Cluster of Differentiation
CI	Confidence Interval
Con A	Concanavalin A
CpG-ODN	Immunostimulatory CpG-Oligodeoxynucleotides
CRTH2	Chemoattractant Receptor-Homologous molecule expressed on Th2 Cells
CXCR3	C–X–C motif chemokine receptor 3
D.p	*Dermatophagoides pteronyssinus*
FITC	Fluorescein isothiocyanate
GAM	Generalized Additive Model
IFN	Interferon
IL	Interleukin
LDL	Lower detection limit
LPS	Lipopolysaccharide
NCDs	Non-communicable diseases
NELA	Nutrition in Early Life and Asthma
NK	Natural Killer cells
NO_2	Nitrogen Dioxide
O_3	Ozone
OR	Odds Ratio
PAMP	Pathogen Associated Molecular Pattern
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein Complex
PG	Peptidoglycan
PHA	Phytohemagglutinin
pI:C	Polyinosinic-polycytidylic acid
PM	Particulate matter
PM_{10}	PM with aerodynamic diameter between 10 and 5 micrometres

$PM_{2.5}$	PM with aerodynamic diameter less than 2.5 micrometres
Tc	Cytotoxic T cells
Th	Helper T cells
Th1	T helper type 1 cells
Th17	T helper type 17 cells
Th2	T helper type 2 cells
Tim-3	T-Cell Immunoglobulin (Ig) And Mucin Domain containing Molecule-3
TNF	Tumour necrosis factor
TRAP	Traffic-Related Air Pollution
Treg	Regulatory T cells
WBC	White blood cells
WHO	World Health Organization
WQS	Weighted Quantile Sum Regression.
WRF-Chem	Weather Research and Forecasting model coupled with Chemistry

PRESENTATION AND SCIENTIFIC UNIT

This doctoral thesis is presented as a compendium of three scientific articles and it applies for the International Doctorate mention in accordance with the guidelines of official doctoral studies of the University of Murcia (RD-99/2011 and R-310/2015) and with the approval of the thesis tutor and supervisor, the Academic Committee of The Doctoral Program in Health Sciences and the General Committee for Doctoral Studies. This document is made up of a compendium of three scientific articles (García-Serna et al, 2020; García-Serna et al, 2021; and García-Serna et al, 2022) published in international journals indexed in the *Journal Citation Reports* (JCR).

This doctoral thesis was developed in the Paediatrics Research group at the Biomedical Research Institute of Murcia (IMIB-Arrixaca), led by Professor Luis Garcia-Marcos, and derived from the research lines led by Dr. Eva Morales that focus on: 1) birth cohorts studies: understanding of the role of genetics, epigenetics, and molecular mechanisms of human complex diseases and early development by analysing data of cutting-edge longitudinal birth cohort studies; 2) environmental epidemiology: effects of air pollutants and endocrine disruptors on maternal and child health; and 3) molecular epidemiology: integration of biomarkers in epidemiological studies. This thesis uses data from the Nutrition in Early Life and Asthma (NELA) cohort study, a prospective population-based birth cohort set up in 2015. The data are derived from the research projects: "Impact of in utero exposure to outdoor air pollution on immune function in newborns: identification of epigenetic mechanisms (PI16/00422)", led by Dr. Eva Morales, and the integrated project of Excellence "Unravelling in utero determinants predicting lung function in infants: a step for prenatal prevention of asthma (PIE15/00051)", led by Prof. Luis Garcia-Marcos.

Azahara M García-Serna joined the Paediatrics Research Group in 2018 as a predoctoral fellow. She has collaborated in the NELA project as a PhD student focusing her research on the identification of immunological markers in relation to the prenatal environment for better prediction and early diagnosis of asthma and allergic manifestations later in life. Furthermore, she has collaborated in two additional NELA projects and she has provided statistical support to other researchers. Additionally, Miss García-Serna has been serving as an assistant scholar with *Venia Docendi* teaching at the Department of Biochemistry and Molecular Biology B and Immunology of the University of Murcia from 2018 to 2021.

This PhD provides a general introduction, which presents the background, the studies carried out and justifies the scientific unity of itself in the fields of cohort studies and environmental epidemiology. Additionally, an overall summary of the aims of this research, methods and the final conclusions are included in this document. Finally, public health implications are also included to highlight the importance of the main findings of the current work. Additionally, this doctoral thesis includes an appendix with three additionally manuscripts related to the objectives of this thesis that are in preparation.

The scientific publications of which PhD is composed are:

Article 1

- **Title: Prenatal and perinatal environmental influences shaping the neonatal immune system: a focus on asthma and allergy origins**
- **Authors**: García-Serna AM, Martín-Orozco E, Hernández-Caselles T, Morales E.
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- Available online at: https://www.mdpi.com/1660-4601/18/8/3962

Article 2

- **Title: Air pollution from traffic during pregnancy impairs newborn's cord blood immune cells**
- **Authors:** García-Serna AM, Hernández-Caselles T, Jiménez-Guerrero P, Martín-Orozco E, Pérez-Fernández V, Cantero-Cano E, Muñoz-García M, Ballesteros-Meseguer C, Pérez de Los Cobos I, García-Marcos L, Morales E; NELA Study group
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- **Journal Category:** Public, Environmental & Occupational Health
- **Quartile:** Q1 and D1 (16/203)
- **DOI:** 10.1016/j.envres.2020.110468
- Available online at: https://www.sciencedirect.com/science/article/pii/S0013935120313657?via%3Dihub

Article 3

- **Title: Cytokine profiles in cord blood in relation to prenatal traffic-related air pollution: the NELA cohort**
- **Authors:** García-Serna AM, Martin-Orozco E, Jimenez-Guerrero P, Hernández-Caselles T, Perez-Fernandez V, Cantero-Cano E, Muñoz-García M, Molina-Ruano MD, Rojo-Atenza E, Garcia-Marcos L, Morales E
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- **Journal Category:** Pediatrics
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SUMMARY

Background: Considered as the second environmental risk factor causing non-communicable diseases, air pollution has become a public health concern worldwide. Alterations in the immune system are proposed as mechanisms underlying these adverse effects, and gestation may represent a period of increased vulnerability. However, the impact of prenatal exposure to traffic-related air pollution (TRAP) on immune system development has been poorly studied.

Aims: To study the impact of prenatal exposure to TRAP on immune system at birth.

Specific objectives:

1. To summarize the evidence on the associations between the prenatal and perinatal environment and changes in immune system cells and cytokine profiles in umbilical cord blood.
2. To characterize the immune system at birth analysing immune cell subpopulations, unstimulated cytokine profiles and cytokine responses to a wide panel of environmental stimuli in umbilical cord blood.
3. To study the effects of prenatal exposure to TRAP on distributions of immune system cell subpopulations in umbilical cord blood.
4. To investigate the effects of prenatal exposure to TRAP on cytokine profiles of unstimulated and stimulated umbilical cord blood cells.
5. To identify gestational windows of higher susceptibility of the developing immune system to prenatal exposure to TRAP.

Methods: For the first objective, a PUBMED search limited to human and English results was conducted to review the available evidence on the associations between the prenatal and perinatal environment and changes in immune system cells and cytokine profiles at birth.

For objectives 2 to 5 we used data from mother-newborn pairs embedded in the NELA study, a prospective population-based birth cohort (2015–2018). Long-term (whole pregnancy and trimesters) and short-term (15 days before delivery) residential exposures to traffic-related nitrogen dioxide (NO_2), particulate matter ($PM_{2.5}$ and PM_{10}), and ozone (O_3) during gestation were estimated using a dispersion/chemical transport modelling based on source apportionment methodologies. In-depth immunophenotyping of cord blood leukocyte subsets including T helper type 1 (Th1), Th2, Th17 and regulatory T (Treg) lymphocyte subsets was performed by flow cytometry. A wide cytokine panel (IFN-α, IFN-γ, IL1β, IL-10, IL-13, IL-17F, IL-2, IL-23, IL-4, IL-5, IL-6 and TNF-α) was assessed by Luminex technology in umbilical cord blood. Associations between TRAP concentrations and immune cell counts were assessed using multivariate Poisson regression models; and the relationships between TRAP exposure and cytokine production by cord blood cells were assessed fitting multivariate linear and logistic regression as well as multi-pollutant models.

Results:

1. Prenatal and perinatal periods seem to represent crucial developmental windows of higher susceptibility of the immune system to diverse environmental influences.
2. Cord blood NK, cytotoxic T and Treg cells decreased in relation to higher prenatal exposure to TRAP; whereas, higher prenatal exposure to traffic-related PM was associated with increased total Th and Th1 cells in cord blood.
3. Prenatal exposure to higher levels of TRAP was associated with increased detection of unstimulated concentrations of pro-inflammatory (IL-1β and IL-6), Th2-related (IL-13), and IL-10 cytokines in newborns.
4. Exposures to NO_2 and PM during pregnancy were associated with higher proinflammatory (IL-6 and IFN-α) and Th1-related (IFN-γ) cytokine responses of umbilical cord blood cells to environmental stimuli.
5. The first and the third trimester of gestation were identified as windows of higher susceptibility of foetal immune system to adverse effects of TRAP.

Conclusions: Prenatal and perinatal periods are crucial developmental windows of higher susceptibility of the immune system to diverse environmental influences. Furthermore, prenatal exposure to TRAP could impair foetal immune system development through disturbances in leukocyte subsets and increased production of proinflammatory cytokines in cord blood. These changes might influence immune system responses and contribute to increase risk of respiratory infections, allergy, and wheezing in early life.

RESUMEN

Antecedentes: Considerada como el segundo factor de riesgo de las enfermedades no transmisibles, la contaminación atmosférica es un problema de salud pública a nivel mundial. Alteraciones del sistema inmunitario han sido propuestas como mecanismos subyacentes de estos efectos, y la gestación como una etapa de mayor vulnerabilidad. Sin embargo, el impacto de la exposición prenatal a contaminación atmosférica derivada del tráfico (TRAP) en el sistema inmunitario ha sido poco estudiado.

Objetivos: Estudiar el impacto de la exposición prenatal a TRAP en el sistema inmunitario al nacimiento.

Objetivos específicos:

1. Resumir las evidencias de la influencia del ambiente prenatal y perinatal sobre las células del sistema inmunitario y los perfiles de citoquinas en sangre de cordón.
2. Caracterizar el sistema inmunitario al nacimiento analizando subpoblaciones celulares y los perfiles de citoquinas sin estimular y en respuesta a estímulos en sangre de cordón.
3. Estudiar los efectos de la exposición prenatal a TRAP en la distribución de subpoblaciones celulares del sistema inmunitario en sangre de cordón.
4. Investigar los efectos de la exposición prenatal a TRAP sobre los perfiles de citoquinas de células de sangre de cordón sin estimular y estimuladas.
5. Identificar ventanas de susceptibilidad del sistema inmunitario a la exposición prenatal a TRAP.

Métodos: Para el primer objetivo se realizó una búsqueda en PUBMED limitada a estudios en humanos publicados en inglés sobre asociaciones entre factores prenatales y perinatales con cambios en células del sistema inmunitario y en los perfiles de citoquinas en sangre de cordón.

Para el resto de objetivos usamos datos de parejas madre-hijo del estudio NELA, una cohorte al nacimiento prospectiva de base poblacional (2015-2018). La exposición residencial durante el embarazo a dióxido de nitrógeno (NO_2), material particulado ($PM_{2.5}$ and PM_{10}) y ozono (O_3) relacionada con tráfico se estimó mediante un modelo de dispersión y transporte químico. Se realizó un inmunofenotipado de los leucocitos en sangre de cordón, incluyendo las subpoblaciones de linfocitos T cooperadores de tipo 1 (Th1), Th2, Th17 y T reguladoras (Treg) mediante citometría de flujo. Además, se analizó un amplio panel de citoquinas con tecnología Luminex. Las asociaciones entre las concentraciones de TRAP y los recuentos de células del sistema inmunitario se analizaron con modelos de regresión de Poisson; y las relaciones entre la exposición a TRAP y la producción de citoquinas se analizó con modelos de regresión lineal y logística así cómo modelos de múltiples contaminantes.

Resultados:

1. Los períodos prenatal y perinatal parecen representar ventanas cruciales de mayor susceptibilidad del sistema inmunitario a diversas influencias ambientales.
2. Los recuentos de células NK, T citotóxicas y Treg disminuyeron en relación a mayores niveles de TRAP; mientras que mayores niveles de PM se asociaron con un aumento del número total de células Th y Th1 en sangre de cordón.

3. La exposición prenatal a mayores niveles de TRAP se asoció con un aumento de los índices de probabilidad de detectar niveles de citoquinas inflamatorias (IL-1β y IL-6), Th2 (IL-13) e IL-10.
4. Las exposiciones a NO_2 y PM durante el embarazo se asociaron a una mayor respuesta de citoquinas inflamatorias y Th1 a estímulos ambientales.
5. El primer y tercer trimestre de gestación se identificaron como ventanas de mayor susceptibilidad del sistema inmunitario a efectos de TRAP.

Conclusiones: Los periodos prenatal y perinatal son etapas de mayor susceptibilidad del sistema inmunitario a factores ambientales. Además, la exposición prenatal a TRAP podría afectar el desarrollo del sistema inmunitario mediante cambios en las subpoblaciones de leucocitos y el incremento de citoquinas proinflamatorias al nacimiento. Estos cambios podrían influir las respuestas inmunitarias y aumentar el riesgo de infecciones respiratorias y manifestaciones alérgicas en la infancia.

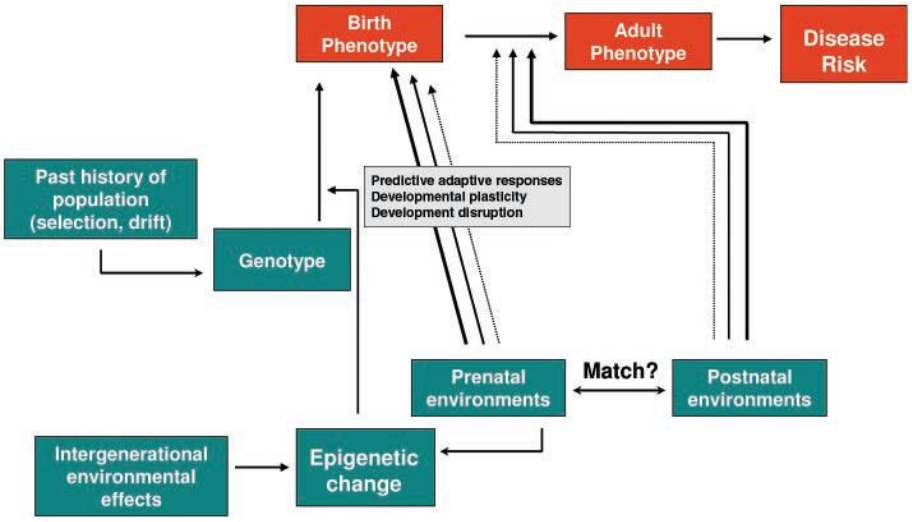
INTRODUCTION

INTRODUCTION

Developmental Origins of Health and Diseases hypothesis

In the 1980s, Barker hypothesized that undernutrition in early life increased susceptibility to diseases later in life (Barker & Osmond, 1986). This observation was the seed of the Developmental Origins of Health and Diseases (DOHaD) paradigm, which postulates that prenatal exposures to an adverse foetal environment could influence human development causing irreversible disturbances that, finally, might translate into altered disease risk later in life (Gluckman & Hanson, 2004).

Figure 1. Scheme of the Developmental Origin of Health and Disease (DOHaD) paradigm.



Source: Gluckman & Hanson, 2004.

Developmental plasticity has been proposed as the framework of the DOHaD hypothesis suggesting that environmental exposures could act during foetal development and trigger a response that induces a chain of changes resulting into alternative phenotypes expressed from a single genotype (Gluckman & Hanson, 2004). These alterations might be the underlying mechanisms of disease risk later in life. Indeed, results from diverse epidemiological studies have supported these hypotheses and have revealed prenatal factors such as nutritional, socio-economic and environmental pollutants to be associated with the occurrence of impaired health conditions in early life (Gluckman & Hanson, 2004; Palmer, 2011).

Exposure to air pollution as a hazard for human health

Air pollution is a public health concern worldwide. In 2016, nine out of ten people breathed air exceeding the air quality standards established by the World Health Organization (WHO) in 2005 (**Table 1**), resulting in 4,2 million premature deaths in the world. Furthermore, after tobacco smoking, air pollution has been identified as the second environmental risk factor causing non-communicable diseases (NCDs) (Prüss-Ustün et al., 2019). In late 2021, WHO has established new air quality recommendations (**Table 1**) based on growing evidence on the

relationship between air pollution exposure and adverse outcomes across the lifespan. However, the European air quality standards are still far away from those recommendations (**Table 1**).

Table 1. Comparison between the European standards of air quality (AQ) and the World Health Organization (WHO) guidelines.

Pollutant	Time	European AQ Standards	AQG (WHO) 2005	New AQG (WHO) 2021
Particulate matter <2.5µm ($PM_{2.5}$)	Annual	25 µg/m^3	10 µg/m^3	5 µg/m^3
	24-hour	-	25 µg/m^3	15 µg/m^3
Particulate matter <10µm (PM_{10})	Annual	40 µg/m^3	20 µg/m^3	15 µg/m^3
	24-hour	50 µg/m^3	50 µg/m^3	45 µg/m^3
Ozone (O_3)	Peek season (6m)	-	-	60 µg/m^3
	8-hour	120 µg/m^3	100 µg/m^3	100 µg/m^3
Nitrogen Dioxide (NO_2)	Annual	40 µg/m^3	40 µg/m^3	10 µg/m^3
	24-hour	200 µg/m^3	-	25 µg/m^3

Sources: Directive 2008/50/EC; World Health Organization, 2021.

The main outdoor air pollutants are the nitrogen dioxide (NO_2), particulate matters (PM), that are classified by the aerodynamic diameter into PM with aerodynamic diameter between 10 and 5 micrometres (PM_{10}) and PM with aerodynamic diameter less than 2.5 micrometers ($PM_{2.5}$), and ozone (O_3). Vehicle traffic emissions constitute a high proportion of all anthropogenic contributions to NO_2 and PM concentrations in the European Union (European Environment Agency, 2021).

Maternal exposure to outdoor air pollution during pregnancy could influence foetal programming and increase the risk of adverse birth outcomes such as preterm birth and low birthweight (Ju et al., 2021; Li et al., 2020), which may contribute to the occurrence of early onset disorders and to disease risk throughout life. Along these lines, adverse neonatal respiratory outcomes (Seeni et al., 2018), impaired lung function (Cai et al., 2020; Morales et al., 2015) and increased risk of asthma (Deng et al., 2016; Rivera Rivera et al., 2021; Yan et al., 2020; Y. Zhang et al., 2021) have been associated with maternal exposure to airborne PM, NO_2 and O_3 across pregnancy. Evidence about the biological mechanisms underlying these adverse effects is growing in the last years and several pathways have been proposed including triggering of oxidative stress (Bontinck et al., 2020; Enweasor et al., 2021; Glencross et al., 2020), epigenetic modifications (Bontinck et al., 2020; Gruzieva et al., 2019) and immune system disturbances (Dietert, 2014; Suzuki et al., 2020).

Immune system development and maturation

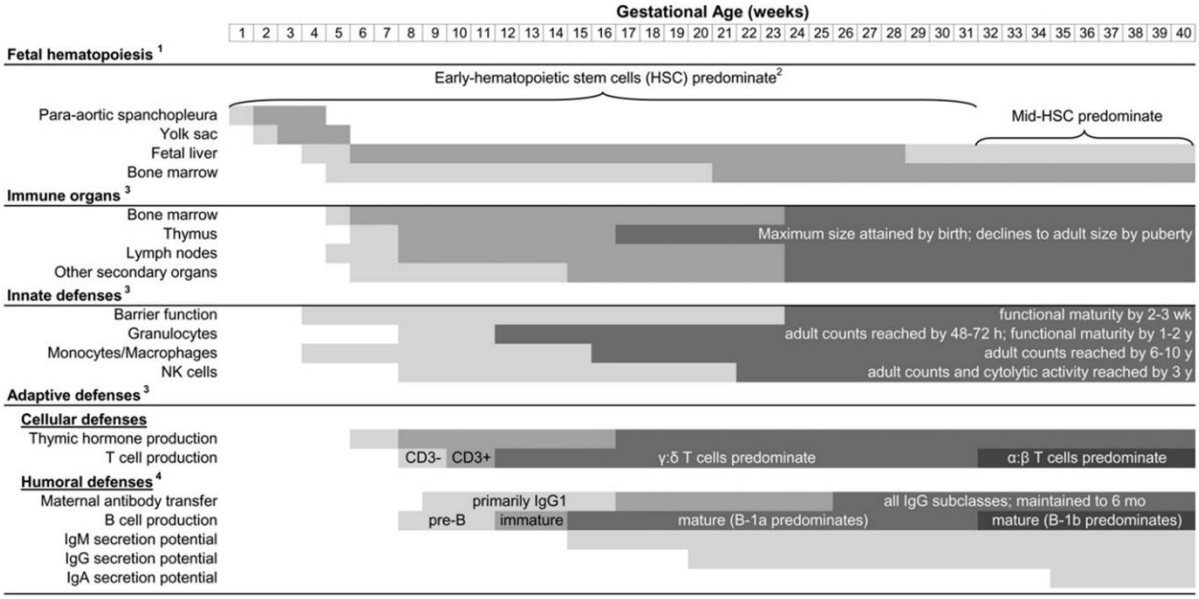
Immune system is a complex network of organs, cells and molecular components that aims to protect the host against organisms and molecules that it recognizes as foreigners and harmful. According to the immune response, two mechanisms can be differentiated: the innate

response, which is the first line of defence characterized for being a fast and non-specific response and by the presence of myeloid leukocytes (granulocytes, monocytes, dendritic cells, etc.…); and the adaptive response, which is only found in vertebrates, specific for each pathogen and characterized by the participation of T and B cells and the production of antibodies.

Multipotential hematopoietic stem cells are the common progenitor of leukocytes, that differentiate into myeloid and lymphoid precursors cells. The main cellular component of the innate response are the myeloid cells: granulocytes (neutrophils, basophils and eosinophils) and monocytes (Wynn et al., 2013; X. Zhang et al., 2017). In presence of different stimuli, monocytes can be mainly differentiated into myeloid dendritic cells (DCs) and macrophages, the main source of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12 or tumour necrosis factor alpha (TNF)-α (Wynn et al., 2013). Lymphoid precursors cells differentiate into Innate Lymphoid cells (ILCs), which do not express the typical linage marker and act in the innate response as the counterpart of T helper (Th) subsets (e.g., ILC2 can produce Th2-cell-associated cytokines) (Vivier et al., 2018); the Natural Killer cells (NK), which are involved in both innate and the adaptive response; and B and T lymphocytes, the main players in the adaptive response. T lymphocytes can be classified into two major subsets depending on the surface marker and the role in the immune response including the cytotoxic T cells (Tc), identified by expressing the cluster of differentiation (CD)8+, which secrete inflammatory cytokines, such as IFN-γ and TNF-α and cytotoxic granules to attack invaders and kill infected or malignant cells; and the Th cells identified as expressing CD4+ surface marker, which promote and control the development of the immune response. Furthermore, Th cells can be activated by dendritic cells and differentiated into Th subpopulations depending on the dominant cytokine on the environment during their activation by the antigen-presenting cells: Th1 (IL-12), Th2 (IL-4), Th17 (Tumor Growth Factor [TGF]-β, IL-1β and IL-6) and T regulatory (Treg, TGF-β) (Patente et al., 2019; Rautajoki et al., 2008).

Five major maturational periods recognised as critical windows of vulnerability to immunotoxicants are proposed during the development of the immune system (**Figure 2**) (Dietert et al., 2000; Kuper et al., 2016): **(1)** initiation of haematopoiesis (8-10 week of gestation), when pluripotent stems cells appear in the yolk sac and in the foetal liver progressively, and myeloid and lymphoid precursors colonize the thymus and the foetal liver; **(2)** migration of stem cells and progenitor expansion to new peripheral tissues (10-16 week of gestation); **(3)** colonization of the bone marrow and the thymus (16 week of gestation to birth); **(4)** maturation to immune competence with increasing immune cell numbers and DCs maturation towards a Th1 response (first year of life); and **(5)** establishment of the immunological memory (from first to eighteenth year of life). However, critical windows for developmental immunotoxicity may depend on specific rather than global developmental immune events, like the differentiation and seeding of macrophages to tissues (6-24 week of gestation) or negative selection and apoptosis of autoreactive thymocytes (15-26 weeks of gestation), among others (Dietert, 2008; Dietert & Piepenbrink, 2006).

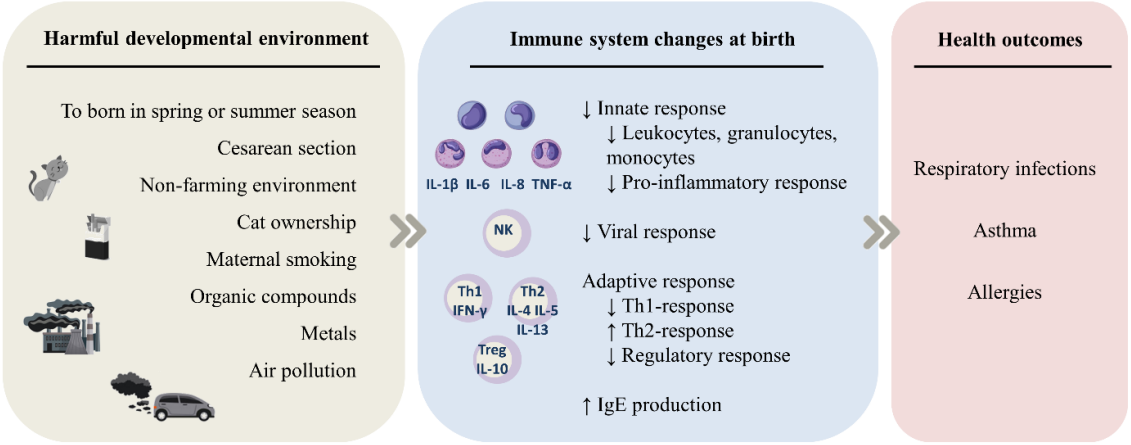
Figure 2. Developmental timeline of foetal immune system.



Source: Palmer, 2011.

Successful pregnancy and foetal development are accompanied by changes in maternal and foetal immune system response, especially in the Th1/Th2 balance, which ensure tolerance and prevent immune-mediated damage of the foetus while protecting against pathogens. Thus, a Th1 response, which is important in pre-implantation period, is inhibited in the maternal-foetal interface along the gestation towards a predominant Th2 response to avoid foetal rejection and adverse pregnancy outcomes (Graham et al., 2021; Wegmann et al., 1993). After birth, the switch to a Th1 environment starts in the neonate to ensure a correct response to intracellular pathogens in early life. However, this balance can be affected by prenatal and perinatal factors that could lead to the development of several diseases later in life (**Figure 3**). For instance, predominant Th1 and Th17 responses are associated with autoimmune diseases (Kamali et al., 2019), while a predominant Th2 response is associated with the occurrence of atopic diseases and allergies (Licona-Limón et al., 2013).

Figure 3. Prenatal environment, changes in the immune system at birth and related health outcomes later in life.



Effects of prenatal air pollution on immune system at birth

Prenatal and perinatal exposures, in particular exposures to outdoor air pollution, during the immune system development could trigger changes in immune system function that might lead to a higher vulnerability to suffer from non-NCDs in early life including respiratory or allergic disorders (Dietert, 2014). However, studies investigating associations between prenatal exposure to air pollution and the immune function at birth in humans are limited and inconsistent (Ashley-Martin et al., 2016; Baïz et al., 2011; Friedman et al., 2021; Hahn et al., 2021; Herr et al., 2010; Hertz-Picciotto et al., 2002, 2005; Latzin et al., 2011; Lura et al., 2018).

According to previous studies on the impact of prenatal exposure to outdoor air pollution on the cells of the immune system, prenatal exposure to short-term NO_2 was associated with decreased leukocytes counts in neonates at birth (Lura et al., 2018); whereas newborns whose mothers were exposed to NO_2 during pregnancy showed increased proportions of Tc and NK cells at birth (Baïz et al., 2011). Reports about the effects of prenatal exposure to PM on immune system cells are inconsistent. First, a cross-sectional study showed that living in a highly PM polluted urban area from Czech Republic was associated with an increased percentage of NKs and a decrease of T cell percentage in cord blood. After that, other studies have suggested that changes on T and NK cells at birth could depend on timing of exposure during gestation and the pollutant (Baïz et al., 2011; Herr et al., 2010; Hertz-Picciotto et al., 2005). For instance, exposure to $PM_{2.5}$ during first trimester of pregnancy was associated with increased T cells but decreased NK cell percentages (Herr et al., 2010), whereas neonates prenatally exposed during the third trimester to $PM_{2.5}$ showed decreased T cells but increased NK cell percentages (Herr et al., 2010). Besides, prenatal exposure to PM_{10} during the first trimester of gestation was associated with decreased T cells but increased NK cell percentages in cord blood at birth (Baïz et al., 2011). Finally, to our knowledge, the impact of prenatal exposure to ozone, a powerful oxidant, during pregnancy on immune system cells at birth has not been yet studied.

Regarding cytokines profiles, neonates whose mothers were exposed to NO_2 during pregnancy showed increased inflammatory markers in cord blood at birth (Ashley-Martin et al., 2016). In this sense, O_3 exposure during the second trimester of pregnancy was associated with increased IL-6 concentrations in cord blood (Friedman et al., 2021). However, exposure to $PM_{2.5}$ during long and short-term was related to decreased production of inflammatory cytokines (TNF-α, IL-6) (Friedman et al., 2021; Hahn et al., 2021) and IL-10 production (Hahn et al., 2021).

In summary, previous studies on the associations between prenatal exposure to outdoor air pollution and the immune system at birth are characterized by a large heterogeneity in the exposure assessment, the gestation periods examined and the immune system outcomes assessed. Furthermore, specific effects of traffic-related air pollution have been poorly studied (Hahn et al., 2021; Latzin et al., 2011). For this reason, we have conducted a more comprehensive study of the effects of prenatal exposure to the complex air pollution mixture derived from traffic on the immune system at birth, by characterising a wide panel of immune cells and cytokines measured in umbilical cord blood of newborns.

HYPOTHESIS

HYPOTHESIS

1. Environmental factors acting during the prenatal and perinatal period may induce changes in the developing immune system, including changes in the immune cell subpopulations and in the cytokine profiles in umbilical cord blood at birth.
2. Prenatal exposure to residential traffic-related air pollutants alters immune system cell subpopulations in newborns.
3. Prenatal exposure to residential traffic-related air pollutants modifies unstimulated cytokine profiles in newborns.
4. Prenatal exposure to residential traffic-related air pollutants disturbs cytokine responses to stimuli of umbilical cord blood cells of newborns.
5. Specific trimesters of pregnancy may be windows of higher susceptibility of immune system to traffic-related air pollutants effects. Consequently, immune cell subpopulations and cytokine profiles at birth could be disturbed.

OBJECTIVES

OBJECTIVES

The main objective of this doctoral thesis was to unravel the impact of prenatal exposure to residential traffic-related air pollution (TRAP) on immune system at birth.

The specific objectives were:

1. To review previous knowledge on the relationship between the prenatal and perinatal environment and changes in immune system cells and cytokine profiles in umbilical cord blood at birth. (**Article 1**)
2. To characterize the immune system at birth analysing immune cell subpopulations, unstimulated cytokine profiles and cytokine responses to a wide panel of environmental stimuli in umbilical cord blood of newborns. (**Article 2**, **Article 3** and **Appendix 1**)
3. To study the effects of prenatal exposure to TRAP on immune cell subpopulations distributions in umbilical cord blood at birth. (**Article 2**)
4. To investigate the effects of prenatal exposure to TRAP on unstimulated cytokine profiles in umbilical cord blood of newborns. (**Article 3**)
5. To examine the associations between prenatal exposure to TRAP and cytokines responses to stimuli of umbilical cord blood in newborns. (**Appendix 2**)
6. To identify gestational windows of higher susceptibility of the developing immune system to prenatal exposure to TRAP. (**Article 1**, **Article 2**, **Article 3**, **Appendix 2**, **Appendix 3**)

METHODOLOGY

METHODOLOGY

This section is divided in two parts to better describe the methodology of Article 1 based on a narrative review, and the methodology of the original articles 2 and 3 that are based on the data from the NELA birth cohort.

Herein, we explain the most relevant methodological aspects of the three articles that are also showed in the Material and methods section of each of them.

Article 1

Search strategy and study selection

A narrative review was performed to provide a comprehensive summary of the previous knowledge about the impact of prenatal and perinatal environmental factors on the neonatal immune system development *in utero*. To this purpose, a Medline search limited to human and English results was conducted using the following keywords: outcome ("leukocytes" OR "lymphocytes" OR "immune system" OR "Th1" OR "Th2" OR "cytokines" OR "IgE") combined with "cord blood" and with the next keywords for exposures ("season of birth" OR "mode of delivery" OR "caesarean section" OR "vaginal delivery" OR "farming environment" OR "farming" OR "pets" OR "dog" OR "cat" OR "indoor allergens" OR "dust mite" OR "mold" OR "dampness" OR "tobacco smoking" OR "persistent organic pollutants" OR "organochlorine pesticides" OR "volatile organic compounds" OR "metals" OR "arsenic" OR "cadmium" OR "lead" OR "chromium" OR "mercury " OR "air pollution" OR "nitrogen dioxide" OR "particulate matter")". Additional inclusion criteria for paper selection were: **(1)** original scientific epidemiologic studies performed in human individuals; **(2)** outcome assessment included phenotyping of immune cells (leukocyte and lymphocyte subsets) or cytokine profile patterns in cord blood of newborns at birth; and **(3)** environmental factors included season of birth, mode of delivery, farming exposures, pets, common allergens, tobacco smoking, persistent and non-persistent pollutants, toxic metals and outdoor air pollution during pregnancy or at delivery.

Seventy-eight publications examining the relationship between prenatal and perinatal environmental factors, immune cells and cytokine production in cord blood published between 1979 and 2020 were identified.

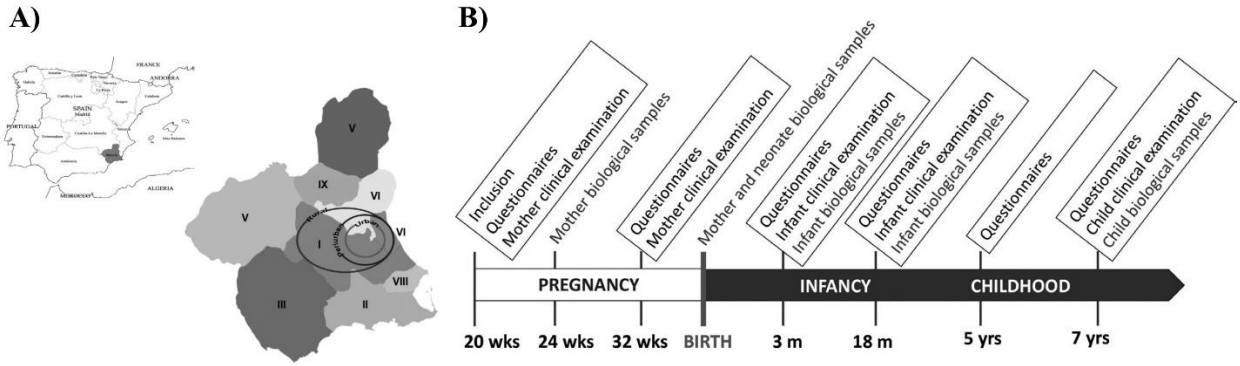
Articles 2 and 3

Study design

The Nutrition in Early Life and Asthma (NELA) study is a prospective population-based birth cohort set up in 2015 in Murcia, a south-eastern Mediterranean region of Spain. The main objective of NELA is to unravel the contribution of **(1)** maternal obesity/adiposity and foetal growth; **(2)** maternal and child nutrition; **(3)** residential outdoor air pollution; **(4)** endocrine disruptors; **(5)** and maternal psychological stress to the inception and mechanisms of asthma and allergy. The study protocol, recruiting methods, and data collection processes has been previously published (Morales et al., 2021).

In brief, 1350 pregnant women who fulfil the inclusion criteria were invited to participate in the study at the time of the control ultrasound at 20 weeks of gestation at the Maternal-Fetal Unit of the Virgen de la Arrixaca University Hospital between March 2015 to April 2018. The inclusion criteria were: women from Health Area I and certain districts of Health Areas VI and VII of the Region of Murcia (**Figure 4**); planning to live in the area of study during at least 2 years; intention to give birth at the reference hospital; Spanish Caucasian origin; 18–45 years of age; singleton pregnancy; non-assisted conception; and normal echography at 20 weeks of gestation (no major malformations). The exclusion criteria were: chronic disease; pregnancy complications (except gestational diabetes and hypertensive disorders); and not intending to deliver in the reference hospital. Finally, 738 (54% of participation) were enrolled in the study. The study protocol was reviewed and approved by the Ethics Committee of the Biomedical Research Institute of Murcia (IMIB-Arrixaca) in accordance with the guidelines of The Declaration of Helsinki (report 9/14; 29/09/2014). Written informed consents were obtained from parents at recruitment.

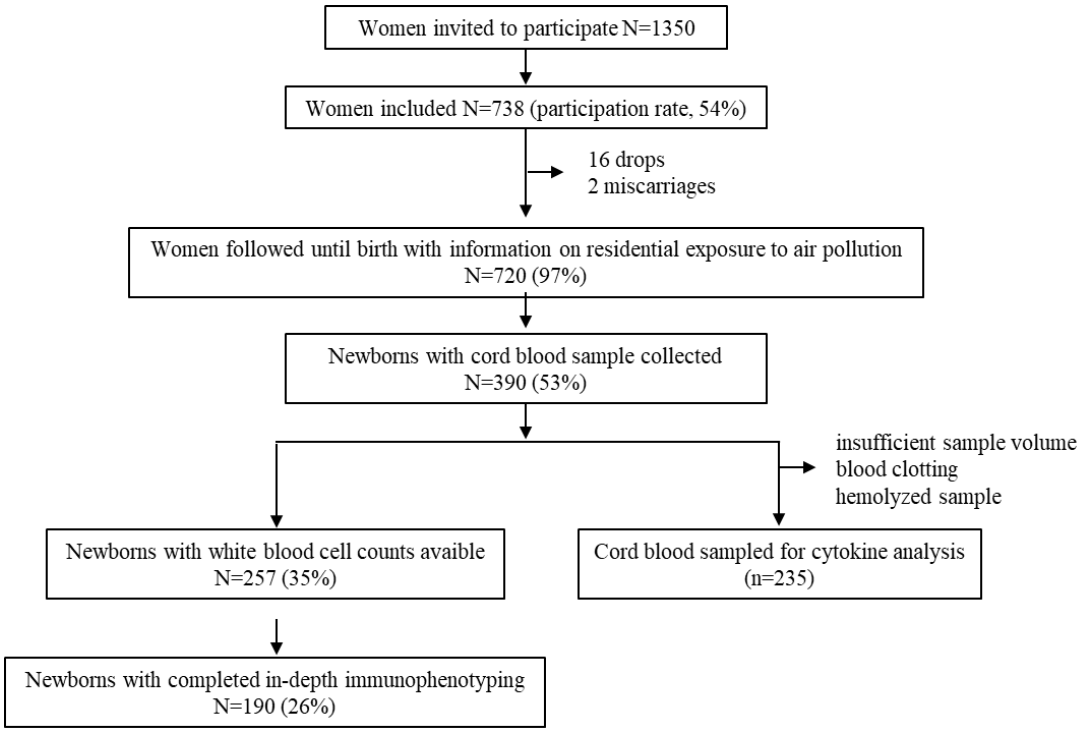
Figure 4. Geographical areas (A) and the follow-up plan (B) of the NELA study. The roman numbers indicate heath areas, and circles indicates the included areas (Morales et al., 2021).



Study participants

Mother-child pairs of the NELA cohort who had information about residential exposure to outdoor traffic-related air pollution during pregnancy and immune system at birth were included in the present studies: 190 with immunophenotyping of cord blood immune cells and 235 with assessed cytokine profiles (**Figure 5**). Compared to excluded participants, those included in **Articles 2** and **3** had older parents, mothers had higher pre-pregnancy body mass index, and newborns had higher birthweight, a slightly longer gestational age and were less frequently born during the summer season (**Table 2**).

Figure 5. Study population flowchart for articles 2 and 3. The NELA study.



Exposure assessment to residential outdoor air pollutants

To assess prenatal exposure to outdoor air pollutants, residential address of mothers during pregnancy was geo-codified using Geographic Information System (GIS). Residential outdoor air pollution exposure during pregnancy was estimated using the AIRNELA modelling system, a dispersion/chemical transport modelling based on source apportionment methodologies (García-Serna et al., 2021; Morales et al., 2021). Briefly, AIRNELA model estimated pollutant concentrations using dispersion and chemical transformations data delivered by different sources, taking into account meteorological factors. To this purpose, the regional meteorological model Advanced Research Weather Research and Forecasting (WRF-ARW) Model v3.6.1 was used to provide the meteorology to the chemistry transport model (Skamarock et al., 2005); and WRF was coupled off-line on an hourly basis to CHIMERE chemistry transport model (Menut et al., 2013). AIRNELA model resolution was 0.5 km for the NELA geographic area.

We estimated the global residential outdoor air pollution emissions caused by all sources (base) and by traffic (TRAP). Base air pollution concentrations were estimated by base-case simulations, including total emissions in WRF+CHIMERE; while TRAP estimations were calculated by the difference between base air pollution concentrations and air pollution concentrations derived from simulations turning off (zeroing-out) traffic emissions.

Prenatal exposure to NO_2, $PM_{2.5}$, PM_{10}, and O_3 were estimated in different windows of exposure: whole pregnancy, trimester-specific, and short-term exposure. Exposure windows were defined as follow: **(1)** whole pregnancy: from conception to birth; **(2)** the first trimester: from conception to 13^{th} weeks of gestation; **(3)** the second trimester: from 15^{th} to 26^{th} weeks of gestation; **(4)** the third trimester: from 27 weeks of gestation until birth, and **(5)** short-term

exposure: 15-days before the delivery date. The conception date was calculated using the date of the last menstrual period reported at recruitment and confirmed using estimates based on ultrasound examination at 12 weeks of gestation.

Immunophenotyping of cord blood immune cells

Venous umbilical cord blood samples were collected at birth and transferred into sterile tubes with ethylenediamine tetraacetic acid (EDTA). White blood cells (WBC) were assayed immediately after collection using an automatized blood cell count.

Lymphocyte subsets were analysed using flow cytometry and phenotypes were defined based on bibliography according to surface markers. For that purpose, within 48 hours 50 µL of whole cord blood were incubated with the appropriate combination of fluorochrome-conjugated antibodies for 15 min at room temperature in dark conditions. Then, red blood cells were lysed using Fixing/Lising solution (Becton Dickinson) for 5 min. Immune cells were analysed using a BD FACSCanto™ flow cytometry system. Immune cells were gated according to size and granularity parameters. Cell percentages were obtained according to phenotypes previously defined and absolute cell numbers were calculated using percentages of lymphocyte subsets and leukocyte counts (WBC/µl).

Unstimulated cytokine profiles and cytokine responses to stimuli in umbilical cord blood

Blood was obtained from the umbilical cord vein, transferred from syringes to sterile heparinized tubes, and kept at room temperature until processing within 48 h. Samples were diluted 1:7 with RPMI 1640 medium, and supernatants were collected after 7 days of culture (López et al., 2014) aliquoted, and frozen at −80°C. Human Cytokine Multiplex-Assay-Kit (ThermoFisher, Viena, Austria) was used to measure cytokine concentrations in supernatants with Luminex technology according to the manufacturer's instructions

Immune response at birth was assessed analysing cytokine production after incubation with and without different stimuli in cord blood cells. A wide panel of cytokines were selected based on the main representative immune response related to them: global innate response related cytokines (IL-6, IFN-α, IL1-β and TNF-α), Th1-related (IFN-γ and IL-2), Th2-related (IL-4, IL-5 and IL-13), Th17-related (IL-17 and IL-23), and one immunomodulatory cytokine, IL-10.

In addition, we used eight stimuli according to their ability to induce innate or adaptive immune response: mitogens (Concanavalin A (Con A) and Phytohemagglutinin (PHA)), pathogen associated molecular patterns (PAMPs; Lipopolysaccharide (LPS), Peptidoglycan (PG), Polyinosinic-polycytidylic acid (pI:C), Immunostimulatory CpG-oligodeoxynucleotides (CpG-ODN)) and common environmental allergen extracts including the *Olea europaea* (olive) pollen extract and the house dust mite *Dermatophagoides pteronyssinus* (D.p.) extract. Finally, to establish detection ranges for cytokine concentrations, standard curves were performed for each cytokine assayed using mixtures of multiple standard cytokines. Detection ranges were defined as the linear range in the curve.

Other variables

Face-to-face questionnaires administered during pregnancy and after delivery were used to obtained information about potential confounding factors (sociodemographic factors, prenatal exposures, and clinical information) (**Table 2**). The variables included in **Articles 2** and **3** were: maternal and paternal age (years); parity (0, nulliparous; vs. 1 or more, non-nulliparous); education level (incomplete secondary or less, complete secondary, and university); maternal pre-pregnancy body mass index (BMI) based on height and pre-pregnancy self-reported weight (kg/m^2) (categorized as normal BMI<25, overweight 25<BMI<30, and obesity BMI>30); maternal and paternal reported history of asthma (yes/no) and atopy (yes/no); maternal smoking during pregnancy (yes/no) and paternal smoking; area of study (urban, residential and rural); parental social class (defined as maternal or paternal occupation during pregnancy based on the highest social class by using a widely used Spanish adaptation of the international ISCO88 coding system: I–II, managers/technicians; III, skilled; IV–V, semiskilled/unskilled; and unemployed) (Domingo-Salvany et al., 2000); pets at home (yes/no) and maternal contact with farming animals during pregnancy (yes/no). Variables related to the child and labour were also considered: newborn's sex; gestational age (weeks); birthweight (g); season at birth (spring, March-May; summer, June-August; fall, September-November; and winter, December-February); mode of delivery (vaginal non-instrumental, vaginal instrumental, and caesarean section); and fever (yes/no) and use of antibiotics during labour (yes/no).

Table 2. Main characteristics of the participants in the NELA study.

	NELA cohort (n=738)	**Garcia-Serna et al., 2021 (n=190)**	**Garcia-Serna et al., 2022 (n=235)**
Maternal age, years, mean ± standard deviation (sd)	32.6 ± 4.6	33.1 ± 4.2	33.3 ± 4.3
Parity, nulliparous, n (%)	374 (50.7)	99 (52.1)	121 (51.5)
Maternal education level, n (%)			
Incomplete secondary or less	146 (19.8)	32 (16.8)	42 (17.9)
Complete secondary	191 (25.9)	49 (25.8)	53 (22.6)
University	401 (54.3)	109 (57.4)	140 (59.6)
Maternal pre-pregnancy BMI (kg/m^2), mean ± sd	23.9 ± 4.4	24.5 ± 4.8	24.4 ± 4.5
Normal, n (%)	513 (69.5)	124 (65.2)	152 (64.7)
Overweight, n (%)	156 (21.1)	44 (23.2)	59 (25.1)
Obesity, n (%)	69 (9.35)	22 (11.6)	24 (10.2)
Maternal asthma, yes, n (%)	81 (11.0)	26 (13.7)	33 (14.0)
Maternal history of atopy, yes, n (%)	310 (42.0)	88 (46.3)	107 (45.5)
Maternal smoking during pregnancy, yes, n (%)	128 (17.3)	31 (16.3)	39 (16.6)
Father age, years, mean ± sd	34.8 ±5.3	35.6 ± 5.2	35.6 ± 5.1
Father education level, n (%)			
Incomplete secondary or less	218 (29.6)	48 (25.3)	57 (24.3)
Complete secondary	229 (31.1)	75 (39.5)	84 (35.7)
University	289 (39.3)	67 (35.3)	94 (40.0)
Father asthma, yes, n (%)	66 (9.0)	17 (9.0)	22 (9.4)
Father history of atopy, yes, n (%)	263 (35.7)	70 (37.0)	88 (37.6)
Father smoking, yes, n (%)	258 (35.1)	71 (37.4)	83 (35.3)
Area of study, n (%)			
Urban	534 (72.4)	147 (77.4)	173 (73.6)
Residential	107 (14.5)	23 (12.1)	34 (14.5)
Rural	97 (13.1)	20 (10.5)	28 (11.9)
Metropolitan area, n (%)	6274 (85.0)	158 (83.2)	199 (84.7)
Parental social class, n (%)			
I-II (managers/technicians)	367 (49.7)	96 (50.5)	126 (53.6)
III (skilled)	162 (21.9)	47 (24.7)	52 (22.1)
IV-V (semiskilled/unskilled)	191 (25.9)	43 (22.6)	54 (23.0)
Unemployed	18 (2.4)	4 (2.1)	3 (1.3)
Pets at home, yes, n (%)	341 (46.2)	93 (49.0)	111 (47.2)
Maternal contact with farming animals, yes, n (%)	132 (17.9)	38 (20.0)	44 (18.7)
Newborn's sex, female, n (%)	363 (50.4)	89 (46.8)	119 (50.6)
Gestational age, weeks, mean ± sd	39.6 ±1.5	39.7 ±1.2	39.8 ± 1.3
Preterm (<37 wks.), n (%)	36 (4.9)	3 (1.6)	6 (2.6)
Birthweight, g, mean ± sd	3242.2 ± 474.8	3261 ± 434.4	3275 ± 430.5
<2500 g, n (%)	42 (5.7)	9 (4.7)	10 (4.3)
Season at birth, n (%)			
Autumn	202 (28.1)	48 (25.3)	74 (31.5)
Spring	186 (25.8)	57 (30.0)	63 (26.8)
Summer	208 (28.9)	36 (19.0)	53 (22.6)
Winter	124 (17.2)	49 (25.8)	45 (19.2)
Mode of delivery, n (%)			
Vaginal non-instrumental	409 (57.4)	110 (57.9)	132 (56.2)
Vaginal Instrumental	146 (20.5)	38 (20.0)	48 (20.4)
Caesarean section	157 (22.1)	42 (22.1)	55 (23.4)
Fever during labour, yes, n (%)	34 (4.9)	9 (4.7)	11 (4.7)
Use of antibiotics during labour, yes, n (%)	178 (28.0)	52 (27.8)	68 (30.2)

Statistical analysis

Descriptive statistic was used to summarize the main characteristics of the study population. Categorical variables were assayed by the Chi-square test and quantitative variables by the Kruskall-Wallis test. Spearman's rank correlation was used to study the relationships between TRAPs across pregnancy periods.

1. Associations between prenatal exposure to residential TRAP and immune cell counts in cord blood at birth

Umbilical cord blood cell counts fitted a Poisson distribution. Thus, multivariate Poisson regression was applied to study the associations between prenatal exposure to TRAP and cord blood cell counts. Air pollutants concentrations were dichotomized based on the median value: "high", equal or up to the median value; and "low", below the median value. Low category was used as the reference. Regression coefficients were presented as incidence relative risk (IRR) and their corresponding 95% confidence interval (CI). IRR can be interpreted as the percentage of change in the mean of cell counts. Models were adjusted by variables that showed marginal significant associations ($p<0.1$) or modified the coefficient of air pollutants by more than 5%. Thus, final models were adjusted by parity, maternal pre-pregnancy BMI, parental social class, season of birth, mode of delivery, newborn's sex, gestational age and birthweight. Additionally, each trimester of gestation was mutually adjusted.

Finally, we used adjusted generalized additive models (GAMs) by graphical examination and likelihood ratio test (Hastie & Tibshirani, 1990) to examine the global linear relationship between continuous air pollutant concentrations and immune cell counts. We considered that models fitted a linear relationship when p-value for gain in linearity (p-gain) was higher than 0.1.

2. Associations between prenatal exposure to residential TRAP and cytokine production in cord blood cells

Unstimulated cord blood cells showed low cytokine concentrations, so we decided to dichotomized concentration values for each cytokine into "detectable", when values were above the lower detection limit (LDL), and "non-detectable", when concentration values were below LDL. "Non-detectable" category was used as the reference. Then, associations between long-term (trimester specific and whole pregnancy) and short-term (15 days before delivery) exposure to TRAP and cytokine production in unstimulated cord blood cells were analysed using multivariate logistic regressions with single pollutants as dependent variables. Coefficients were presented as odds ratios (OR) and their corresponding 95% confidence interval (CI); and results were interpreted as the odds of being above the LDL for each cytokine per 10µg/m^3 concentration of NO_2, PM_{10} and O_3, and per 5 µg/m^3 concentration of $PM_{2.5}$.

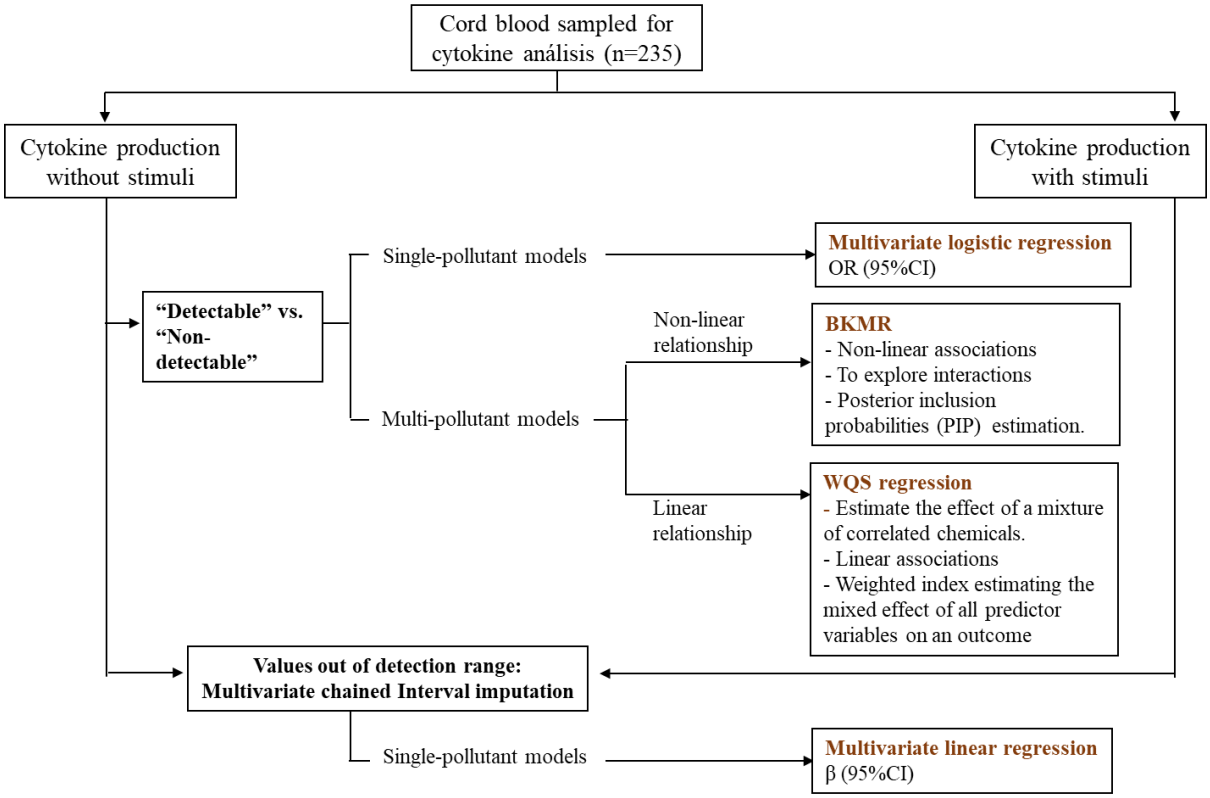
Air pollution results from a mixture of several chemicals that could establish interactions and modify single-exposure effects on health outcome. Although several statistical approaches have been developed in the last years to manage this statistical challenge (Joubert et al., 2022; Tanner et al., 2020), mixtures analysis approaches to study binary outcomes are limited (Gibson et al., 2019). The Bayesian kernel machine regression (BKMR) method estimates multivariable

exposure-response function using a Gaussian kernel machine (Bobb et al., 2015). This Bayesian model allows to estimate the joint effects of mixture components on a particular health binary outcome to identify nonlinear and nonadditive associations and potentially interactions among mixture components. BKMR models are estimated by Markov chain Monte Carlo (MCMC), using the Metropolis-Hastings algorithm and a Gibbs sampler for the remaining parameters. Weighted quantile sum (WQS) regression is another approach for mixtures analysis that allows to assess overall effects of correlated exposures categorized into quartiles among a particular health outcome and estimate weights for each exposure (Carrico et al., 2015). The main limitations of the WQS approach are the previous assumptions of relationships between predictors and the outcome of interest: **(a)** all exposures contribute to the outcome in the same directions and **(b)** associations follow a linear tendency without interactions.

To explore the effects of exposure to air pollutant mixtures during the whole pregnancy on cytokine production in unstimulated cord blood cells we designed the analysis plan showed in **Figure 6**. First, we applied probit BKMR models using the R package *bkmr* (Bobb et al., 2018) to test for non-linear associations between air pollutants and cytokine production ("detectable" vs. "non-detectable"), and to identify interactions among mixture components. Models were fitted with 20000 iterations and the parameter *r.jump2* was set to 0.5 as recommended (Bobb et al., 2018). Results from BKMR models did not show neither non-linear associations nor important interactions between studied air pollutants. Therefore, WQS regression models were applied to identify individual exposure weights on the main associations identified in logistic regressions models. Data were divided into two data sets: a training data set (40%) and a validation data set (60%) and 1000 bootstrap iterations were performed to parameter estimation. The β_1 parameter was constrained to positive values based on previous results derived from single-pollutant models showed associations between all air pollutants assayed and each cytokine. First, period-specific evaluation was performed running WQS models were for each window of susceptibility. After that, all times were included in the same models to rank important associations of each pollutant in all critical windows examined through weighted linear index of predictors included in the final model.

Categorization of a continuous variable could lead to loss of information and the consequently loss of statistic power. Multiple imputation methods are recommended when measurement values are constrained by detection limits and data show different percentages of non-detectable values (Uh et al., 2008). Accordingly, cytokine values out of detectable ranges were imputed using multivariate multiple imputation. Interval regression imputation method and chained equations (Royston, 2007; van Buuren et al., 1999) were performed conditioning the imputation to the range of (0, LDL) and (>HDL), and twenty data sets were generated. We used multivariate linear regression models with air pollutant concentrations categorized into quartiles and log-transformed cytokine concentrations to assess the trend of the main associations found in previous analyses. Regression models were applied to each data set separately, and, finally, results were combined using the Rubin's rules (Little & Rubin, 2014).

Figure 6. Statistical analysis plan for cytokine data.



Rstudio (version 1.1.463, RStudio, Boston, Mass), Stata (version 15.1, Stata Corp, College Station, Texas, USA), and GraphPad Prism software (version 8.0.2, GraphPad Software Inc., USA) were used to perform the statistical analyses.

RESULTS

Prenatal and Perinatal Environmental Influences Shaping the Neonatal Immune System: A Focus on Asthma and Allergy Origins

García-Serna AM, Martín-Orozco E, Hernández-Caselles T, Morales E.

It is suggested that programming of the immune system starts before birth and is shaped by environmental influences acting during critical windows of susceptibility for human development. Prenatal and perinatal exposure to physiological, biological, physical, or chemical factors can trigger permanent, irreversible changes to the developing immune system, which may be reflected in cord blood of neonates. The aim of this narrative review is to summarize the evidence on the role of the prenatal and perinatal environment, including season of birth, mode of delivery, exposure to common allergens, a farming environment, pet ownership, and exposure to tobacco smoking and pollutants, in shaping the immune cell populations and cytokines at birth in humans. We also discuss how reported disruptions in the immune system at birth might contribute to the development of asthma and related allergic manifestations later in life.

https://www.mdpi.com/1660-4601/18/8/3962

Air pollution from traffic during pregnancy impairs newborn's cord blood immune cells.

García-Serna AM, Hernández-Caselles T, Jiménez-Guerrero P, Martín-Orozco E, Pérez-Fernández V, Cantero-Cano E, Muñoz-García M, Ballesteros-Meseguer C, Pérez de Los Cobos I, García-Marcos L, Morales E; NELA Study group.

Background: Hazards of traffic-related air pollution (TRAP) on the developing immune system are poorly understood. We sought to investigate the effects of prenatal exposure to TRAP on cord blood immune cell distributions; and to identify gestational windows of susceptibility.

Methods: In-depth immunophenotyping of cord blood leukocyte and lymphocyte subsets was performed by flow cytometry in 190 newborns embedded in the Nutrition in Early Life and Asthma (NELA) birth cohort (2015–2018). Long-term (whole pregnancy and trimesters) and short-term (15-days before delivery) residential exposures to traffic-related nitrogen dioxide (NO_2), particulate matter ($PM_{2.5}$ and PM_{10}), and ozone (O_3) were estimated using dispersion/chemical transport modelling. Associations between TRAP concentrations and cord blood immune cell counts were assessed using multivariate Poisson regression models.

Results: Mean number of natural killer (NK) cells decreased 15% in relation to higher NO_2 concentrations (≥36.4 μg/m3) during whole pregnancy (incidence relative risk (IRR), 0.85; 95% CI, 0.72, 0.99), with stronger associations in the first trimester. Higher $PM_{2.5}$ concentrations (≥13.3 μg/m3) during whole pregnancy associated with a reduced mean number of cytotoxic T cells (IRR, 0.88; 95% CI, 0.78, 0.99). Newborns exposed to higher PM_{10} (≥23.6 μg/m3) and $PM_{2.5}$ concentrations during the first and third trimester showed greater mean number of helper T type 1 (Th1) cells ($P < 0.05$). Decreased number of regulatory T (Treg) cells was associated with greater short-term NO_2 (IRR, 0.90; 95% CI, 0.80, 1.01) and PM_{10} (IRR, 0.88; 95% CI, 0.77, 0.99) concentrations.

Conclusions: Prenatal exposure to TRAP, particularly in early and late gestation, impairs fetal immune system development through disturbances in cord blood leukocyte and lymphocyte distributions.

https://www.sciencedirect.com/science/article/pii/S0013935120313657?via%3Dihub

Cytokine profiles in cord blood in relation to prenatal traffic-related air pollution: The NELA cohort

García-Serna AM, Martín-Orozco E, Jiménez-Guerrero P, Hernández-Caselles T, Pérez-Fernández V, Cantero-Cano E, Muñoz-García M, Molina-Ruano MD, Rojo-Atenza E, García-Marcos L, Morales E; NELA Study Group.

Background: Outdoor air pollution may disturb immune system development. We investigated whether gestational exposure to traffic-related air pollutants (TRAP) is associated with unstimulated cytokine profiles in newborns.

Methods: Data come from 235 newborns of the NELA cohort. Innate response–related cytokines (IL-6, IFN-α, IL1-β, and TNF-α), Th1-related (IFN-γ and IL-2), Th2-related (IL-4, IL-5, and IL-13), Th17-related (IL-17 and IL-23), and immunomodulatory cytokine IL-10 were quantified in the supernatant of unstimulated whole umbilical cord blood cells after 7 days of culture using the Luminex technology. Dispersion/chemical transport modeling was used to estimate long-term (whole pregnancy and trimesters) and short-term (15 days before delivery) residential exposures to traffic-related nitrogen dioxide (NO_2), particulate matter ($PM_{2.5}$ and PM_{10}), and ozone (O_3). We fitted multivariable logistic regression, Bayesian kernel machine regression (BKMR), and weighted quantile sum (WQS) regression models.

Results: NO_2 during the whole pregnancy increased the odds of detection of IL-1β (OR per 10 µg/m3 increase = 1.37; 95% CI, 1.02, 1.85) and IL-6 (OR per 10 µg/m^3 increase = 1.32; 95% CI 1.00, 1.75). Increased odds of detected concentrations of IL-10 was found in newborns exposed during whole pregnancy to higher levels of NO_2 (OR per 10 µg/m3 increase = 1.30; 95% CI 0.99, 1.69), PM10 (OR per 10 µg/m^3 increase = 1.49; 95% CI 0.95, 2.33), and $PM_{2.5}$ (OR per 5 µg/m^3 increase = 1.56; 95% CI 0.97, 2.51). Exposure to O_3 during the whole pregnancy increased the odds of detected IL-13 (OR per 10 µg/m3 increase = 1.22; 95% CI 1.01, 1.49). WQS model revealed first and third trimesters of gestation as windows of higher susceptibility.

Conclusions: Gestational exposure to TRAP may increase detection of pro-inflammatory, Th2-related, and T regulatory cytokines in newborns. These changes might influence immune system responses later in life.

https://onlinelibrary.wiley.com/doi/10.1111/pai.13732

CONCLUSIONS

CONCLUSIONS

Here we enumerate the conclusions derived from each of the studies that constitute the present doctoral thesis:

1. Prenatal and perinatal periods seem to represent crucial developmental windows of higher susceptibility of the immune system to diverse environmental influences.
2. Cord blood NK, T cytotoxic and T regulatory cells decreased in relation to higher prenatal exposure to traffic-related pollutants.
3. Higher prenatal exposure to traffic-related PM_{10} and $PM_{2.5}$ was associated with increased total Th and Th1 cells in newborns' cord blood.
4. Prenatal exposure to higher levels of traffic related air pollutants was associated with increased detection of unstimulated concentrations of pro-inflammatory (IL-1β and IL-6), Th2-related (IL-13), and immunomodulatory (IL-10) cytokines in newborns' cord blood.
5. Exposure to residential traffic-related NO_2 and PM *in utero* may promote higher proinflammatory (IL-6 and IFN-α) and Th1-related (IFN- γ) cytokine responses of umbilical cord blood cells to environmental stimuli such as mitogens, pathogen associated molecular patterns (PAMPs) and common allergens.
6. Early and late pregnancy may be windows of higher susceptibility of foetal immune system to adverse effects of traffic-related air pollutants.
7. The identified changes in immune cell subpopulations and cytokine profiles at birth in relation to prenatal exposure to traffic-related air pollutants could lead to higher susceptibility to respiratory infections, asthma, and allergic manifestations later in life.

DISCUSSION AND PUBLIC HEALTH IMPLICATIONS

DISCUSSION AND PUBLIC HEALTH IMPLICATIONS

Pregnancy is a critical period of higher vulnerability to environmental exposures. In **Article 1**, we summarized the available evidence until 2020 on the role of prenatal and perinatal environment factors, including, season of birth, mode of delivery, exposure to common allergens, farming environment, pet ownership, and exposure to tobacco smoking and pollutants on foetal development of immune cell populations and cytokine production in neonates at birth. This review showed that perinatal factors such as season of birth and mode of delivery may skew Th1/Th2 balance, increasing the risk of diseases, such as asthma and respiratory infections (Darabi et al., 2019; Kristensen & Henriksen, 2016). Furthermore, there is growing evidence that certain changes in immune cell distribution and cytokine productions are associated with exposure *in utero* to tobacco smoke and to organic or inorganic environmental pollutants. These immune changes could play a key role in the mechanism underlying the adverse respiratory outcomes found in children prenatally exposed to these pollutants (Gibbs et al., 2016; Morales et al., 2015). However, the heterogeneity in exposure assessment, study design, and immune parameter studied, together with the scarce bibliography linking immune system biomarkers in cord blood at birth and health outcomes in early life, make it difficult to disentangle the specific mechanisms triggered by environmental exposures during the foetal stages.

The results derived from the present thesis advised that the pregnant women in Murcia were exposed to residential outdoor air pollution concentrations above the European air quality standards (European Commission, 2022); and traffic-related exposures exceeded the new World Health Organization (WHO) air quality recommendations established in 2021. This high air pollution exposure could have several adverse health effects on them and, even, on their offspring. In this sense, this thesis concludes that pregnancy is a vulnerable period to environmental exposures and provides extensive information about how maternal exposure to residential outdoor air pollution during pregnancy impacts on immune system foetal development, inducing changes in lymphocyte subpopulation counts and on cytokine production in neonates at birth. Moreover, these disruptions on immune function at birth could lead to increased risk of respiratory infections, asthma or atopic manifestations in early life.

Th1 cells generate important proinflammatory cytokines whose changes in serum may be indicative of an increased systemic inflammation (Zhou et al., 2009). Thus, **Article 2** (García-Serna et al., 2021) and **3** (García-Serna et al., 2022) found that those newborns whose mothers were exposed to higher levels of traffic-related air pollution during pregnancy showed a pro-inflammatory response with increased Th1-related cells counts and increased pro-inflammatory cytokine production (specially IL-6) in cord blood. Although the release of pro-inflammatory cytokines may activate cells of the innate and adaptive immune system to deal with infections, too high concentrations could induce an immune hyper-reaction. Indeed, high concentrations of pro-inflammatory mediators like IL-6 have been shown to be associated with severity in patients suffering respiratory diseases such as asthma exacerbation in childhood (Jackson et al., 2020) and COVID-19 (Gao et al., 2021).

Article 2 (García-Serna et al., 2021) showed that maternal exposure to higher traffic-related air pollution concentrations during pregnancy was associated with decreased counts of

Tc and NK cells in cord blood. Tc cells are involved in the defence against respiratory viruses (Schmidt & Varga, 2018), and consequently, we hypothesized that changes in those cells may be involve in the mechanism explaining the association between prenatal air pollution exposure and respiratory viral infections in childhood previously reported (Rice et al., 2015; Soh et al., 2018).

Additionally, **Article 2** (García-Serna et al., 2021) found decreased Treg cell counts in relation to higher maternal air pollution exposure during the last days of gestation. Tolerance to allergens critically depends on the generation of allergen-specific Treg cells, which mediate a state of sustained non-responsiveness to allergens and suppress inflammatory Th2 responses responsible for allergic manifestations (Abdel-Gadir et al., 2018). Hence, we hypothesized that prenatal exposure to traffic-related air pollution might increase the risk of allergy in childhood through disruption of Treg cells, based on previous studies that found defective Treg cells in cord blood of neonates with a family history of allergy (Haddeland et al., 2005; Schaub et al., 2008) and in allergic subjects themselves (Černý et al., 2020) including asthmatics (Huang et al., 2017; Lloyd & Hawrylowicz, 2009).

In summary, the results from this PhD have revealed that Murcia is among the most polluted regions in Spain, showing that traffic is the main source of outdoor air pollution in urban areas. This should be considered in order to revise policies about traffic mobility and to pursue established air quality standards in order to protect maternal and child health. Finally, promoting a proper immune system development during early life, including the prenatal period, should be recognized as a major element in the public health agenda to prevent NCDs, especially asthma and allergic manifestations.

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APPENDIX

APPENDIX

Three additional manuscripts were derived from the research carried out during this PhD project and we consider them part of it. Some of these scientific articles have been submitted for peer review in JCR journals or are planned to be submitted.

APPENDIX 1:

Cytokine production by newborns. Influence of sex and season of birth

Introduction

Cytokine network is the main component of immunity, acting as a general coordinator/regulator of the overall immune response. Prenatal factors could impact the immune system development at critical windows of vulnerability[1],[2] and contribute to the development of adverse health outcomes later in life, including allergies[3] and asthma[4,]. The aim of our study was to determine the cytokine profiles of newborns from the NELA cohort and to analyse the relationship between cytokine profiles at birth and sex and season of birth, two factors related to asthma incidence and severity[5,6].

Methods

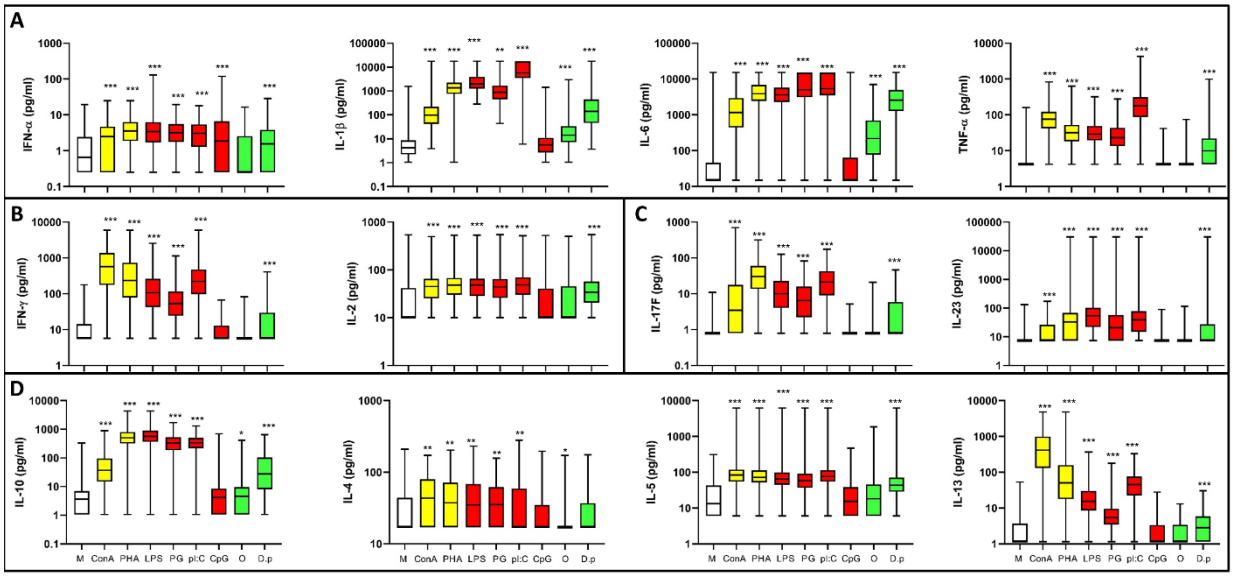
Data come from 235 newborns from the Nutrition in Early Life and Asthma (NELA) study. Cord blood samples were collected from the umbilical cord vein and transferred into sterile heparinized tubes at delivery. Whole cord blood was diluted 1:7 with RPMI 1640 medium and cultured for 7 days unstimulated and in the presence of 8 different stimuli as follows: Concanavalin A (Con A), Immunostimulatory CpG oligonucleotides (CpG-ODN), Polyinosinic-polycytidylic acid (pI:C), Peptidoglycan (PG), Lipopolysaccharide (LPS), Phytohemagglutinin (PHA) and Olea europaea pollen (olive, O) and the dust mite *Dermatophagoides pteronyssinus* (*D.p.*) Production of cytokines by stimulated cord blood samples was determined using the Human Cytokine Multiplex-Assay-Kit according to the manufacturer's instructions (ThermoFisher, Viena, Austria) and Luminex technology. We analysed general inflammatory response cytokines (IL-6, IFN-α, IL1-β, TNF-α, IL-8, IL-33), T helper 1 (Th1)-related cytokines (IL-12p70, IFN-γ, IL-2, IL-18, MIG), T helper 2 (Th2)-related cytokines (IL-4, IL-5, IL-13), T helper 17 (Th17)-related cytokines (IL-17A, IL-17F, IL-23), T helper 9 (Th9)-related cytokine (IL-9) and T helper 2/T regulatory (Th2/Treg)-related cytokines (IL-10, TGF-β). Mixes of multiple standard cytokines were used to generate standard curves for each cytokine. Measurements out of the detection range were censored: values below the detection range were given a value that corresponded to a half of the lowest value of the detection range of the respective cytokine, and values above the detection range were given a value that corresponded to the highest value of the detection range.

Cytokine concentrations were categorized into high responders (H, >60th percentile of each cytokine concentrations) and low responders (L, <40th percentile of each cytokine concentrations) due to their non-Gaussian distribution and low detection rates presented in several conditions. Associations between sex and season of birth with cytokine response patterns were examined using multivariate logistic regression models. Due to the small sample size, season of birth was dichotomized as cold seasons (winter and autumn births) and warm seasons (spring and summer). Coefficients of associations are presented as odds ratio (OR) and 95% confidence interval (CI). Final models were adjusted for maternal age, maternal body mass index (BMI) pre-pregnancy, maternal history of atopy, gestational age and birthweight.

Results

Response to mitogens and PAMPs was vigorous (**Figure** S**1**), inducing high levels of inflammatory cytokines (IL-1β, IL-6, TNF-α) and intermediate levels of Th1 (IFN- γ), Th2 (IL-5 and IL-13) and Treg (IL-10) related cytokines in cord blood. CpG-ODN only induced a significant secretion of IFN-α (**Figure S1A**); and allergens induced a mild cytokine response.

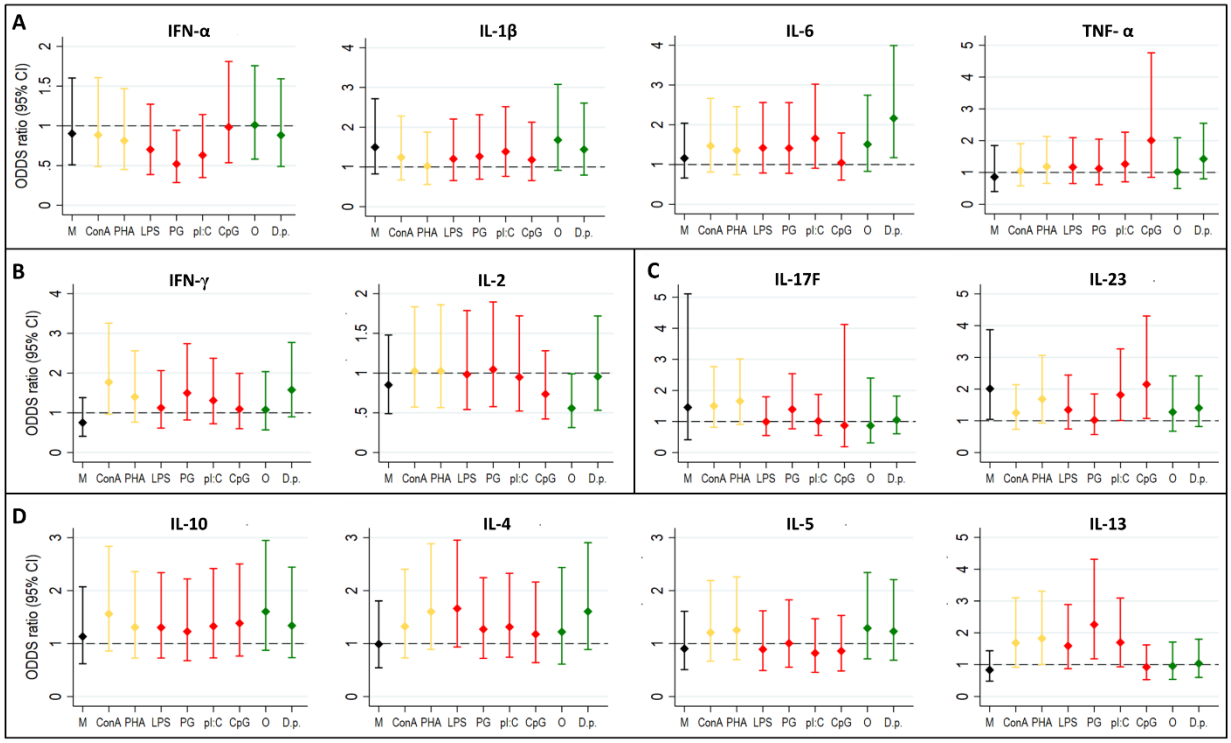
Figure S1. Cytokine concentrations and patterns of response in cord blood cells.



Box-plots represent concentration levels of different cytokines produced by cells unstimulated (white filled boxes) or stimulated with different mitogens (yellow filled boxes), PAMPs (red filled boxes) or allergenic extracts (green filled boxes). Box plots represent the concentration values (pg/ml) included in the 10th to 90th percentiles. Median values are represented by the horizontal line inside each box. The whiskers represent the highest and the lowest cytokine values. P-values derived from Mann-Whitney test with correction for multiple comparisons using the Benjamini-Hochberg method. *p-value<0.05 **p-value<0.01 ***p-value<0.005. (**A**) Inflammatory cytokine production in cord blood cells; (**B**) Th1 cytokines production in cord blood cells; (**C**) Th17 cytokines production by cord blood cells; (**D**) regulatory and Th2 cytokines production by cord blood cells.

Analysis of sex differences in cytokine production revealed that male sex was associated with higher IL-6 response to D.p (**Figure S2A**); and higher IL-23 response in cells incubated with pI:C compared to females. Furthermore, increased odds of IL-13 response to mitogens and PAMPs were found in males compared to females (**Figure S2D**). However, a decreased of IFN-α response to PG and IL-2 response to olive pollen were found among males compared to females (**Figure S2A-B**). These results suggest that sex could influence the immune system development through biasing the cytokine balance. This might explain the higher prevalence of asthma or allergic diseases among males during childhood as compared to females[7].

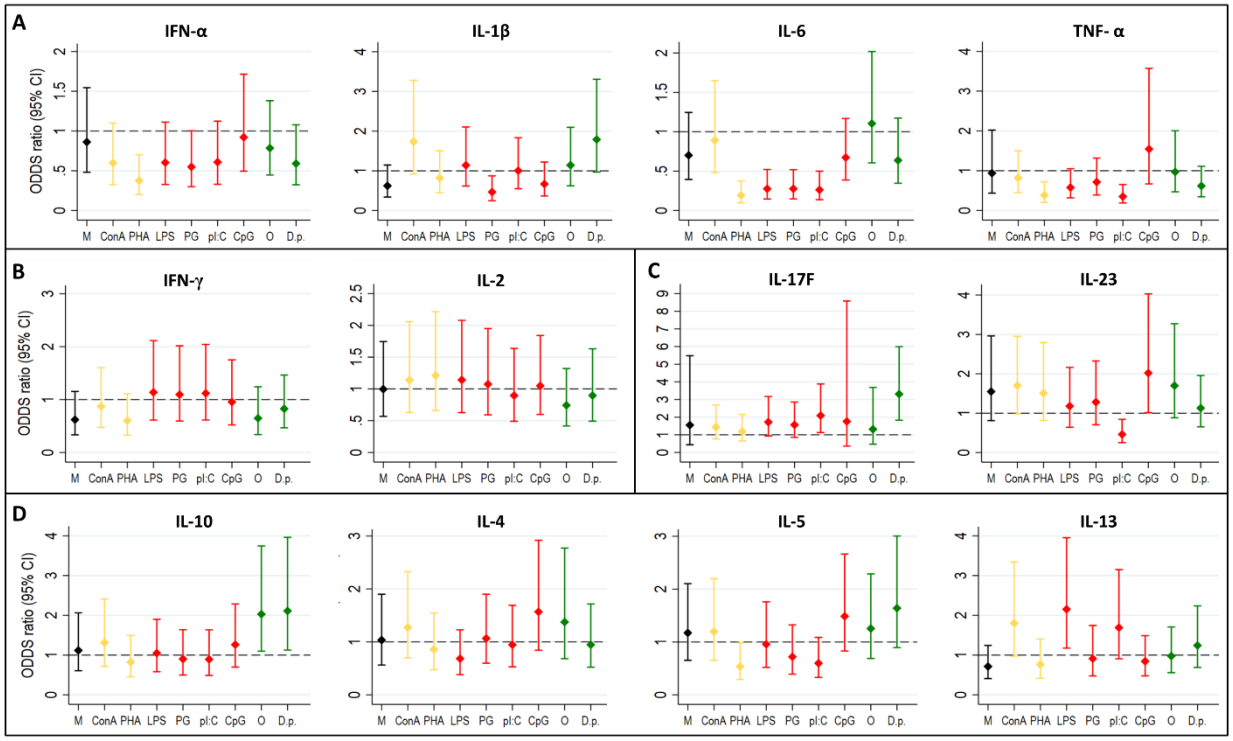
Figure S2. Cytokine response in unstimulated and stimulated cord blood cells according to newborn's sex.



Graphs show results derived from associations between production of (A) inflammatory, (B) Th1-related, (C) Th17-related and (D) Th2-related and immunomodulatory cytokines. A code of colours has been used depending on the type of stimulus used to culture the cells: unstimulated cells (black), cells stimulated with mitogens (yellow), cells incubated with pathogen associated molecular patterns (red) and cells cultured with common allergens extracts (green). Coefficients were derived from a multivariate logistic regression comparing high (>60th percentile cytokines concentrations) with low (<40th percentile cytokines concentrations) responder samples Female low responders were used as the reference group. Models were adjusted by maternal age, pre-pregnancy body mass index, maternal atopy, gestational age and birthweight. Diamonds represent coefficients and horizontal bar with whiskers represent 95% confidence intervals.

Birth in warm seasons (summer and spring) was associated with lower production of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α and IFN-α) (**Figure S3A**) as compared to birth in cold seasons (autumn or winter). Conversely, IL-17 and IL-13 responses, especially to LPS and pI:C, were higher in newborns born in warm seasons (**Figure S3C-D**). Finally, we found a significant increased odds of IL-10 release in response to D.p and olive extracts in children born during warm seasons as compared to those born during cold seasons (**Figure** S**3D**). Although some authors have suggested that children born during the spring season show a predisposition to develop autoimmune diseases[8,9] and children born during fall or winter suffer more frequently from allergic diseases[10,11,12]; others have obtained different results connecting the prevalence of certain diseases and season of birth[13]. For this reason, further studies are needed to confirm the relationship between cytokine release patterns, season of birth and immune-related diseases during early life.

Figure S3. Cytokine response in unstimulated and stimulated cord blood cells according to season. Association between cytokine response and season of birth.



Graphs show results derived from associations between production of (**A**) inflammatory, (**B**) Th1-related, (**C**) Th17-related and (**D**) Th2-related and immunomodulatory cytokines. A code of colours has been used depending on the type of stimulus used to culture the cells: unstimulated cells (black), cells stimulated with mitogens (yellow), cells incubated with pathogen associated molecular patterns (red) and cells cultured with common allergens extracts (green). The seasons of birth have been categorized into warm (March, April, May, June, July, August) and cold (September, October, November, December, January, February) months. Coefficients are derived from a multivariate logistic regression comparing high (>60th percentile cytokines concentrations) with low (<40th percentile cytokines concentrations) responder samples. Low responders born in cold months were used as the reference group. Models were adjusted by maternal age, pre-pregnancy body mass index, maternal atopy, gestational age and birthweight. Diamonds represent coefficients and horizontal bar with whiskers represent 95% confidence intervals.

Conclusions

In conclusion, cytokine profile at birth in the NELA cohort was characterized by a high production of inflammatory cytokines, moderate production of specific Th1, Th2 and Tr cytokines and low levels of Th17 cytokines. Furthermore, our results showed how sex and season of birth impact cytokine response to a wide panel of stimuli in cord blood cells at birth. Male newborns showed higher response of inflammatory and Th2 (IL-13) related cytokines, but lower response of Th1 (IL-2) related cytokines as compared to females; and births in warm seasons were associated with lower inflammatory and higher Th2 cytokine levels as compared to those in warm seasons.

To conclude, sex and season of birth are two factors that might skew the Th1/Th2/Th17 balance, affecting the response to microbes and allergens during the first years of life and, therefore, potentially modifying the risk of respiratory infections or allergy manifestations during childhood.

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APPENDIX 2:

Traffic-related air pollution *in utero* modifies cytokine responses to stimuli of umbilical cord blood cells: The NELA cohort

Background

Prenatal exposure to outdoor air pollution may disturb immune system responses to stimuli in the offspring and program the occurrence of non-communicable diseases later in life; however, studies are limited. We aimed to examine the associations between *in utero* exposure to traffic-related air pollutants (TRAP) and cytokine responses to stimuli in newborns.

Methods

Data come from 235 mother-offspring pairs of the NELA cohort. Inflammatory related cytokines (IL-6, IFN- α, IL1-β and TNF-α), Th1-related (IFN-γ and IL-2), Th2-related (IL-4, IL-5 and IL-13), Th17-related (IL-17 and IL-23) and Treg-related IL-10 were assessed by Luminex technology in umbilical cord blood of newborns cultured for 7 days with mitogens, pathogen associated molecular patterns (PAMPs) stimuli and common environmental allergens. Dispersion/chemical transport modelling was used to estimate long-term (whole pregnancy and trimesters) and short-term (15-days before delivery) residential exposures to traffic-related nitrogen dioxide (NO_2), particulate matter ($PM_{2.5}$ and PM_{10}) and ozone (O_3). Multivariable linear regression models were fitted.

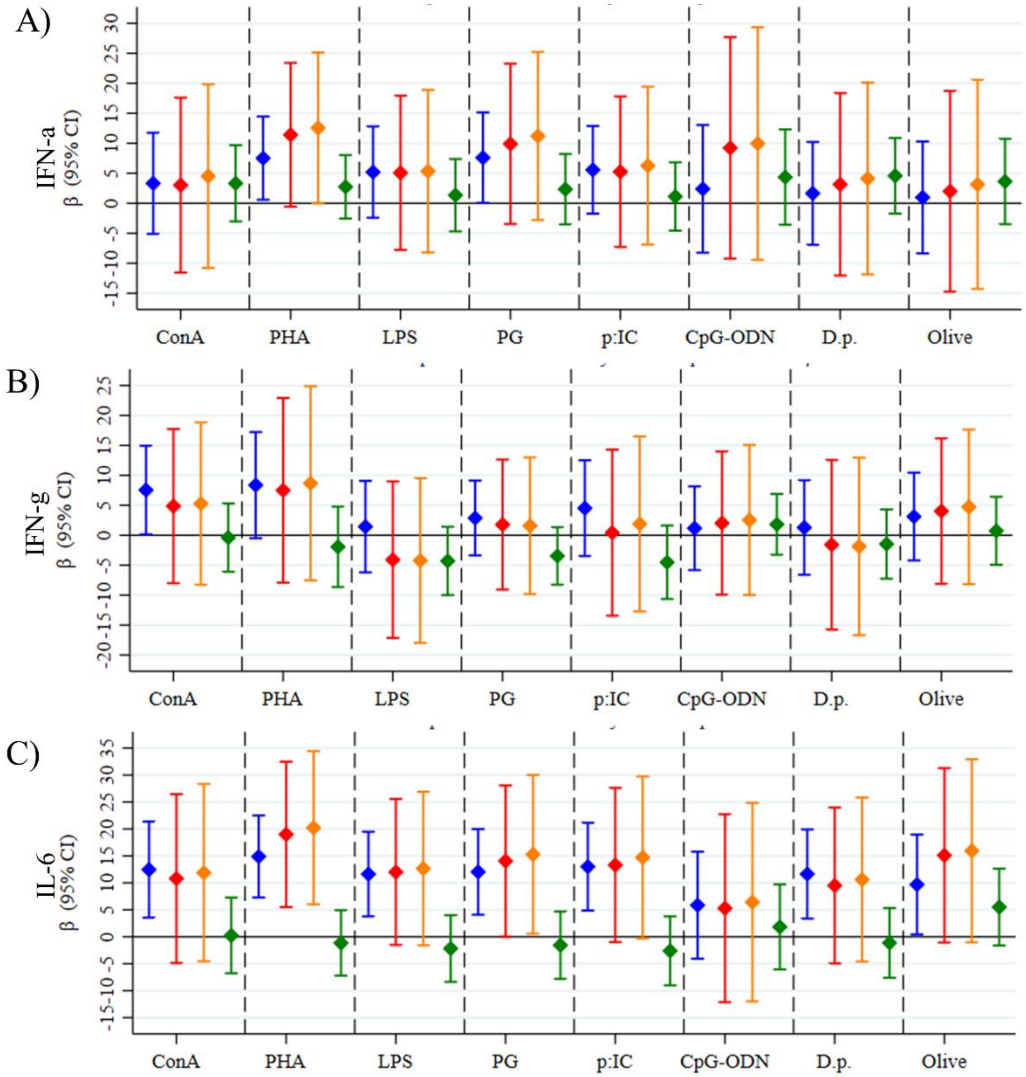
Results

Figure 1 summarizes the main associations found between prenatal exposure to TRAP and cytokine response to immunogenic an allergic stimulus. For every 10μg/m^3 increase in prenatal exposure to NO_2, we found an increased IL-6 response to mitogens Concanavalin A (12.5%, 95% CI: 3.6, 21.4) and Phytohemagglutinin (PHA) (14.9%, 95% CI: 7.3, 22.5); to PAMPs Lipopolysaccharide (LPS) (11.6%, 95% CI: 3.8, 19.5), Peptidoglycan (PG) (12.0%, 95% CI: 4.1, 20.0) and p:IC (13.0%, 95% CI: 4.9, 21.2); and to allergens D.p (11.6%, 95% CI: 3.4, 19.9) and olive extract (9.7%, 95% CI: 0.4, 19.0). IL-6 response to PHA also increased in relation to PM (19.0% per 5μg/m^3 increase in $PM_{2.5}$, 95% CI: 5.5, 32.5; and 20.2% per 10μg/m^3 increase in PM_{10}, 95% CI: 6.0, 34.4). Per 10μg/m^3 increase in NO_2, IFN-α responses to PHA and PG increased by 7.5% (95% CI: 0.6, 14.5) and 7.6% (95% CI: 0.1, 15.1), respectively. In utero NO_2 also induced an increased Th1-related IFN-γ response to Concanavalin A (7.5% per 10μg/m^3 increase in NO_2, 95% CI: 0.1, 14.9).

Conclusion

Prenatal exposure to TRAP may promote higher proinflammatory and Th1-related cytokine responses to stimuli in the offspring.

Figure 1. Associations between prenatal exposure to traffic-related air pollution and cytokine response stimulated cord blood cells.



Graphs show results derived from associations between prenatal exposure to traffic-related NO_2 (blue), $PM_{2.5}$ (red), PM_{10} (yellow) and O_3 (green) and inflammatory cytokines response. Coefficients derived from linear regression represent percentage of change in concentrations. Cytokine concentrations were log transformed and values below detection limits were imputed. All models were adjusted for maternal pre-pregnancy BMI, parental social class, maternal atopy, smoking father, season of birth, newborn's sex, gestational age and birthweight. Diamonds represent coefficients and vertical bar with whiskers represent 95% confidence intervals.

APPENDIX 3:

Pregnancy exposure to outdoor air pollution impacts miRNA expression in cord blood at birth: the NELA cohort study

Background

Gestation is a sensitive period of foetal development to maternal exposures that could impact health in life. Air pollutants can be translocated through the placenta toward the foetus[1] inducing adverse health effects in newborns, promoting oxidative stress and inflammation[2] and increasing the risk of respiratory childhood diseases[3,4]. Among the biological mechanisms suggested that could be triggered by air pollutions, changes in microRNA (miRNA) expression could have a crucial role[5].

Micro-RNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. Exposure to PM and traffic-related air pollutants has been associated with miRNA expression changes in the blood[5,6]. However, evidence is scarce about the relationship between prenatal exposure to outdoor air pollution and miRNA patterns in cord blood. To our knowledge, only one study[7] has examined the relationship between exposure to $PM_{2.5}$ during gestation and the expression of members of the miR-17/92 cluster in cord blood, finding a down-regulation of miR-17 and miR-20a expression in neonates exposed to $PM_{2.5}$ during the entire pregnancy and showing more substantial effects in the first and third trimester of pregnancy.

Therefore, this study aimed to investigate the effects of exposure to outdoor air pollution during gestation on miRNA expression in the cord blood of newborns and identify critical windows of higher vulnerability.

Methods

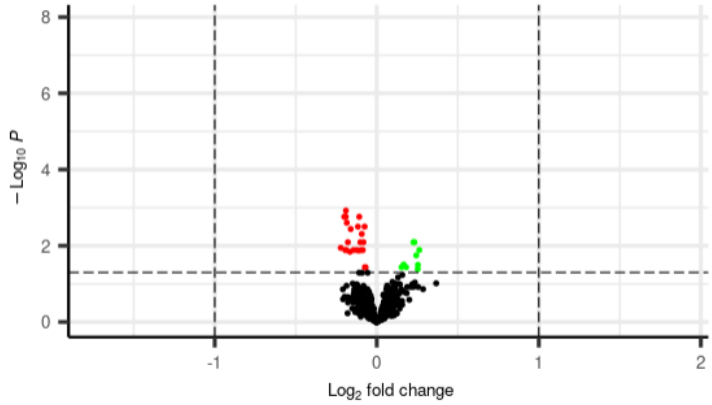
Cord blood RNA samples (n=362) from the NELA cohort study were collected at birth, and miRNAs were hybridized and analysed using SurePrint miRNA microarray. The corresponding data quality assessment, pre-processing, and normalizations were performed using the *AgiMicroRNA* (v2.38.0) R package[8,9] in R version 4.0.3. Residential outdoor air pollution exposure was estimated across pregnancy using the dispersion/chemical transport modelling described previously (**Methodology section**). Exposures showed high correlation coefficients and seasonal distribution, so a cluster analysis was performed to reduce dimensionality and avoid multi-collinearity. Associations between groups of exposure and miRNA expression in cord blood at birth were studied using multivariate linear regression models and over-representation analysis. Air pollutant mixture analyses were performed using the Bayesian kernel machine regression model, to assess mixture effects and the relative contribution of each pollutant, and the Lagged kernel machine regression, to identify windows of vulnerability.

Results

We found that miRNA variance was mainly associated with O_3 and PM_{10} exposures during the first and third trimester of gestation. Afterwards, to simplify the miRNA analysis and avoid disguising pollutants' effects periods, subjects were categorized into two groups **(Figure**

S2A) according to the relationship between season of birth, pollutants concentrations, and miRNA variance. Group1 (G1, n=191) was exposed to lower PM and O_3 outdoor concentrations in the first trimester of pregnancy and higher PM and O_3 outdoor concentrations during the third trimester of gestation (**Figures S2 B-C**); while the other group of neonates (G2, n=171) was exposed to higher PM_{10} and O_3 concentrations during the first trimester but low concentrations during the third trimester of pregnancy. Thus, combined exposures to O_3 and PM_{10} during the first and third trimester of pregnancy showed 32 miRNAs differentially regulated in cord blood at birth (Table 2). Enrichment analysis revealed adipocytokine signalling pathway, Gap junction and growth hormone synthesis, secretion, and action pathways under-represented in relation to prenatal air pollution.

Figure S2. Differential expression of microRNAs in newborns exposed to high O_3 and PM_{10} levels during first trimester of gestation compared to those exposed to high levels during third trimester of gestation.



Volcano plot including adjusted p-values derived from multivariate lineal regression adjusted by sex, gestational age and season of birth. Dash horizontal line represents p-value cut-off adjusted p (FDR=0.05). FDR values above cut-off and with positive coefficients were represented as green circles; FDR above cut-off and with negative coefficients were represented as red circles; and FDR below the cut-off were coloured in black.

Mixture analysis estimated that maternal exposure to O_3 changed hsa-miR-30a-5p and hsa-miR-30c-5p expression, in the second and third trimester of pregnancy, respectively; whereas $PM_{2.5}$ exposure during first and third trimester of pregnancy was associated with changes in hsa-miR-425-3p.

Conclusions

In conclusion, this study shows for the first time how outdoor air pollution mixture exposure during pregnancy impacts the expression of miRNA in cord blood at birth. Furthermore, miRNAs analysed showed different windows of susceptibility, depending on the air pollutant that leads the effects.

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APPENDIX 4:

This appendix includes communications in scientific meetings.

- **“How does traffic-related air pollution influence on immune system development in utero? The NELA cohort study”.** ISEE Young 2021. ISEE Europe and the Swiss Tropical and Public Health Institute (Swiss TPH). 2021. Switzerland. Oral communication.
- **[La Exposición A Contaminación Del Aire Modifica La Distribución De Las Células Inmunitarias En Sangre De Cordón: La Cohorte NELA]**. V Jornadas Científicas del IMIB-Arrixaca. Instituto Murciano de Investigación Biosanitaria. 2020. Spain. Poster
- **[Niveles De Detección De Citoquinas En Sangre De Cordón Al Nacer Y Exposición Prenatal A Contaminantes Del Aire: Estudio NELA]**. V Jornadas Científicas del IMIB-Arrixaca. Instituto Murciano de Investigación Biosanitaria. 2020. España. Awarded oral communication.
- **“Influence of residential exposure to traffic-related fine particulate matter on umbilical cord blood cytokine levels: a birth cohort study”** ISEE 2019 - 31st Annual Conference Of The International Society Of Environmental Epidemiology. Utrecht, The Netherlands 2019. Poster
- **"Neonatal cytokine profiles in the NELA cohort study: production of cytokines by umbilical cord blood cells incubated with mitogens, pathogen-associated molecular patterns (PAMPS) and allergenic extracts".** III Jornadas Científicas del IMIB-Arrixaca. Murcia, Region of Murcia, Spain, November 2018.

APPENDIX 5:

RESUMEN EXTENDIDO EN CASTELLANO

Presentación y unidad científica

Esta tesis doctoral se presenta como un compendio de publicaciones y opta a la mención internacional de acuerdo a las recomendaciones de los estudios oficiales de la Universidad de Murcia y con la aprobación de la directora y el tutor de la misma, el Comité Académico del programa de doctorado en Ciencias de la Salud y de la Comisión General de Doctorado. Así, el presente documento consta de tres estudios científicos (García-Serna et al, 2020; García-Serna et al, 2021; y García-Serna et al, 2022) publicados en revistas internacionales indexadas en el *Journal Citation Reports* (JCR).

La estructura del documento se presenta con una introducción al inicio, donde se muestran los antecedentes, los estudios previos relacionados con los objetivos de la tesis y que justifica la unidad científica de la misma en el campo de los estudios de cohortes y la epidemiología ambiental. A continuación, se muestran las hipótesis y los objetivos de esta investigación, seguidos de los métodos empleados y las conclusiones finales. Por último, se incluye un apartado de discusión e implicaciones en Salud Pública, que pone de manifiesto la importancia de los resultados obtenidos; y un apéndice que contiene estudios derivados de esta tesis doctoral, que están en proceso de ser publicados.

Introducción

En los años 80, Barker postuló que la exposición prenatal a distintos factores podía afectar al desarrollo fetal y, finalmente, podría traducirse en un mayor riesgo de desarrollar enfermedades en la infancia y a lo largo de la vida. Esta hipótesis la conocemos hoy cómo *Los orígenes de salud y enfermedad durante el desarrollo* (DOHaD). Diversos estudios epidemiológicos han apoyado esta hipótesis y se han identificado factores prenatales tales como nutricionales, socioeconómicos y medioambientales asociados a eventos adversos en edades tempranas.

La contaminación atmosférica es un problema mundial de salud pública. En 2016, nueve de cada diez personas respiraban aire que excedía las recomendaciones de la Organización Mundial de la Salud (OMS) de 2005, resultando en 4,2 millones de muertes prematuras en el mundo. Los principales contaminantes atmosféricos son el dióxido de nitrógeno (NO_2), el material particulado (PM), que puede ser clasificado según su diámetro aerodinámico en PM con diámetro aerodinámico entre 10 y 5 micrómetros (PM_{10}) y PM con diámetro aerodinámico menor que 2,5 micrómetros ($PM_{2.5}$), y el ozono (O_3). Las emisiones de tráfico de vehículos representan un gran porcentaje de las contribuciones antropométricas de las concentraciones de NO_2 y PM en la Unión Europea. Entre los efectos adversos en nuestra salud que tiene la exposición a estos contaminantes, encontramos el incremento del riesgo de enfermedades respiratorias en la infancia y procesos inflamatorios.

Las exposiciones maternas a contaminación atmosférica durante el embarazo pueden influir en la programación fetal desencadenando eventos adversos asociados al nacimiento, como nacimientos prematuros y bajo peso, y contribuir al aumento del riesgo de eventos respiratorios adversos, disminución de la función pulmonar y un aumento en el riesgo de asma en la infancia y en la vida adulta. Las evidencias acerca de los mecanismos biológicos responsables de estos efectos adversos han incrementado en los últimos años y se han propuesto diversas vías: estrés oxidativo, modificaciones epigenéticas y cambios en el sistema inmunitario.

El sistema inmunitario es una red compleja de órganos, células y componentes moleculares cuya función es proteger al organismo de agentes y moléculas que reconoce como extraños y perjudiciales. De acuerdo con la respuesta inmune, se pueden distinguir dos mecanismos: la respuesta innata, que constituye la primera línea de defensa y está caracterizada por ser un mecanismo de respuesta rápido e inespecífico, con alta presencia de leucocitos mieloides (granulocitos, monocitos, células dendríticas, etc…); y la respuesta adaptativa, que sólo se encuentra en los animales vertebrados, es específica de cada patógeno y está caracterizada por la participación de las células T y B y por la producción de anticuerpos.

En el desarrollo y maduración del sistema inmunitario se han identificado cinco periodos principales de maduración que podrían representar ventanas críticas de vulnerabilidad a compuestos inmunotóxicos: (1) inicio de la hematopoyesis (de la semana 8 a la 10 de embarazo); (2) migración de las células madre y expansión de las células progenitoras (semana 10-16 de embarazo); (3) colonización de la médula marrón y el timo (de la semana 16 de embarazo al nacimiento); (4) maduración de la competencia inmunológica (primeros años de vida); y (5) establecimiento de la memoria inmunológica (desde los primeros años de vida hasta los 18). Sin embargo, la inmunotoxicidad durante el desarrollo también podría depender de eventos inmunológicos específicos como la diferenciación y colonización de macrófagos en diferentes tejidos (semanas 6-24 de gestación) o la selección negativa y apoptosis de timocitos autorreactivos (semanas 15-26 de gestación), entre otros.

En estudios previos se observó que la exposición a NO_2 en los últimos días del embarazo estaba relacionada con una disminución de leucocitos en recién nacidos; mientras que niños recién nacidos cuyas madres estuvieron expuestas a NO_2 durante todo el embarazo mostraron un incremento en porcentajes de células T citotóxicas (Tc) y células NK. Sin embargo, los resultados sobre los efectos de la exposición a PM durante el embarazo en el sistema inmunitario son inconsistentes. Primero, se observó que los recién nacidos cuyas madres habían vivido en áreas altamente contaminadas con PM, mostraban un descenso de porcentajes de células T y un aumento de células NK en sangre de cordón. Sin embargo, otros estudios han sugerido que los cambios en células T y NK en el momento del nacimiento podrían depender del período de la exposición durante el embarazo y el contaminante específico. Por ejemplo, la exposición a $PM_{2.5}$ durante el primer trimestre de embarazo se asoció con un aumento de células T y un descenso en los porcentajes de células NK, mientras que la exposición a $PM_{2.5}$ durante el tercer trimestre de embarazo se relacionó una disminución de células T y un aumento de células NK. Por último, no existen estudios que hayan evaluado el impacto de la exposición prenatal a ozono, un potente oxidante, en la distribución de células inmunes en sangre de cordón tras el nacimiento.

Con relación a los perfiles de citoquinas, el impacto de la exposición prenatal a contaminación atmosférica en la producción de citoquinas no está claro. Por un lado, se observó que los recién nacidos de madres que estuvieron expuestas a niveles altos de NO_2 durante el embarazo mostraban un aumento de marcadores relacionados con inflamación en sangre de cordón. En este sentido, la exposición a ozono durante el embarazo también se asoció con un aumento de IL-6 en sangre de cordón. Sin embargo, otros estudios mostraron que la exposición prenatal a $PM_{2.5}$ durante los últimos días del embarazo y todo el embarazo se relacionó con una disminución de citoquinas inflamatorias (IL-6 y TNF-a) y de IL-10.

En definitiva, los estudios previos sobre la asociación de la exposición prenatal a contaminación atmosférica y el sistema inmunitario en el momento del nacimiento están caracterizados por una gran heterogeneidad en el método de exposición, los periodos de gestación examinados y los parámetros inmunológicos estudiados. Además, los efectos específicos de la contaminación derivada del tráfico han sido poco estudiados. Por todo ello, en este trabajo se ha llevado a cabo un estudio más detallado de los efectos de la contaminación atmosférica relacionada con el tráfico en el sistema inmunitario al nacimiento, caracterizando un amplio panel de células inmunitarias y citoquinas medidas en sangre venosa de cordón umbilical de recién nacidos.

Hipótesis

1. Los factores ambientales que actúan durante el periodo prenatal y perinatal podrían inducir cambios en el sistema inmunitario en desarrollo, generando cambios en las subpoblaciones celulares del sistema inmunitario y en los perfiles de citoquinas en sangre de cordón umbilical en el momento del nacimiento.
2. Las exposiciones prenatales a contaminación ambiental derivada del tráfico alteran las subpoblaciones celulares del sistema inmunitario en recién nacidos.
3. Las exposiciones prenatales a contaminación ambiental derivada del tráfico modifican los perfiles de citoquinas en recién nacidos.
4. Las exposiciones prenatales a contaminación ambiental derivada del tráfico perturban la respuesta de citoquinas ante ciertos estímulos en recién nacidos.
5. Ciertos trimestres en el embarazo podrían ser ventanas de mayor susceptibilidad a los efectos de la contaminación atmosférica relacionada con el tráfico en células del sistema inmunitario y la producción de citoquinas en el momento del nacimiento.

Objetivos

El principal objetivo de esta tesis doctoral ha sido desvelar el impacto de la contaminación atmosférica relacionada con el tráfico durante la gestación en el sistema inmunitario al nacimiento. Los objetivos específicos han sido:

1. Revisar el conocimiento previo sobre la relación entre la contaminación atmosférica y factores prenatales y perinatales medioambientales y cambios en células del sistema inmunitario y perfiles de citoquinas en sangre de cordón umbilical al nacimiento (**Artículo 1**).

2. Caracterizar el sistema inmunitario al nacimiento, analizando las subpoblaciones celulares, los perfiles de citoquinas en ausencia y en presencia de un amplio panel de estímulos inmunogénicos (**Artículo 2**, **Artículo 3**, **Apéndice 1**).
3. Estudiar los efectos de la exposición a contaminación atmosférica relacionada con el tráfico en la distribución de las subpoblaciones celulares del sistema inmunitario en sangre de cordón umbilical al nacimiento (**Artículo 2**).
4. Examinar las asociaciones entre la exposición prenatal a contaminación atmosférica relacionada con el tráfico y la respuesta de citoquinas a estímulos en sangre de cordón umbilical de recién nacidos (**Apéndice 2**).
5. Identificar ventanas gestacionales de mayor susceptibilidad para el desarrollo del sistema inmunitario frente a la contaminación atmosférica relacionada con el tráfico durante el embarazo (**Artículo 1, Artículo 2**, **Artículo 3**, **Apéndice 2, Apéndice 3**).

Metodología

La metodología empleada en esta tesis se puede dividir en dos partes: la metodología empleada para el **Artículo 1**, que se basó en una revisión narrativa; y la metodología empleada para los **Artículos 2** y **3** que está basada en datos que provienen de la cohorte al nacimiento "Nutrition in Early Life and Asthma" (NELA).

Artículo 1

Estrategia de búsqueda y selección de estudios

El Artículo 1 consta de una revisión narrativa basada en la búsqueda en PubMed de estudios que relacionasen la exposición a factores prenatales y perinatales con el sistema inmunitario, concretamente subpoblaciones de células inmunitarias y citoquinas en sangre de cordón. Para ello, se empleó una serie de palabras clave relacionadas con el evento de interés (leucocitos, linfocitos, y citoquinas) y la exposición a distintos factores y/o moléculas ambientales. Los criterios de inclusión fueron los siguientes: **(1)** estudios científicos epidemiológicos originales llevados a cabo en humanos; **(2)** la evaluación del resultado debía incluir el fenotipado de células relacionadas con el sistema inmunitario (leucocitos y subpoblaciones de linfocitos) o perfiles de citoquinas medidos en sangre de cordón al nacimiento; **(3)** las exposiciones incluidas debían ser estación del año al nacimiento, tipo de parto, exposición a alérgenos comunes, un ambiente granjero, poseer mascotas, tabaco, contaminantes orgánicos persistentes y no persistentes, metales tóxicos y contaminantes atmosféricos durante el embarazo o al nacimiento.

Artículos 2 y 3

Diseño del estudio

El estudio NELA es una cohorte prospectiva al nacimiento de base poblacional que se inició en 2015 en Murcia (España). El principal objetivo de NELA es evaluar la relación entre la nutrición materna y la nutrición en los primeros años de vida, así como otros factores como la

exposición a contaminantes atmosféricos y disruptores endocrinos, y los orígenes del asma y la alergia.

Se invitó a participar en el estudio a 1350 mujeres embarazadas que cumplían los criterios de inclusión cuando asistían a la visita de la ecografía de las 20 semanas de gestación en la Unidad Materno-fetal del Hospital Clínico Universitario Virgen de la Arrixaca, entre marzo de 2015 y abril de 2018. Los criterios de inclusión fueron los siguientes: mujeres que pertenecían al Área de Salud I y ciertas pedanías de las Áreas de Salud VI y VII de la Región de Murcia; planear vivir en el área de estudio durante al menos 2 años; intención de dar a luz en el hospital de referencia; origen caucásico español; tener 18-45 años de edad; embarazo único; concepción no asistida; y ecografía normal de las 20 semanas de gestación (ausencia de malformaciones mayores). Los criterios de exclusión eran los siguientes: sufrir una enfermedad crónica; complicaciones en el embarazo (excluyendo preeclampsia y diabetes gestacional); y no tener intención de dar a luz en el hospital de referencia. Finalmente, se reclutaron 738 participantes (54% de participación). Los consentimientos informados se obtuvieron de los padres en el reclutamiento; y el protocolo de estudio fue revisado y aprobado por el Comité Ético del Instituto Murciano de Investigación de Murcia (IMIB-Arrixaca) de acuerdo con las recomendaciones de La Declaración de Helsinki.

Estimación de la exposición a contaminación atmosférica residencial

La exposición a contaminación atmosférica residencial durante el embarazo se estimó empleando un sistema de modelado denominado AIRNELA. Este sistema estima las concentraciones de diferentes contaminantes usando datos de dispersión y transformaciones químicas que son liberados por diferentes fuentes de emisión, y que, además, para ello tiene en cuenta la posible influencia de factores meteorológicos. Para determinar la exposición individual de cada participante, se empleó la dirección de la residencia familiar que fue geo-codificada usando el Sistema de Información Geográfica (GIS). La resolución del modelo AIRNELA es de 0.5 km en el área geográfica NELA.

Para los **artículos 2** y **3**, se estimaron las concentraciones residenciales de NO_2, $PM_{2.5}$, PM_{10}, and O_3 relativas a todas las fuentes de emisión (base) y las estimadas específicamente por el tráfico (TRAP). Además, se obtuvo la exposición prenatal durante diferentes ventanas de exposición: exposición a largo plazo (el promedio de todo el embarazo y el promedio de cada uno de los trimestres) y exposición a corto plazo (exposición durante los últimos 15 días del embarazo). La fecha de la concepción se calculó en base a la fecha de la última menstruación reportada en el reclutamiento, y se confirmó usando las estimaciones de la ecografía de las 12 semanas.

Inmunofenotipado de células de sangre de cordón umbilical

Para el estudio del sistema inmunitario se tomaron muestras de sangre venosa de cordón umbilical al nacimiento y se realizó un hemograma para obtener los contajes de células sanguíneas de la serie blanca. A continuación, se incubaron 50 µl de sangre completa con la combinación apropiada de anticuerpos conjugados a un fluorocromo durante 15 minutos en condiciones de temperatura ambiente y oscuridad. Después, se empleó un tampón para lisar los eritrocitos y fijas las células. La adquisición de las células se analizó usando un citómetro de flujo

BD FACSCanto™. Las principales poblaciones celulares se definieron en función de tamaño y granularidad y los porcentajes se calcularon de acuerdo a los fenotipos definidos por la combinación de marcadores de superficie y la bibliografía. Para el cálculo de los recuentos celulares se emplearon los porcentajes de las subpoblaciones de linfocitos y los datos obtenidos en el hemograma (WBC/µl).

Medición de perfiles de citoquinas de sangre de cordón umbilical

Por otro lado, para analizar la producción de citoquinas al nacimiento, se cultivaron muestras de sangre venosa completa de cordón umbilical diluidas en medio de cultivo, en presencia y ausencia de diversos estímulos. Los estímulos empleados se eligieron en función de la habilidad de inducir distintos tipos de respuesta inmunitaria: mitógenos (Concanavalina A (Con A) y fitohemaglutinina (PHA)), patrones moleculares asociados a patógenos (PAMPs; lipopolisacárido (LPS), peptidoglicano (PG), ácido poliinosinico-policitidilico (pI:C), desoxi-oligonucleótidos inmunoestimuladores CpG (CpG-ODN)) y extractos de alérgenos ambientales comunes incluyendo el extracto de polen de *Olea europaea* (olivo) y el extracto del ácaro del polvo *Dermatophagoides pteronyssinus* (D. p).

Tras 7 días de incubación, se recogieron los sobrenadantes y se congelaron a -80ºC. Para medir la concentración de citoquinas en los sobrenadantes recogidos se empleó el kit Human Cytokine Multiplex-Assay-Kit (ThermoFisher, Viena, Austria) y tecnología Luminex de acuerdo a las instrucciones del fabricante. Se examinó un amplio panel de citoquinas, seleccionadas por la respuesta inmunitaria más representativa con la que están asociadas: citoquinas relacionadas con la respuesta innata global (IL-6, IFN-α, IL1-β and TNF-α), Th1 (IFN-γ and IL-2), Th2 (IL-4, IL-5 and IL-13), Th17 (IL-17 and IL-23), y la citoquina inmunoreguladora IL-10. Finalmente, se emplearon estándares de las distintas citoquinas para elaborar curvas patrón, que se usaron para estimar las concentraciones de las citoquinas y establecer los límites de detección de cada una de ellas.

Análisis estadísticos

Los análisis estadísticos incluidos en esta tesis se dividen en tres tipos: la estadística descriptiva que se empleó para resumir las principales características de la población de estudio; los análisis utilizados para estudiar las asociaciones entre la exposición de interés y los contajes de células del sistema inmunitario; y la estadística aplicada al estudio de las asociaciones entre la exposición de interés y los perfiles de citoquinas.

1. <u>Análisis descriptivo</u>

Para la estadística descriptiva se aplicó el test de la Chi-cuadrado, para las variables categóricas; y el test de Kruskall-Wallis para aquellas variables continuas. La información sobre posibles variables confusoras se obtuvo en cuestionarios que se realizaron cara a cara durante el embarazo o después del parto.

2. Estudio de las asociaciones entre la exposición prenatal a contaminación atmosférica derivada del tráfico y células del sistema inmunitario

Tras evaluar la distribución de los recuentos celulares, se observó que éstos seguían una distribución de Poisson. Así, se emplearon modelos de regresión de Poisson para el análisis de las asociaciones entre la exposición prenatal a contaminación atmosférica derivada del tráfico y los recuentos celulares. Los coeficientes de la regresión se presentaron como riesgo de incidencia relativa (IRR) y su correspondiente intervalo de confianza al 95%. El IRR se puede interpretar como el porcentaje de cambio en la media de los recuentos celulares cuando se resta el IRR a la unidad. Además, los modelos se ajustaron por terceras variables, incluyendo aquellas que tuviesen asociación marginal ($p<0.1$) y modificasen el coeficiente al menos un 5%. Las variables de ajuste que se incluyeron en los modelos finales se enumeran a continuación: ser madre primeriza, el índice de masa corporal de la madre antes del embarazo, clase social de la familia, estación del año al nacimiento, tipo de parto, sexo del recién nacido, edad gestacional y peso al nacer. Además, cada trimestre se ajustó por la exposición del mismo contaminante en los otros trimestres. Por último, la linealidad en las asociaciones se evaluó de forma gráfica aplicando modelos aditivos generalizados (GAM).

3. Estudio de las asociaciones entre la exposición prenatal a contaminación atmosférica derivada del tráfico y células del sistema inmunitario

Para evaluar los efectos de la contaminación atmosférica durante todo el embarazo en la producción de citoquinas se diseñó un plan de análisis exhaustivo. En primer lugar, se observó que la producción de citoquinas en células de sangre de cordón era muy baja, por lo que se decidió categorizar la concentración de citoquinas sin estimular: "no detectable", si la concentración era menor que el límite inferior de detección; y "detectable", si la concentración de citoquinas estaba por encima del límite inferior de detección para cada una de las citoquinas testadas. A continuación, la relación entre la exposición prenatal a contaminación atmosférica derivada del tráfico y los perfiles de citoquinas sin estimular se evaluó aplicando modelos de regresión logística para cada uno de los contaminantes evaluados, y ajustando los modelos por terceras variables confusoras (el índice de masa corporal de la madre antes del embarazo, clase social de la familia, historial de atopia materna, padre fumador, la estación del año al nacimiento, sexo del recién nacido, edad gestacional y peso al nacer.). Los coeficientes se presentaron como odds ratios (OR) y su correspondiente intervalo de confianza al 95%; y los resultados se pueden interpretar como el índice de probabilidad de estar por encima del límite inferior de detección para cada citoquina por cada incremento de 10µg/m^3 de NO_2, PM_{10} y O_3, y por 5 µg/m^3 de $PM_{2.5}$.

La contaminación atmosférica es el producto de la mezcla de diversos compuestos químicos que pueden establecer interacciones y modificar los efectos de la exposición a un solo contaminante en eventos relacionados con salud. El estudio del efecto de la contaminación atmosférica como mezcla en la producción de citoquinas en células de cordón sin estimular se evaluó mediante los modelos BKMR y WQS, sólo para las principales citoquinas afectadas por la exposición prenatal a contaminación atmosférica según los modelos de regresión logística. En primer lugar, se evaluó la no-linealidad en las asociaciones entre los contaminantes y las citoquinas de interés aplicando modelos BKMR para eventos binarios, implementando una regresión de probit. La regresión de la máquina de kernel bayesiana (BKMR) es un método que

estima los efectos globales de una mezcla de contaminantes en un tiempo determinado, usando para ello cadenas de Monte Carlo Markov (MCMC), algoritmos de Metropolis-Hastings y un contador Gibbs para los parámetros restantes. Además, el BKMR permite identificar efectos no lineares y no aditivos en las asociaciones entre las exposiciones y el evento estudiado, así como posibles interacciones entre los componentes de las mezclas.

Tras comprobar que los resultados de los modelos BKMR no mostraban asociaciones no lineales ni interacciones importantes entre los contaminantes incluidos, se aplicaron modelos de regresión de suma cuantílica ponderada (WQS) para identificar la contribución individual de cada una de las exposiciones a las principales asociaciones identificadas en los modelos de regresión logística. El método WQS permite estimar los efectos generales de mezclas correlacionadas, categorizando en cuartiles las exposiciones individuales y estimando el peso de cada exposición sobre un determinado evento en salud. La mayor limitación del método WQS es que antes de aplicarlo se deben asumir los siguientes supuestos: (a) todas las exposiciones contribuyen en la misma dirección al evento estudiado, y (b) las asociaciones siguen una tendencia lineal sin interacciones entre ellas.

En los modelos WQR, los datos se dividieron en dos grupos al azar: un grupo de entrenamiento (40%) y un grupo de validación (60%); y el parámetro $\beta 1$ se forzó hacia valores positivos. En primer lugar, se aplicaron modelos WQS para cada periodo de exposición por separado para identificar los contaminantes más relevantes en cada periodo; y a continuación, se construyó un modelo final para identificar las ventanas de mayor susceptibilidad y jerarquizar las asociaciones, analizando conjuntamente todos los periodos de exposición.

Una de las limitaciones de los análisis previos se fundamenta en que la categorización de una variable continua puede conducir a la pérdida de información y una pérdida de poder estadístico. Además, la bibliografía recomienda aplicar métodos de imputación múltiple en datos biológicos con medidas que contienen valores por debajo de los límites de detección. Teniendo en cuenta lo anterior, se aplicaron métodos de imputación múltiple limitada a intervalos y con ecuaciones en cadena para tratar los valores censurados fuera de los límites de detección. Para ello, se condicionó las imputaciones a los intervalos fijados entre 0 y el límite inferior de detección, y el límite de detección superior y el doble del máximo valor encontrado para cada citoquina. A continuación, se evaluó la tendencia de las asociaciones entre la exposición prenatal a contaminación ambiental y las principales citoquinas estudiada aplicando modelos de regresión lineal con los contaminantes categorizados en cuartiles.

Los programas informáticos empleados para el análisis estadístico y la representación de los datos presentados fueron Rstudio (version 1.1.463, RStudio, Boston, Mass), Stata (version 15.1, Stata Corp, College Station, Texas, USA), y GraphPad Prism software (version 8.0.2, GraphPad Software Inc., USA).

Resultados

Los resultados se muestran según los objetivos planteados al inicio del estudio:

1. El **Artículo 1** resume la evidencia previa sobre el impacto de la estación del año, el tipo de parto, la exposición prenatal a alérgenos comunes y químico, como el tabaco, contaminantes orgánicos, metales y contaminantes atmosféricos, sobre las células del sistema inmunitario y la producción de citoquinas en sangre de cordón umbilical de recién nacidos. Se identificaron 78 estudios científicos publicados entre 1979 y 2020 que evalúan la relación entre la exposición a factores prenatales y perinatales durante el embarazo con células del sistema inmunitario y perfiles de citoquinas al nacimiento.
    a. Los nacimientos en inviernos se asociaron a un aumento de leucocitos, células NK, células Th activadas, una respuesta Th1 y Th17 predominante con aumento de citoquinas relacionadas con inflamación; mientras que los nacimientos en primavera se relacionaron con una respuesta Th2 predominante en recién nacidos.
    b. Los partos vaginales se asociaron a un incremento de leucocitos y de ciertas subpoblaciones (neutrófilos, monocitos, NK y células Treg) junto con un incremento de citoquinas relacionadas con inflamación (IL-1β, IL-6 e IL-8) y una tendencia a una respuesta aumentada de citoquinas relacionadas con Th1. Por el contrario, aquellos recién nacidos que nacieron por cesárea mostraron una disminución de leucocitos, linfocitos y células dendríticas.
    c. La exposición a un ambiente rural y animales de granja durante el embarazo se asoció a una mayor producción de citoquinas inflamatorias (IL-6 y TNF-α) y respuesta Th1 (IFN-γ), junto con una disminución de la respuesta Th2 (IL-5) y Treg (IL-10).
    d. Fumar durante el embarazo se relacionó con una disminución de leucocitos, linfocitos, y células T reguladoras. Además, se observó menor respuesta Th1 (IFN-γ) y Treg (IL-10) en sangre de cordón de niños expuestos a tabaco durante el embarazo.
    e. La exposición a contaminantes orgánicos persistentes durante el embarazo se asoció a una disminución de células dendríticas, T y NK; junto con un aumento de células B y mayor producción de IgG. Además, se observó una menor respuesta inflamatoria en recién nacidos expuestos a contaminantes orgánicos persistentes y no persistentes durante el embarazo.
    f. La exposición prenatal a metales tóxicos como el arsénico, mercurio o plomo, entre otros, se relacionó con una mayor respuesta Th2 (IL-13) pero una disminución de células Th.
    g. La exposición prenatal a contaminación atmosférica afecta las distribuciones de leucocitos y linfocitos en sangre de cordón al nacimiento. El inicio y el final del embarazo podrían ser ventanas críticas de mayor susceptibilidad del sistema inmunitario en desarrollo a contaminación atmosférica.
2. En el **Artículo 2** se caracterizaron las poblaciones celulares de los recién nacidos incluidos en el estudio NELA, se observó mayor número de células que expresaban marcadores Th2 (Th CCR4+) que células relacionadas con fenotipo Th1 (Th CXCR3+). Además, se encontró en sangre de cordón un subtipo celular que expresaba marcadores

relacionados con células Th1/Th2 (Th CXCR3+CCR4+). Sin embargo, no se detectaron células de sangre venosa de cordón umbilical que expresasen los marcadores clásicos Th2, el CD294; Th1, el Tim-3; ni Th-17, el receptor de IL-23.

   a. En el **apéndice 1** se evaluaron los perfiles de citoquinas en células sin estimular y en presencia de diversos estímulos inmunogénicos. Se observó una respuesta mayoritaria de citoquinas relacionadas con inflamación (IL-1β, IL-6, TNF-α), y niveles medios de citoquinas relacionadas con las respuestas Th1 (IFN-γ), Th2 (IL-5 e IL-13) y Treg (IL-10).

3. Los resultados del **Artículo 2** mostraron cómo la exposición prenatal a contaminación atmosférica relacionada con tráfico está relacionada con cambios en los recuentos de células del sistema inmunitario en sangre de cordón al nacimiento.
   a. La exposición prenatal a niveles de NO_2 superiores a 36.4 µg/m^3 se asoció a una disminución del 15% en los recuentos de células NK (IRR: 0.85; IC al 95%: 0.72, 0.99) en sangre de cordón. Los mayores efectos se observaron cuando la exposición se dio en el primer trimestre de embarazo (IRR: 0.77; IC al 95%: 0.57, 1.00).
   b. La exposición a concentraciones de $PM_{2.5}$ mayores de 13.3 µg/m^3 durante todo el embarazo se relacionó con un descenso del 12% de células Tc (IRR: 0.88; IC al 95%: 0.78, 0.99).
   c. La exposición prenatal a niveles altos de PM_{10} (>23.6 µg/m^3) y $PM_{2.5}$ se asoció con una disminución de los recuentos de células que co-expresaban marcadores Th1 y Th2 (Th CXCR3+CCR4+).
   d. La exposición a niveles altos de PM_{10} y $PM_{2.5}$ en el primer y tercer trimestre de gestación se asoció con un aumento de células relacionadas con fenotipo Th1 (Th CXCR3+).
   e. Se observó una disminución en los recuentos de células Treg (Th CD25+CD127-) en recién nacidos cuyas madres habían estado expuestas a altos niveles de NO_2 (IRR: 0.90; IC al 95%: 0.80, 1.01) y PM_{10} (IRR:0.88; IC al 95%: 0.77, 0.99) los últimos 15 días de embarazo.
   f. Los recién nacidos expuestos a mayores niveles de O_3 (>20.3 µg/m^3) durante todo el embarazo mostraron menores recuentos de células Th17 (Th CCR6+CD161+) al nacimiento.

4. Los resultados del **Artículo 3** mostraron que los recién nacidos cuyas madres estuvieron expuestas a altos niveles de contaminación atmosférica relacionada con tráfico presentaban cambios en la producción de citoquinas en células sin estimular de sangre de cordón umbilical al nacimiento.
   a. La exposición prenatal a NO_2 durante todo el embarazo se asoció a un incremento en la detección de las citoquinas proinflamatorias IL1β (OR por un aumento de 10 µg/m^3=1.37; IC al 95%: 1.02, 1.85) e IL-6 (OR por un aumento de 10 µg/m^3=1.32; IC al 95%: 1.00, 1.75).
   b. La exposición a ozono se relacionó con un aumento en la detección de IL-13 (OR por un aumento de 10 µg/m^3=1.22; IC al 95%: 1.01, 1.49), una citoquina relacionada con la respuesta Th2.

c. Se encontraron mayores índices de probabilidad de detección de IL-10 en sangre de cordón de recién nacidos con relación a las exposiciones prenatal a NO_2 (OR por un aumento de 10 $\mu g/m^3$ = 1.30; IC al 95% 0.99, 1.69), $PM_{2.5}$ (OR por un aumento de 5 $\mu g/m^3$ = 1.56; IC al 95% 0.97, 2.51), y PM_{10} (OR por un aumento de 10 $\mu g/m^3$ = 1.49; IC al 95% 0.95, 2.33) relacionadas con el tráfico.

5. En el **apéndice 2** se evaluaron los efectos de la exposición prenatal a contaminación atmosférica relacionada con el tráfico y la respuesta de citoquinas a estímulos inmunogénicos al nacimiento, encontrando una respuesta aumentada de citoquinas relacionadas con inflamación (IFN-α IFN-γ e IL-6) en sangre de cordón de recién nacidos expuestos a mayores niveles de NO_2 durante el embarazo.
6. El primer y el tercer trimestre de embarazo se identificaron como ventanas de mayor susceptibilidad del sistema inmunitario fetal a la exposición de contaminación atmosférica derivada del tráfico.

Conclusiones

Las conclusiones derivadas de los estudios que forman parte de esta tesis doctoral se enumeran a continuación:

1. Los períodos prenatal y perinatal parecen representar ventanas cruciales en el desarrollo de mayor susceptibilidad del sistema inmunitario a efectos de diversas influencias ambientales.
2. Las células NK, T citotóxicas y T reguladoras en sangre de cordón disminuyeron en relación con una mayor exposición prenatal a contaminación atmosférica relacionada con el tráfico.
3. Mayor exposición prenatal a $PM_{2.5}$ y PM_{10} relacionada con el tráfico se asoció a un incremento del total de células Th y de células Th1 en sangre de cordón de recién nacidos.
4. La exposición prenatal a altos niveles de contaminantes atmosféricos relacionados con el tráfico se asoció con un aumento en la detección de citoquinas proinflamatorias (IL-1 β e IL-6), Th2 (IL-13), e inmunoreguladoras (IL-10) en recién nacidos.
5. La exposición a altos niveles de NO_2 y PM relacionados con tráfico *in utero* podría promover una mayor respuesta de citoquinas inflamatorias (IL-6 y IFN-α) y Th1 (IFN-γ) en respuesta a estímulos ambientales como mitógenos, patrones moleculares asociados a patógenos (PAMPs) y alérgenos comunes.
6. El inicio y el final de la gestación podrían representar ventanas de mayor susceptibilidad del sistema inmunitario fetal a efectos adversos de contaminantes atmosféricos relacionados con tráfico.
7. Las alteraciones en los recuentos de células del sistema inmunitario y los perfiles de citoquinas al nacimiento en relación con la exposición prenatal a contaminación atmosférica podrían conducir a una mayor susceptibilidad a infecciones respiratorias, asma, y manifestaciones alérgicas a lo largo de la vida.