The role of environmental/dietary mercury exposure in autoimmune response associated with T helper 17 (Th17) axis

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I confirm the word count of this thesis is less than 100,000 words

I dedicate this thesis to my Dear Parents, who supported me along my education journey

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Abstract

Autoimmunity encompasses a range of chronic inflammatory conditions resultant from inappropriate immune response against self-antigens. The exact etiology of autoimmune conditions remains elusive, with many genetic and environmental factors implicated. One of proposed environmental factor is mercury (Hg). Humans are primarily exposed to Hg predominantly through the consumtion of fish which contain methylmercury (MeHg). The impact of Hg exposure through fish consumption on autoimmunity has gained public health interest, owing to the high importance of fish in the human diet. Fish are a rich source of nutrients, especially the n-3 long chain polyunsaturated fatty acids (LCPUFA) which have immunomodulatory properties and have been proposed to mitigate against adverse effects of MeHg. Although, human studies were focused on the impact of Hg exposure on autoantibody production there is increasing interest in how Hg may influence Th17 response, an immune cell lineage implicated in autoimmune pathogenesis, notably in Systemic Lupus Erythematosus (SLE). Therefore, this PhD thesis aimed to investigate associations between Hg exposure and cytokines associated with the Th17 axis in both fish consumers and autoimmune patients with SLE. A narrative literature review was undertaken to evaluate the contribution of dietary sources to Hg/MeHg exposure. This chapter provided a comprehensive review of foods that may contain Hg/MeHg, including both plant and animal products and discussed how other nutrients present within these foods may influence the adverse effects of Hg exposure. A study of a high fish-consuming young adults from the Republic of Seychelles, found no association between MeHg exposure (hair Hg concentrations 10.21 (5.98) ppm) and Th17 response, with or without adjustment for LCPUFA status. In this study, IL-17F was negatively associated with female status in models which controlled for total n-6 and n-3 LCPUFA (β = -0.292; 95% CI: -0.532, -0.052; p = 0.017) and n-6:n-3 LCPUFA ratio (β = -0.290; 95% CI: -0.529, -0.051; p = 0.018). Also, there was an association between IL-27 and maternal socioeconomic status (β = -0.005; 95% CI: -0.010, 0.000; p = 0.044) in the model adjusted for n-6:n-3 LCPUFA ratio and IL-27 concentrations were negatively associated with smoking status in adjusted for total n-6 and n-3 LCPUFA (β = -0.157;

95% CI: -0.284, -0.029; p = 0.016) and for n-6:n-3 LPCUFA ratio (β = -0.158; 95% CI: -0.285, -0.031; p = 0.015). A separate study investigated the relationship between Hg exposure and disease activity in a cohort of SLE patients, and demonstrated that exposure to Hg, measured as inorganic Hg (iHg) in urine (median 1.99 ng/g creatinine) and as MeHg in hair (median 0.59 ppm), did not significantly influence the relationship between Th17 cytokines and clinical measures of disease activity and disease-associated damage. Furthermore, in this study IL-17E was positively associated with SLICC/ACR+ in unadjusted (β = 0.465, 95% CI: 0.432, 0.786, p = 0.005) and adjusted (β = 0.355, 95% CI: 0.050, 0.660, p = 0.023) models. Finally, the influence of fish consumption on MeHg status and IL-17A and IL-22 was determined following an 8-week long intervention, in which women of child-bearing age consumed either one or two portions of fish high in MeHg (tuna), fish low in MeHg (sardines) or a meal without fish. Whilst the intervention with tuna resulted in a significant increase in MeHg status, no significant changes in Th17 cytokines were noted between the intervention groups, thereby indicating that MeHg exposure did not alter the Th17 response. Overall, results from this thesis suggest that exposure to MeHg, through regular fish intake, does not contribute to Th17-associated autoimmunity in young adults and has no effect on the cytokines associated with Th17 response in SLE or young women of child-bearing age. These findings contribute to the evidence supporting a public health message recommending regular fish intake, as a part of well-balanced diet among general population.

Keywords

Fish consumption; autoimmunity; autoimmune disease; long chain polyunsaturated fatty acids; mercury; methylmercury; diet; SLE, Lupus; nutrition, toxicology

Abbreviations

% percentage

α alpha

β beta

γ gamma

µg micrograms

µL microlitres

AA arachidonic acid

ALA α-linolenic acid

ANA antinuclear antibody

ANoA anti-nucleolar antibodies

BILAG British Isles Lupus Assessment Group Index

BMI body mass index

CAT catalase

CI confidence interval

cm centimetres

CRP C-reactive protein

CV coefficient of variation

DHA docosahexaenoic acid

DNA deoxyribonucleic acid

ELISA enzyme Linked Immunosorbent Assay

EPA eicosapentaenoic acid

FAME fatty acid methyl esters

FAO Food and Agriculture Organization of the United Nations

fg/ml fentograms per millilitre

g grams

GC-MS Gas chromatography–mass spectrometry

GPx glutathione peroxidase

GSH glutathione

IFN interferon

IL interleukin

iHg inorganic mercury

IQR interquartile range

Kg kilograms

LA linoleic acid

LCPUFA long chain polyunsaturated fatty acids

LLOD lower limit of detection

MeHg methylmercury

mg milligrams

mg/L milligrams per litre

MSD Meso Scale Discovery

n number of samples/sample size

n-3 LCPUFA n-3 Long Chain Polyunsaturated Fatty Acids

n-6 LCPUFA n-6 Long Chain Polyunsaturated Fatty Acids

NFκB Nuclear factor kappa Beta

ng Nanograms

NICHE Nutrition Innovation Centre for Food and Health

p level of significance

pg/mL picograms per millilitre

PGE2 prostaglandin E2

ppb parts per billion

ppm parts per million

PTWI provisional tolerable weekly intake

ROS reactive oxygen species

SCDS Seychelles Child Development Study

SCFA short-chain fatty acids

SD standard deviation

SES socioeconomic status

SLAM Systemic Lupus Activity Measure

SLE systemic lupus erythematosus

SLICC/ACR+ disease associated damage was assessed using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology

SOD superoxide dismutase

SPSS Statistical Package for Social Science

TGF-β Transforming growth factor beta

Th1 T helper type 1 cells

Th2 T helper type 2 cells

Th17 T helper type 17 cells

THg total mercury

THQ total target hazard quotient

TNF Tumour necrosis factor

Treg Regulatory T cells

TXNRD thioredoxin reductase

UK United Kingdom

USA United States of America

Declarations

Note on access to contents

"I hereby declare that with effect from the date on which the thesis is deposited in Ulster University Doctoral College, I permit

the Librarian of the University to allow the thesis to be copied in whole or in part without reference to me on the understanding that such authority applies to the provision of single copies made for study purposes or for inclusion within the stock of another library.

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Statement of Collaboration

The research study within this thesis (chapter 3) is part of the wider research known as Seychelles Child Development Study (SCDS). The SCDS is a collaborative research project between the Nutrition Innovation Centre for Food and Health (NICHE), Ulster University, the Ministry for Health, Republic of Seychelles, the University of Rochester (USA), the Karolinksa Institute (Sweden) and Lund University (Sweden).

In chapter 3, the preexisting data (hair Hg status, PUFA status, information on BMI, sex, fish consumption, maternal SES) was included in statistical analyses, and pre-collected serum samples were used to measure concentrations of cytokines associated with Th17 response).

In chapter 4, the convenient sample of SLE patients was used and statistical analyses were based on the pre-collected data, which included age, sex, Hg exposure (hair Hg status, Hg concentrations in urine, number of dental amalgams) and clinical assessment (disease activity and disease-associated damage measures). In this PhD, the frozen serum samples of these patients were used to measure concentrations of Th17 cytokines.

In chapter 5, the pre-collected plasma samples of childbearing-age women, who participated in the iFish trial were used to measure concentrations of IL-17A and IL-22 cytokines. The statistical analyses were conducted on the pre-existing data, including age, BMI, hair Hg and LCPUFA status of participants.

Signed: …….

Joanna Michalina Jurek

Impact of COVID-19 pandemic

This PhD was carried out during COVID-19 pandemic (2019-2021), which has significant impact on the ability to conduct planned initially research. Consequently, certain study components were changed, including aims, study design, and data collection.

Before COVID-19 pandemic the intense training has been performed, that included the phlebotomy training (October 2018 – June 2019; certificate obtained), training in laboratory animal handling (January 2019 – December 2019) and personal animal license (obtained in March 2019).

Because of pandemic, the initially planned experimental ex vivo study designed to investigate the role of mercury chloride exposure on the cytokines release associated with Th 17 axis from isolated from blood human peripheral blood mononuclear cells have been postponed due to restrictions incorporated to prevent COVID-19 spread. These restrictions significantly influenced the ability to visit the clinics, take blood samples from patients and conduct experiment in the university laboratory. However, during PhD duration, the study protocol has been developed and ethical permissions have been granted. Study protocol with study design is outlined in full in appendix 1.

Presentations / Publications / Abstracts

This PhD work, including rationale, objectives, methodology, and initial findings have been presented in the following conferences:

- Oral presentation at The Nutrition Society Postgraduate Meeting (February 2019), Portrush Northern Ireland, UK.
- Poster and oral presentation at Annual SCDS meeting (November 2019), Victoria, Republic of Seychelles.
- The impact of mercury exposure on the relationship between Th17 cytokines and disease activity and disease-associated damage in Systemic Lupus Erythematosus, Jurek J, McSorley E, Oktapodas Feile M, Armstrong D, van Wijngaarden E, Doherty L, Crowe W, Strain S,

Allsopp P. e-poster and pre-recorded online presentation (October 2021) in LUPUS CORA 2021 Conference, Venice, Italy.

Awards and certifications:

- Associate Fellow of the Higher Education Academy, ref. PR183308, UK, 2020.
- Laureate of Royal Society of Biology Northern Ireland Outreach Champion Award, Northern Ireland, 2019.
- Personal Animal License, 2019.
- Phlebotomy certification, 2019.

Membership of professional associations, CPD:

- The Nutrition Society,
- The Ulster Immunology Group,
- American Society for Nutrition,
- The Royal Society of Biology,
- The Irish Society of Immunology.

CHAPTER 1:

General Introduction

Mercury (Hg)

Mercury (Hg) is a chemical element which occurs naturally in the environment as a result of atmospheric emissions and volcanic eruptions. Furthermore, industrial emissions through anthropogenic activity (e.g., small-scale gold mining, chloralkali use, coal and fossil fuel burning) also contribute to atmospheric Hg. Hg exists in various chemical forms, including elemental Hg (Hg^0), inorganic Hg (iHg) and organic Hg (oHg) to which humans can be exposed through the use of Hg containing products (creams, vaccinations), inhalation of Hg vapor from dental amalgams, involvement in the artisanal activities and the consumption of foods contaminated with iHg and methylmercury (MeHg), of which fish is the dominant MeHg source in the human diet (Rice *et al.,* 2014).

The progressive release of industrial Hg into the environment, contributes substantially to Hg deposition in both aquatic and terrestrial ecosystems. Environmental iHg, present in the soil and water sources, can be transformed by the methylating activity of bacteria, to MeHg, and then subsequently accumulated along the aquatic and terrestrial food chains, including fish, plant crops and livestock. Although MeHg can be found in all food sources, the MeHg concentrations in fish tends to be much higher than those in plant and animal products, which makes the consumption of marine products a primary route of Hg exposure in humans (Rice *et al.,* 2014). The incorporation of methylated Hg in the aquatic sediments is more pronounced when compared with terrestrial ecosystems, as the anoxic conditions of the marine environment facilitate transformation of iHg by sulphur-reducing bacteria into MeHg which then can be readily taken up by plankton and algae. The plankton and algae are ingested by the primary consumers of pelagic food chain, such as small fish (e.g., sardines, anchovy), which then serve as a food source for predatory fish (e.g., shark, tuna, swordfish, mackerel) at the top levels of the chain, leading to subsequent bioaccumulation and biomagnification of MeHg in the marine ecosystems (Beckers and Rinklebe, 2017). Frequent consumption of any type of fish can increase MeHg exposure as determined in hair and blood of the consumers, however, individuals who regularly consume large long-lived predatory fish, might be at greater risk of potential adverse health consequences linked with MeHg exposure (Rice *et al.,* 2014).

Although exposure to all forms of Hg can cause adverse effects on human health, the presence of MeHg in fish is of particular concern, since MeHg is the most toxic form of Hg (Rice *et al.,* 2014). MeHg, in contrast to iHg, is a lipid-soluble molecule with high affinity for thiol (SH)-containing molecules, such as cysteine (Cys) or glutathione (GSH). This facilitates its rapid distribution in the body and its accumulation in erythrocytes, brain and placenta tissues (Kershaw *et al.,* 1980; Ye *et al.,* 2016; Rand and Caito, 2019). MeHg conjugated with GSH, undergoes slow demethylation in the gut lumen, liver and peripheral tissues to iHg, which facilitate its elimination in iHg, mainly in the faeces (> 90%); and also, a small fraction of about 10% in urine (Rand and Caito, 2019) at the rate of 1% of the body burden per day (Rice *et al.,* 2014).

The health hazards linked with Hg exposure were initially discovered through the Hg poisoning incidents reported in Japan, Iraq, Iran and Tanzania. The local residents, who experienced high dietary MeHg exposure (Hg hair ranged 5.61 to 35.7 μg/g) through the ingestion of fish contaminated with MeHg (Harada, 1995) or bread made with MeHg polluted wheat grain (Bakir *et al.,* 1973), were affected by severe neurological disturbances (e.g. ataxia, dysarthria, sensory and auditory impairments). Adverse effects were also evident in later generations, including children of women who consumed the contaminated food source during pregnancy (Igata, 1993; Harada, 1995). The long-term consequences of MeHg exposure-initiated research into the health impacts of chronic, low to moderate grade Hg exposure in high fish consuming populations with a primary focus on exposure during pregnancy owing to the developing fetus being most susceptible to MeHg neurotoxicity (Choi *et al.,* 2008).

Low to moderate exposure to Hg, through the consumption of Hg/MeHg containing foods has been associated with adverse health effects, including neurological (Bakir *et al.,* 1973; Davis *et al.,* 1994; Harada, 1995; Auger *et al.,* 2005; Philibert *et al.,* 2008), cardiovascular (Salonen *et al.,* 1995; Guallar *et al.,* 2002; Virtanen *et al.,* 2005) and immune function (Silva *et al.,* 2004; Alves *et al.,* 2006; Gardner *et al.,* 2010; Nyland *et al.,*2011a; Somers *et al.,* 2015), nevertheless the severity and observed symptoms

of these effects varies amongst populations. This variation is potentially explained because of individual factors (age, sex, genetic susceptibility) and differences in the Hg food source, dose, timing and pattern of exposure as well as presence of other environmental contaminants (Karagas *et al.,* 2012).

Autoimmune disease

Autoimmunity can develop as the result of aberrant changes in the immune response, which are mistakenly directed against their own genetic material/self-antigens. Dysregulation of immune homeostasis can lead to loss of immune tolerance and subsequent development of chronic inflammation and resultant damage to tissue and organs (Martin *et al.,* 2014). There are many factors implicated in autoimmunity development. The multifactorial contribution of both genetic susceptibility and environmental exposures are of particular interest, as they appear to be a critical for the initiation and onset of autoimmune disease. For instance, genetic alterations within the genes, that have been implicated in the antigen presentation, T cell selection and immune signalling pathways, including the Major Histocompatibility Complex (MHC) genes (HLA-DR2 (DRB1*1501) and HLA-DR3 (DRB1*0301)), as well as the presence of polymorphisms in the components of T cell receptor, such as Fcγ - FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), have been shown to be associated with both susceptibility and severity of autoimmune disease (Ramos-Casals *et al.,* 2010). Similarly, polymorphisms present in the extrinsic pathway of the Fas and Fas ligand systems, have been suggested as modifying factors of autoimmune disease susceptibility, because of their involvement in the initiation of apoptosis for maintaining peripheral immune tolerance and T cell selection (Ramos-Casals *et al.,* 2010). In autoimmunity, defective cell death mechanisms (phagocytosis, apoptosis) and increased rates of the secondary necrosis (Mok and Lau, 2003; Shao and Cohen, 2011; Cojocaru *et al.,* 2012) have been associated with the release of cellular debris (e.g. double-stranded DNA (dsDNA), ribonucleoproteins, histones), which are then recognized as a source of self-antigens and can stimulate a localized immune response, thereby promoting the release of pro-inflammatory cytokines and the formation of autoantibodies directed against nuclear components.

The maintenance of immune tolerance is critical to the prevention of autoimmune responses and is regulated by both regulatory T (Treg) and T helper (Th) 17 cells. Th17 cells and Treg cells share a common signaling pathway mediated by transforming growth factor beta (TGF-β). The presence of proinflammatory cytokines interleukin (IL)-1β, IL-6 or IL-21, together with TGF-β, promote Th17 cells formation, while TGF-β alone drives differentiation into Treg cells (Lee, 2018). The main function of Treg cells is to produce anti-inflammatory cytokines IL-10 and TGF-β and to downregulate the immune response. These processes can be influenced by many factors, such as cytokine signals and metabolic activities, which can cause Th17/Treg imbalance and lead to an autoimmune response (Lee, 2018) (see figure 1). Nevertheless, chronic activation of immune responses resultant from prolonged exposure to self-antigens or aberrant antigen presentation can lead to loss of tolerance, disruption of immune homeostasis and development of autoimmune disorders (Rosenblum *et al.,* 2015).

There is growing evidence to support the role of Th17 cells and their cytokines in the development of various autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis as well as in Systemic lupus erythematosus (SLE) (Marinoni *et al.,* 2014; Abou Ghanima *et al.,* 2012). Th17 cells activity is regulated by set of the cytokines such as IL-17, IL-21, IL-22, IL-23, IL-27 (Schmidt *et al.,* 2018), which increased levels have been consistently found in the autoimmune patients (Meka *et al.,* 2015; Eyerich *et al.,* 2017; Raymond *et al.,* 2017; Yago *et al.,* 2017; Gharibi *et al.,* 2019). Briefly, IL-17 and IL-22 are main effector cytokines in Th-17 axis (Raymond *et al.,* 2017). IL-17 is a signature cytokine of Th 17 response, with main roles indicated in neutrophil recruitment, apoptosis and production of inflammatory mediators, such as IL-6, IL-8, IL-10, IL-1α, matrix metalloproteinases (MMP), nitric oxide (NO) and prostaglandins (PGE) (Khan and Ahmed, 2015). There are six known IL-17 isoforms IL-17A, IL-17B, IL-17C, IL-17E/IL-25, IL-17 F, among those IL-17A and IL-17F are produced by Th 17 cells (Tabarkiewicz *et al.,* 2015). IL-17A and IL-17F synergize with other pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1, and activate inflammatory response through NFkB, MAPKs, C/EBPs pathways, followed by production of anti-microbial peptides, cytokines, and chemokines

(Schmidt *et al.,* 2018). On the other hand, IL-22 is released in the early stages of Th-17 differentiation, in the result of innate immune response against extracellular pathogens (Li *et al.,* 2014) and environmental toxins (Ramirez *et al.,* 2010). Compared to IL-17, IL-22 exhibits mild pro-inflammatory, although with noticeable regenerative effects, which depend on tissue microenvironment. In addition, IL-22 helps to restore balance between Th17 and T-reg cell populations, increases transepithelial resistance to injury and promotes barrier function by inducing epithelial cell proliferation in the lung. IL-22 has been shown to contribute to cell injury by enhancing pro-inflammatory capacity of TNF-α on keratinocytes (Eyerich *et al.,* 2010; Tabarkiewicz *et al.,* 2015).

Furthermore, IL-21, IL-23, IL-27, IL-31 and IL-33 are cytokines involved in modulating Th17 cells responses. IL-21 is produced by CD4+ T cells, including Th-17. IL-21 promotes differentiation and enhance Th17 cell activity. In addition, IL-21 has been suggested to promote Th 17 cells formation and contribute to Th17/T-reg imbalance (Shi *et al.,* 2019). IL-23 is a key regulatory Th17 cytokine implicated in their expansion (Schmidt *et al.,* 2018) and stabilization (Li *et al.,* 2015). During injury, IL-23, is released from peripheral tissues, such as skin, gastrointestinal tract and lung, what promotes local IL-17 production, and resulting in proinflammatory response, neutrophil recruitment to the infection/injure site and loss of immune tolerance to 'self' antigens (McKenzie *et al.,* 2006). IL-27 is an immunomodulatory cytokine, which inhibit Th17 and Treg development, by suppressing production of Th 17 effector cytokines (IL-17 and IL-22), IL-27 decreases Th17 activity (Meka *et al.,* 2015), however release of danger signal, such as ATP or PGE-2 appears to suppress IL-27 actions (Schnurr *et al.,* 2005). IL-31 is a pro-inflammatory cytokine, produced in the surface tissues (keratinocytes (skin), bronchial epithelium (lung), intestinal lining) following exposure to physical and/or bacterial triggers, including reactive oxygen species (Cornelissen *et al.,* 2012). Enhanced expression of IL-31 has been implicated in autoimmune and inflammatory diseases, including atopic dermatitis, asthma, allergic rhinitis and inflammatory bowel disease (Cornelissen *et al.,* 2012; Huang *et al.,* 2016). In addition, by stimulating release of proinflammatory cytokines, such as IL-6, IL-31 could trigger inflammatory response in SLE disease. Also, IL-31 has been shown to stimulate and delay apoptosis of eosinophils,

what might result in chronic inflammation (Huang *et al.,* 2016). IL-33 is an alarmin cytokine, which is released from damaged or necrotic cells upon exposure to environmental stresses, such as allergens, pathogens or environmental contaminants (Ashley-Martin *et al.,* 2015). Compared to other cytokines of Th17 axis, IL-33 can be passively released during cell necrosis/damage accompanying (Huang *et al.,* 2016; Chan *et al.,* 2019), as well as the result of unresolved tissue damage (Liew *et al.,* 2016).

Evidence obtained from experimental research conducted on genetically predisposed animal models, demonstrated that increased concentrations of both IL-17A and IL-21 can induce lupus-like disease or rheumatoid arthritis (RA)-like symptoms (Biswas *et al.,* 2012), whereas inhibition of Th17 response mediated through IL-17, by decreasing formation of autoantibody (Doreau *et al.,* 2009), effectively suppressed the development of encephalomyelitis and multiple sclerosis (MS) (Komiyama *et al.,* 2006). These findings are consistent with evidence obtained from autoimmune patients, which demonstrate that Th17 cells, by releasing a subset of the proinflammatory cytokines IL-17, IL-22, and IL-23, can promote inflammation in autoimmune conditions, such as psoriasis, inflammatory bowel disease (IBD), RA, MS and Systemic Lupus Erythematosus (SLE) (Rother and van der Vlag, 2015).

Pathogenesis of Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE) is an example of systemic autoimmune condition, predominantly affecting women, with no specific symptoms and great inter-individual variance in disease severity. Among patients, the most common symptoms are systemic manifestations, such as fever, malaise, arthralgias, myalgias, headache, and loss of appetite and weight (Cojocaru *et al.,* 2011), nevertheless some individuals can also develop site-specific symptoms, varying from mild erythematous (malar) rash over the cheeks and nasal bridge (Ben-Menachem, 2010), arthritis, osteonecrosis and myopathy affecting small joints of the hands, wrists, and knees (Zoma, 2004). Renal and cardiovascular complications, resultant from increasing overload of immune complex depositions in the kidney, may lead to organ failure and sepsis, the two main causes of the death in SLE (Cervera *et al.,* 2003).

The clinical manifestations of SLE can vary between patients depending on the disease onset and the mechanism implicated in the disease development. Although a variety of immunological defects have been shown to contribute to SLE pathogenesis, dysregulation of immune responses followed by increased production of autoantibodies directed against cellular components, including nucleic acids (anti-double stranded DNA), and protein components (anti-SM/RNP, anti-Ro/La) (Han *et al.,* 2015) are the most common features of SLE pathogenesis. Loss of tolerance together with alerted immune signalling have been shown to promote the generation of highly reactive T and B cells (Han *et al.,* 2015), that contribute to dysregulated immune responses (Cojocaru *et al.,* 2012). Chronic inflammation presents in SLE, together with increased availability of cellular antigens, may induce Th17 differentiation and promote the release of their cytokines (Robert and Miossec, 2020). This is supported by evidence obtained from observational studies, which indicates that SLE patients have elevated concentrations of cytokines associated with Th17 response, including IL-17A (Raymond *et al.,* 2017), IL-21 (Gharibi *et al.,* 2019), IL-22 (Eyerich *et al.,* 2017), IL-23 (Yago *et al.,* 2017), IL-27 (Meka *et al.,* 2015), IL-31 (de J Guerrero-García *et al.,* 2018) and IL-33 (Zhao and Chen, 2014). In addition, IL-17A, by inducing IL-21 release, can promote Th17 response and stimulate the formation of autoantibodies in B cells (Doreau *et al.,* 2009; Ahmed *et al.,* 2010). This might be of particular importance for SLE pathology, as increased production of autoantibodies can contribute to disease severity and organ damage (Cojocaru *et al.,* 2012).

The diagnosis of SLE is based on the physical examination of organ systems (e.g. presence of rash, skin lesions, hypertension, arthritis, ulcers) and laboratory testing, which involve routine (e.g. blood cell count, creatinine, serum protein) and specific immunological tests, that include C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), components of complement cascade (C3, C4) and serological testing of antibody concentrations (ANA, anti-double-stranded DNA (dsDNA)), and immunoglobulins (Ig) levels (e.g., IgG, IgM). When the diagnosis criteria are not met, the SLE classification criteria are used to identify silent clinical features. To date, several classification criteria

have been developed; the most widely used are those based on the disease activity and disease-associated scoring systems. Nevertheless, variation among presented clinical symptoms together with imperfect sensitivity and specificity of the classification criteria often require use of a few different clinical measures of disease to systematically document and evaluate effectiveness of the treatment.

The SLE Disease Activity Index (SLEDAI) and the British Isles Lupus Assessment Group Index (BILAG 2004) are the most common methods used in the clinical setting (Thanou *et al.,* 2019). The SELENA-SLEDAI tool is a cumulative and weighted index used to assess disease activity across 24 different disease descriptors used in the monitoring and diagnosis of SLE patients. The SELENA-SLEDAI consists of a list of organ manifestations, each with a definition. The physician decides whether each manifestation is “present” or “absent” in the last 10 days. It is a weighted instrument, in which descriptors are multiplied by that organ's “weight”, and nervous system symptoms have been attributed the highest score. The weighted organ manifestations are calculated into the final score, which is a sum of all marked SLE-related descriptors. A total score can fall between 0 and 105, with a higher score representing a more significant degree of disease activity (Petri, 2007). A SELENA-SLEDAI score being above 4 indicates active disease (Gladman *et al.,* 2000) and score falling between of 5 to 12 is classified as those with stable active disease. Using SELENA-SLEDAI allows for the detection of a disease flare, which is reflected by sudden change in the SELENA-SLEDAI score of 3 points of more, but no more than 12 for mild or moderate flare; whilst a difference of 12 points or more, would indicate a severe flare (Petri *et al.,* 2005). Compared to the SELENA SLEDAI index, BILAG is a transitional index system, which through assigning a letter-based grade (A-E), assesses the contribution of each organ system (constitutional, mucocutaneous, neuropsychiatric, musculoskeletal, cardiorespiratory, gastrointestinal, ophthalmic, renal and haematological) to the disease activity in the last 4 weeks. Grade A represents very active disease, which requires high dose of immunosuppressive treatment, grade B represents moderate disease activity, that can be managed with a lower dose of corticosteroids, topical steroids, topical immunosuppressive drugs, or non-steroidal anti-inflammatory drugs, whereas grade C indicates mild-stable disease. Grade D and E implies no disease activity with E score indicating no

current or previous disease activity (Mikdashi and Nived, 2015). Similar to BILAG, the SLAM-R index also enables the assessment of the SLE disease activity within last 4 weeks, while covering symptoms that occurred during the previous month. SLAM includes 24 clinical manifestations (e.g., constitutional, integument, eye, reticuloendothelial, pulmonary, cardiovascular, gastrointestinal, neuromotor, joints), with additional 7 laboratory features (e.g., hematocrit, white blood cell count (WBC), lymphocyte count, platelet count, ESR, serum creatinine/creatinine clearance, urine sediment), and manifestations are determined as either active or not active (Liang *et al.,* 1989). The organ involvements present within the last month are assigned a score ranging between 0–3 points, with higher score indicating a higher severity (Romero-Diaz *et al.,* 2011). A score above 7 is considered clinically significant and distinguishes active disease from remission periods (Liang *et al.,* 1989; Lam and Petri, 2005; Romero-Diaz *et al.,* 2011).

The SLICC/ACR+ index is a system used to assess disease-associated damage and measures any irreversible damage that is a consequence of the disease activity and its treatment. All damage is scored from the time of diagnosis onward, regardless of the damage is attributed to SLE (Ghazali *et al.,* 2018). SLICC/ACR+ assesses the disease damage for at least 6 months with the exception of manifestations such as myocardial infarction and stroke which are recorded once they occur. Damage over time is assessed for 12 organ systems and given a unique score depending of the system (e.g. ocular (range 0–2), neuropsychiatric (0–6), renal (0–3), pulmonary (0–5), cardiovascular (0–6), peripheral vascular (0–5), gastrointestinal (0–6), musculoskeletal (0–7), skin (0–3), endocrine (diabetes) (0–1), gonadal (0–1), and malignancies (0–2)), with maximum theoretical score of 47 points (Gladman *et al.,* 1997).

Role of Hg exposure in development of autoimmune disease

The clinical heterogeneity of many autoimmune diseases, including SLE, indicates that a combination of genetic and environmental factors may influence autoimmune risk. One of proposed stimuli for

autoimmunity is the potent environmental toxicant, Hg (Hong *et al.,* 2012). Depending on the dose and form of Hg, exposure to Hg can dysregulate immune responses and through changing the cytokine profile, can disrupt immune homeostasis and promote development of autoimmune disease (Crowe *et al.,* 2017). Results obtained from *in vitro* studies have shown that exposure to either iHg or oHg can decrease lymphocyte proliferation and affect cytokine and antibody production (cytokines TNF-α, IL-1β, IL-17; antibody IgM and IgG) (Ohi *et al.,* 1976; Shenker *et al.,* 1992; Gardner *et al.,* 2010a). These effects appear to be more pronounced for iHg, than oHg, as treatment with equal iHg concentration (200 nM) increases the pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-17), whereas MeHg only increases IL-1β (Gardner *et al.,* 2010a). *In vivo* studies using animal models, also demonstrated that exposure to high doses of both iHg and MeHg can result in immune dysfunction, including increased titres of autoantibodies (Martinsson *et al.,* 2010) and immune complex depositions (Hultman *et al.,* 1992; Havarinasab *et al.,* 2007), disturbed redox homeostasis and cellular signaling (Grotto *et al.,* 2011), which can promote the spontaneous development of autoimmunity and the induction of a lupus-like condition (Pollard *et al.,* 1999) with symptoms of nephritis (Hirsch *et al.,* 1982), arthritis, cerebritis, skin rash and vasculitis (Pollard *et al.,* 1999). However, consistent with *in vitro* studies, the exposure to iHg appears to exhibit stronger immune-stimulating properties than MeHg, demonstrated by immune deposits formation and increase in IgE production (Havarinasab *et al.,* 2007).

Whilst results from experimental research support Hg role in the immune dysfunction that could lead to the development of autoimmune disease, evidence from human studies is conflicting (Crowe *et al.,* 2017). To date, results of observational studies suggests that individuals, who are occupationally exposed to various forms, mainly through gold mining activities (iHg), fish consumption (MeHg) or having dental amalgams (Hg^0), may have an increased concentration of antibodies which are detectable in circulation, including ANA (Silva *et al.,* 2004; Alves *et al.,* 2006; Gardner *et al.,* 2010b; Nyland *et al.,* 2011a; Ong *et al.,* 2014; Somers *et al.,* 2015; McSorley *et al.,* 2020) and anti-glutathione S-transferase antibody (Motts *et al.,* 2014). Individuals who were exposed to Hg-containing air pollution were observed to have a significantly higher prevalence of autoimmunity, when compared to those who

were not exposed to the vapor (Dahlgren *et al.,* 2007) which may indicate a role of Hg in inducing systemic autoimmunity (Pollard *et al.,* 2019). Research in those with autoimmune conditions, although limited by a low number of studies, suggests that Hg status is correlated with higher disease activity (Arnett *et al.,* 2001) and the development of acute autoimmune disease (Kawasaki disease) (Yeter *et al.,* 2016). Therefore, results of these studies suggests that exposure to Hg may negatively impact on immune system function and may stimulate the development of autoimmune conditions (Somers *et al.,* 2015), however the clinical consequences still need to be investigated.

The complexity of SLE pathogenesis indicate that environmental factors (Choi *et al.,* 2012; Rosenblum *et al.,* 2015) such as exposure to Hg, may be a considerable trigger for aberrant immune responses implicated in autoimmune pathology (Crowe *et al.,* 2017). Cytotoxic effects from Hg exposure may have considerable impact on SLE pathology, as impaired apoptosis has been indicated in the disease development. Consequently, Hg exposure may result in increased necrosis rate and the formation of necrotic bodies and may contribute to the pool of the intra-cellular components, such as danger signals (e.g., adenosine triphosphate (ATP), dsDNA) which act as self-antigens and promote the production of autoantibodies and immune deposit formation within organs. The inefficient clearance of cellular debris, owing to defective apoptosis, may also trigger the release of potent proinflammatory mediators, including alarmin cytokines (IL-33) (Li *et al.,* 2014) that can promote Th17 response and the production of IL-17/IL-22, which in turn can increase inflammation and finally exacerbate disease severity.

Hg like other toxicants, can directly stimulate immune responses through binding to immune cell receptors, such as aryl hydrocarbon receptor (AhR), which are found in many immune cells, including the Th17 and Treg subsets (Stockinger *et al.,* 2011). Results obtained from both *in vivo* and *ex vivo* studies, indicate that exposure to low Hg^{2+} concentrations can induce a robust Th17 response and is associated with increased concentrations of IL-17 in individuals who were occupationally exposed to Hg (Hemdan *et al.,* 2013). Furthermore, Hg^{2+} ions, through the AhR, may also influence the balance between Th17 and Treg cell responses, essential in preserving immune tolerance. iHg by binding to

AhR, can promote Th17 differentiation followed by increased secretion of their potent proinflammatory cytokines, including IL-6 (DiNatale *et al.,* 2010) IL-17, and IL-22 (Ramirez *et al.,* 2010). An increased activity of Th17 cells arising from IL-17, IL-21 and IL-22 actions (Hoe *et al.,* 2017) may impair immune tolerance and promote immunopathology, through increased oxidative stress and reduced activity of antioxidant enzymes (e.g., catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH)). Increased formation of free radicals and nitrogen species, together with compromised antioxidant defenses, have been implicated in promoting inflammation and damage in autoimmunity (Khan *et al.,* 2015), as excessive IL-17 has been shown to initiate tissue inflammation by stimulating proinflammatory cytokines and matrix metalloproteinases (Konya *et al.,* 2015; Ruiz de Morales *et al.,* 2019; McGinley *et al.,* 2020).

Although, these findings indicate that Hg exposure may promote the pro-inflammatory phenotype of the disease (Hedrich *et al.,* 2012), an observational study conducted in the cohort of SLE patients reported no associations between Hg exposure, determined either in hair (biomarker of MeHg exposure) and urine (biomarker of iHg) and measures of disease activity and disease-associated damage, thereby suggesting that Hg exposure has no impact on SLE severity (Crowe *et al.,* 2015). Interestingly, this study found an inverse association between hair MeHg and SLICC/ACR+ score, a measure of cumulative damage, which suggests that higher hair MeHg has a protective effect against disease-damage in SLE. The authors proposed that the dietary intake of the anti-inflammatory n-3 LCPUFA, known to be beneficial in autoimmune conditions and also present in fish, may explain this finding (Crowe *et al.,* 2015).

Impact of fish-derived nutrients on MeHg exposure and autoimmunity

Fish is one of the richest sources of n-3 LCPUFA (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) (Oken, 2015), which have been proposed to mitigate against potential adverse effects of MeHg toxicity (Strain *et al.,* 2008; Nøstbakken *et al.,* 2012; Crowe *et al.,* 2018; McSorley *et al.,* 2018;

McSorley *et al.,* 2020). The immunomodulatory effects of marine n-3 LCPUFA may explain why some observational studies conducted among regular fish consumers, who consume at least 2 portions (140g) of fish a week (Mozaffarian and Rimm, 2006), including general population of Long Island, NY in US (Monastero *et al.,* 2017), as well as cohort of pregnant women from Brazilian Amazon (Nyland *et al.,* 2011b), found no relationship between MeHg exposure and autoimmunity biomarkers Nyland *et al.,* 2011b; Monastero *et al.,* 2017).

A higher dietary intake of the n-3 LCPUFA, DHA and EPA leads to their incorporation within the cell membrane with the displacement of n-6 LCPUFA, arachidonic acid (AA). This can decrease the production of pro-inflammatory AA-derived metabolites (e.g. PGE2 and LTB4), while increasing the formation of less pro-inflammatory eicosanoids and a series of specialized pro-resolving mediators (e.g. resolvins, lipoxins, protectins, maresins, and lipoxins) from the n-3 LCPUFA, that are responsible for preventing an indefinite prolongation of a localised immune response (Calder, 2015; Lee, 2018; Li *et al.,* 2019). This suggest that n-3 LCPUFA may protect against a dysregulated immune response induced by autoimmune conditions and/or exposure to environmental toxicants. Experimental studies have shown pro-resolving mediators, in particular resolvin D1, resolvin D2 and maresin 1, by direct binding to the fatty acid receptor GPR32, may prevent differentiation of naïve T-cells into Th1 and Th17 cells (Mendivil, 2021). This can be of particular importance to those with autoimmune disease, as consumption of oily fish and its provision of significant amounts of dietary EPA and DHA (Tacon and Metian, 2013), may help in the prevalence and management of autoimmune and inflammatory conditions (Swanson *et al.,* 2012; Li *et al.,* 2019). Consequently, increasing n-3 LCPUFA intakes through fish consumption and/or supplementation with marine oils, has been shown to reduce disease activity and improve patients-reported outcomes, including sleep quality and prevalence of comorbid fibromyalgia in SLE (Charoenwoodhipong *et al.,* 2020). Similarly, supplementation with n-3 LCPUFA from fish oil can significantly decrease disease activity score and help with management of disease symptoms (Duffy *et al.,* 2004).

Nevertheless, when assessing the impact of fish intake on health outcomes, other factors like cooking and processing methods should be also considered, as there is evidence which suggests that different culinary practices may impact on the nutritional value of fish. For example, using extreme temperatures during frying/deep frying, especially with the addition of vegetable oils may decrease n-3 LCPUFA in the fish filets (Weber *et al.,* 2008; Gladyshev *et al.,* 2014; Karimian-Khosroshahi *et al.,* 2016), while 'concentrating' MeHg content due to water loss (Perelló *et al.,* 2008; Marmelo *et al.,* 2020). In contrast, using salt (James *et al.,* 2005) or citric acid (Hajeb and Jinap, 2009) solutions, as well as using grilling or boiling to cook fish, have shown opposite effects (He and Wang, 2011).

Health implications

A diet including regular fish intake is encouraged by current dietary guidelines (EFSA, 2014) worldwide, given that fish is an important source of high-quality nutrients, including protein, n-3 LCPUFA, vitamins and minerals, that support health across the lifecycle. Fish intake is also associated with increased MeHg status among the consumers, which raises health concerns, especially for pregnant women and children, as they are the most vulnerable to neurotoxic effects of MeHg exposure owing to its ability to cross the placenta and also the blood-brain barrier. There is accumulating evidence suggesting that MeHg exposure may also contribute to autoimmune dysfunction in genetically predisposed individuals, such as SLE patients, owing to the ability of Hg to perturb immune homeostasis and promote pro-inflammatory Th17 response. Nevertheless, investigations into the impact of MeHg exposure on autoimmune markers and clinical features of autoimmunity in regular fish consumers are limited to date. The limited evidence from previous studies to support a role for Hg exposure through fish intake in autoimmunity may be due to the mitigating potential of fish-derived nutrients, especially n-3 LCPUFA, as it has been shown that the immunomodulatory effects of EPA and DHA, through balancing between Treg and Th17 response, may help to restore tolerogenic immune profile and manage symptoms of autoimmune conditions.

Thesis Aims

The overall aim of this thesis was to examine associations between dietary Hg exposure and cytokines associated with Th17 axis in high fish-consuming populations and patients with SLE.

Schematic representation of study hypothesis is presented on the figure 2. Briefly, the PhD hypothesis is built on the evidence obtained from high fish consuming populations, which have shown associations between exposure to mercury (Hg)/methylmercury (MeHg) through fish intake and increased concentrations of pro-inflammatory cytokines, including Interleukin (IL)-17, IL-6, IL-1β, Tumour necrosis factor (TNF)-α, Interferon (INF)-γ (Nyland *et al.,* 2011a), as well as increased titres of anti-nuclear antibodies (ANA) and anti-nucleolar antibodies (ANoA) (McSorley *et al.,* 2020). In addition, further experimental studies demonstrated that exposure to MeHg can increase release of radioactive oxygen species (ROS), thus contribute to oxidative damage, ultimately leading to necrosis. Upon necrosis, intracellular components, including self-antigens and alarmins, such as IL-33 are released, what additionally stimulate proinflammatory response (Pollard *et al.,* 2019). Establishment of proinflammatory environment may induce formation of T helper (Th) 17 cells in expense of T regulatory (reg) cell subsets, and therefore promote autoimmune response, followed by increased production of effectors Th17 cytokines (IL-17 and IL-22) (Bjørklund *et al.,* 2020). Nevertheless, fish is also a rich source of health-promoting nutrients, such as n-3 long chain polyunsaturated fatty acids (LCPUFA), in particularly eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), which immunomodulatory properties may help to restore immune homeostasis and by producing anti-inflammatory EPA and DHA-derivatives (pro-resolving mediators) and modulating T cell differentiation (increase Treg formation and decrease Th17 activity) might counteract MeHg-induced autoimmune/pro-inflammatory response (Li *et al.,* 2019).

Thesis Objectives

1. Conduct a narrative literature review assessing the contribution of dietary sources of Hg/MeHg, while assessing the potential of their nutritional components to mitigate adverse effects of Hg exposure, in combination, as part of the meal in emphasis on age groups. (**Chapter 2**)

2. Investigate associations between Hg exposure through fish consumption on concentrations of cytokines associated with the Th17 axis, with and without adjusting for LCPUFA status, in a cohort of high fish consuming young adults. (**Chapter 3**)

3. Investigate the impact of environmental Hg exposures on the relationship between cytokines associated with Th17 axis and disease outcomes in Systemic Lupus Erythematosus. (**Chapter 4**)

4. To investigate the effect of how consumption low Hg fish (sardines) or high Hg fish (tuna) after 8 weeks may influence the MeHg status and concentrations of Th17 cytokines in women of child-bearing age. (**Chapter 5**)

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Figure 1. Role of Th17 axis in the autoimmunity and autoimmune disease.

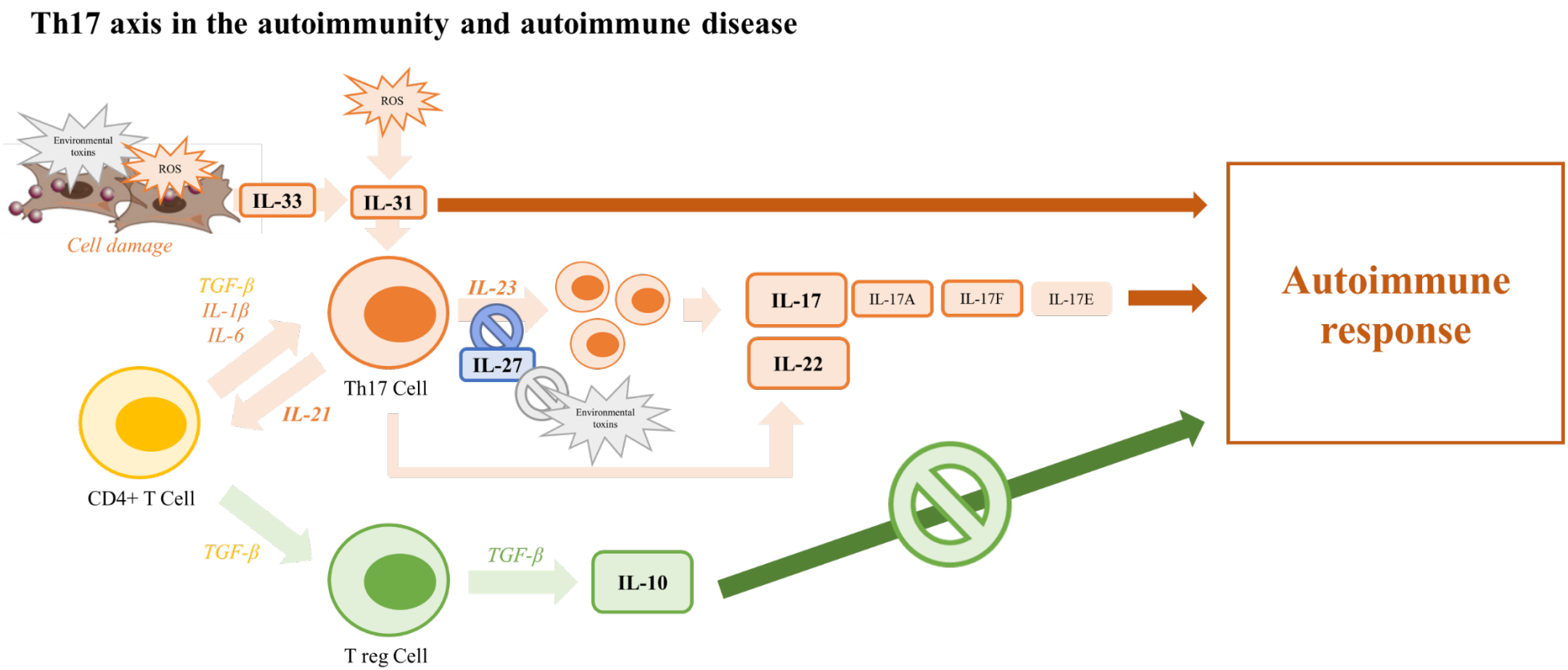
Figure description:

Figure 1 is a graphical representation decribing role and function sytokies involved in Th17 axis in autoimmunity.

The role of Th17 cells and their cytokines (IL-17A/E/F, IL-21. IL-22, IL-23, IL-27, IL-31, IL-33) has been recently implicated in the development of many autoinflammatory conditions, owing to studies reporting an increased levels of these cytokines in the autoimmune patients.

Upon exposure to environmental factors, such as toxins and heavy metals, through increasing oxidative damage and ROS production, can lead in necrotic cell damage, which result in the release of intercellular material and proinflammatory mediators, including alarmins IL-31 and IL-33, what may trigger autoimmune response and induce Th17 formation. Cytokines of Th17 axis, in particularly IL-21, IL-23, IL-27, IL-31 and IL-33 are involved in modulating Th17/Treg differentiation through TGF-β. Establishment of proinflammatory environment, characterized by presence of proinflammatory cytokine IL-6 and alarmin IL-1, can shift CD4+ T cells differentiation into Th17 and consequently decrease formation of Treg cells, which through producing anti-inflammatory cytokine IL-10 help to maintain immune homeostasis.

Th17 cells, like other CD4+ T cells are a source of cytokine IL-21 which additionally promotes differentiation and enhance Th17 cell activity, and together with IL-23 contribute to Th17 expansion and Th17/T-reg imbalance. Th17 cells response is mediated by their effector cytokines, IL-17 (isoforms IL-17A, IL-17E and IL-17F, of which IL-17A is dominant one) and IL-22, that main roles have been implicated in neutrophil recruitment, apoptosis and production of inflammatory mediators, such as IL-6, IL-8, IL-10, IL-1α, matrix metalloproteinases (MMP), nitric oxide (NO) and prostaglandins (PGE). Compared to IL-17, IL-22 exhibits mild pro-inflammatory, although with noticeable regenerative effects, which depend on tissue microenvironment. Th17 actions can be suppresed by IL-27, an immunomodulatory cytokine, which can inhibit Th17 and Treg development, through downregulating production of Th17 effector cytokines. Nevertheless, the cellular injury consequent from exposure to environmental toxins, can suppress IL-27 actions.



CD cluster of differentiation; MeHg methyl mercury; ROS radioactive oxygen species; ANA anti-nuclear antibodies; ANoA anti-nucleolar antibodies; IL interleukin; TGF-β - Transforming growth factor beta; TNF-α Tumour necrosis factor alpha; INF-γ Interferon gamma; Th17 T helper 17 cells; T reg T regulatory cells.

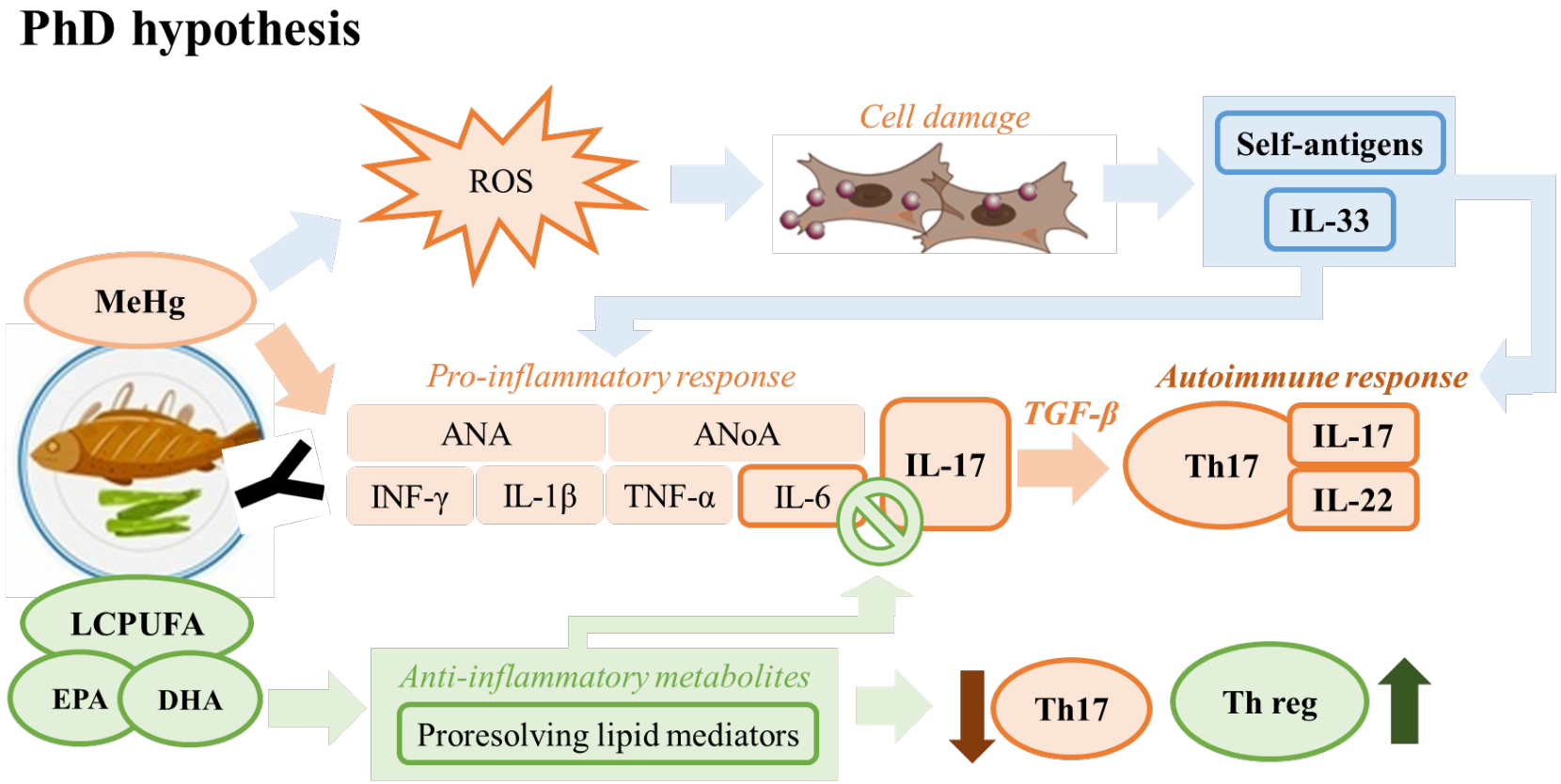
Figure 2. Outline of PhD hypothesis.

Figure description:

Figure 2 is a graphical representation of PhD hypothesis.

The hypothesis of this PhD research is based on the existing evidence obtained from high fish consumers, which demonstrated associations between dietary exposure methylmercury (MeHg) and increased concentrations of pro-inflammatory cytokines, including IL-17, which is a main effector cytokine of Th17 response, as well as proinflammatory mediators, including IL-6, IL-1β, TNFα, INFγ and increased titres of anti-nuclear and anti-nucleolar antibodies. Establishment of proinflammatory environment following MeHg exposure may further increase oxidative damage, which may lead to continuous release of self-antigens and alarmins, such as IL-33 that additionally stimulate proinflammatory response. In addition, this proinflammatory environment may induce formation of Th17 cells in expense of Treg cell subsets, and promote autoimmunity and development of autoimmune disease.

Nevertheless, fish is also a rich source of health-promoting nutrients, such as n-3 LCPUFA, including EPA and DHA which through promoting formation of anti-inflammatory metabolites, such as pro-resolving lipid mediators may help to restore immune homeostasis through modulating T cell differentiation - increasing Treg formation, while decreasing Th17 activity.



MeHg methyl mercury; LCPUFA long chain polyunsaturated fatty acids; EPA eicosapentaenoic acid (EPA, C20:5n-3); DHA docosahexaenoic acid (DHA, C22:6n-3); ROS radioactive oxygen species; ANA anti-nuclear antibodies; ANoA anti-nucleolar antibodies; IL interleukin; TNF-α Tumour necrosis factor alpha; INF-γ Interferon gamma; Th17 T helper 17 cells; T reg T regulatory cells.

CHAPTER 2:

Dietary sources of mercury and their potential impact on health, a review.

Abstract

Mercury (Hg) is a toxic heavy metal which accumulates in both aquatic and terrestrial food chains. In humans, fish intake is the main exposure route of the neurotoxin methylmercury (MeHg), albeit the consumption of grain/vegetable crops and animal products obtained from Hg-polluted sites can also contribute to Hg and MeHg intakes. MeHg is readily absorbed through the GI tract, has the potential to exert immunotoxic effects and can cross the blood-brain barrier, and thereby posing a significant neurotoxic risk. The health impact of Hg exposure has predominantly focused on the mother-child cohorts owing to the neurotoxic risk of MeHg exposure to the developing fetal brain during pregnancy. In adults, Hg exposure has been linked with cardiovascular disease and immune dysfunction, albeit the results of these studies are frequently conflicting. Most of the research to date is based around fish as the main dietary source of MeHg. Less is known about the health impact of plant and animal foods which also contribute to dietary MeHg intake. Furthermore, the cumulative amount of total reported Hg, (inorganic mercury (iHg) and organic methylmercury (MeHg)) obtained from all food sources in the diet has not been investigated. Plant foods, similar to fish, are rich sources of n-3 polyunsaturated fatty acids (LCPUFA), selenium (Se), flavonoids and dietary fibre which may counteract Hg toxicity and potentially protect against adverse health outcomes/symptoms of Hg intoxication. The aim of this review is to evaluate the contribution of Hg sources (fish, rice, vegetables and meat products) present in the diet and assess the potential of other nutrients present in these foods, as well as in the overall diet, to ameliorate adverse effects of Hg toxicity in their consumers.

Keywords

Nutrition, mercury exposure, health, diet, food safety, toxicology

Introduction

Mercury (Hg) is a metal widely distributed in the environment as a result of volcanic eruptions, weathering of rocks and sea vent activity. Atmospheric Hg, being mostly in the elemental form (Hg^0), undergoes transformation to divalent gaseous (Hg^{2+})/inorganic mercury (iHg), which can be subsequently deposited in terrestrial (soil) as well as aquatic (oceans, waterways) systems. This process is mediated by iron/sulphate-reducing bacteria and methanogens which, under anoxic conditions of aquatic sediments and flooded soils, facilitates the transformation of iHg to organic methylmercury (MeHg) and its subsequent incorporation into the food webs. This is especially pronounced in the aquatic environments as microbially-produced MeHg, initially taken up by phytoplankton/algae, can be bioaccumulated along the trophic levels of the food web, thereby leading to high concentrations in the top predators such as shark, swordfish and king mackerel (Silbernagel *et al.,* 2011; Driscoll *et al.,* 2013).

The presence of MeHg in fish is a long-standing public health concern which has been investigated for years, owing to the fact that MeHg has the potential to cross the blood-brain and placental barriers and has been associated with adverse effects to fetal neurodevelopment (Igata, 1993; Grandjean *et al.,* 1997; Oken *et al.,* 2005; Jedrychowski *et al.,* 2007; Lederman *et al.,* 2008; Oken *et al.,* 2008; Freire *et al.,* 2010; Grandjean *et al.,* 2014; Rice *et al.,* 2014; Marques *et al.,* 2015; Prpić *et al.,* 2017). Furthermore, observations of occupationally exposed adults, including those who consumed MeHg-polluted foods and/or were involved in artisanal activities (source of iHg), have reported disturbances in neurological (Bakir *et al.,* 1973; Davis et al., 1994; Harada, 1995; Auger *et al.,* 2005; Philibert *et al.,* 2008), cardiovascular (Salonen *et al.,* 1995; Guallar *et al.,* 2002; Virtanen *et al.,* 2005) and immunological (Silva *et al.,* 2004; Alves *et al.,* 2006; Gardner *et al.,* 2010; Nyland *et al.,* 2011; Somers *et al.,* 2015) functions. Dietary guidelines for the safe consumption of fish during pregnancy exist across the world with a focus on limiting fish intake, especially high MeHg fish (Taylor *et al.,* 2018). More recently, research has proposed the importance of considering other dietary sources

of MeHg and iHg which may also be providing appreciable amounts of this heavy metal when consumed (Wang *et al.,* 2005; Gebeyehu and Bayissa, 2020). Risk of heavy metal contamination of certain foods, lead to development of policy and legal regulations on the permissible level of Hg with particular emphasis not only fish and seafood, but also plant-derived foods, canned products dairy (milk, eggs) and animal meat and organs (liver) (see table 1). Although these safety limits of Hg/MeHg in marine products appears to be consistent across the countries, allowed levels of Hg in other foods may vary or information are lacking. Nevertheless, for majority of count tires the Hg safety limits in fish and fish-derived products is between 0.05 - 1 μg MeHg/g; while for plant foods is ranging from 0.01 μg Hg/g for vegetables to 0.1 μg Hg/g in fungi (China). For other foods as well as canned products this limits are ranging from 0.5 μg Hg/g (canned baby foods and canned dietary foods for special purposes) to 1 μg Hg/g for canned beverages (EU). Certain countries, such as Canada, India, as well as EPA/FDA the Hg limits for drinking water has been developed, and they the lowest in Canada (0.001 μg Hg/mL), and the highest in India (0.01 μg Hg/mL) (see table 1.)

The rapid increase in industrialization across the globe, and the subsequent accumulation of Hg pollution in the environment including agricultural soils, has resulted in plant and animal products obtained from this land being contaminated with Hg (Li *et al.,* 2017; Rai *et al.,* 2019). Few studies have investigated the concentration of Hg in these plants and animal products, albeit those that have demonstrated that the consumption of grains and vegetables obtained from Hg-polluted areas are associated with potential health risks (Wang *et al.,* 2005) and potentially contribute to cardiovascular and neurological complications (Bakir *et al.,* 1973; Davis *et al.,* 1994; Gebeyehu and Bayissa, 2020). The studies are, however, limited in sample size and often based on theoretical calculations of the average Hg content of the foods.

In recent years, epidemiological studies that have investigated the relationship between MeHg and health outcomes have included covariates which may mitigate against the toxic effects of MeHg. Nutrients such as the n-3 long chain polyunsaturated fatty acids (LCPUFA), selenium

(Se), dietary fibre and phytochemicals have all been suggested to have a role in reducing MeHg toxicity. Similarly, the use of certain cooking methods (canning, grilling, freezing, boiling, soaking before cooking) or processing methods (rice polishing) may also impact favorably on Hg toxicity and confound reported outcomes.

Therefore, the aim of this review is to identify and summarize recent evidence on potential dietary sources of Hg exposure and to determine if habitual intake of Hg-contaminated foods, including fish, plant and animal products, impact on the consumer's health, especially if eaten in combination. Additionally, this review will outline potential confounding factors which should be considered when assessing the risks and benefits associated with dietary intake of these foods.

Hg exposure from fish and seafood

Fish is an important component of the human diet as it provides high-quality protein, LCPUFA, vitamins and minerals that are important for optimal health and disease prevention (Gill and Gill, 2015). All fish and shellfish contain MeHg (see table 2) with large, long-living predatory species, such as sharks, swordfish or king mackerel, having the largest concentration and small herbivorous fish, such as sardines, herring, pollock, flounder, sole or plaice, having lower concentrations (Mahaffey *et al.,* 2011). The concentrations of MeHg/Hg in fish may also vary depending on the location where fish was caught. For example, total Hg mean of fish obtained in Tapajos, Brazil was 0.66 µg Hg/g, whereas in Slovak Republic, total Hg mean was 1.17 µg Hg/g. In USA, Hg content in fish was varying depending on if fish was obtained from sea or lake. In this case, freshwater fish (Grand Lake, Oklahoma) had lower total Hg content ranging from 0.024-0.276 µg Hg/g; compared to seafish (Long Island), in which total Hg means ranged from 0.013 to 1.123 µg Hg/g. Among all listed countries, the lowest Hg/ concentrations were in fish obtained in Canada (mean of total Hg ranged from 8.6e-5 µg Hg/g in whitefish to 0.00063 µg Hg/g in pike) (see table 2).

Although MeHg exposure in fish consumers is determined mainly by the fish choice and actual Hg content in the fish, as well as frequency and number of consumed fish meals, it can also depend on individual characteristics, including socioeconomic status, age, sex and ethnicity (Castaño *et al.,* 2015; Faial *et al.,* 2015; Kusanagi *et al.,* 2018; Wiseman *et al.,* 2019) (see table 2). Consequently, populations consuming locally caught fish, in which Hg contents are reported to be high, may account higher Hg exposure, than populations eating the same amount of fish obtained from less polluted with Hg areas (see table 2). Observational studies have shown that individuals who consume fish more than five times per month have higher MeHg exposure compared to those who eat fish occasionally (one to two fish meals per month) (Nielsen *et al.,* 2014) (see table 2). Similarly, a 10-day long intervention with daily consumption of dishes containing ~160g of fish fillets (cod, coalfish, pollock) obtained from a Polish market conducted in disease-free men with initial fish intake (the majority of participants) being ≤ one fish meal a month increased the average Hg concentration in the blood from 0.62 to 1.28 μg/l. The estimated weekly MeHg intake through fish intake in these volunteers was 0.62 μg/kg of body weight (bw)/week, which is still below the provisional tolerable weekly intake (PTWI) for MeH, (1.6 μg/kg bw/week) established by the Joint Food and Agriculture Organization of the United Nations and World Health Organization FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2003). Therefore, the risk of adverse effects from MeHg exposure from consumption of commercially available fish products is these individuals is substantially low, even though its frequent consumption can increase Hg status (Kuras *et al.,* 2017).

Women of childbearing age, pregnant women and children are most vulnerable to MeHg exposure as MeHg can cross the blood-brain barrier and placental membranes and potentially result in adverse neurodevelopment (Choi *et al.,* 2008). First reports of an adverse effect of dietary MeHg exposure were obtained from studies describing cases of Hg poisoning in populations that were acutely exposed to high MeHg concentrations through the ingestion of fish (Harada, 1995) or wheat grain (Bakir *et al.,* 1973) contaminated with MeHg. Observations on the Minamata outbreak in Japan indicated that extremely high MeHg exposure (Hg hair ranged 5.61 to 35.7 μg/g) from consumption of fish polluted

with MeHg (MeHg in fish >35 μg/g) lead to severe neurological disturbances (e.g. ataxia, dysarthria, sensory and auditory impairments) in local residents and cerebral palsy-like symptoms (e.g. mental retardation, physical growth disorder, limb deformities) in children of mothers who ate contaminated fish during pregnancy (Igata, 1993; Harada, 1995). Similar signs of MeHg poisoning were also reported in other countries, such as Iraq (1973), Guatemala (1960) and Pakistan (1969), in which consumption of bread made from MeHg-polluted grains, following the misuse/careless handling of MeHg-containing antifungal seed dressing agents, resulted in the brain damage among infants who acquired MeHg exposure *in utero* and/or from maternal milk (Bakir *et al.,* 1973). Early evidence obtained from these fatal incidences promoted further research investigating the risks of MeHg/Hg exposure through dietary sources, such as fish and seafood, which lead to the introduction of appropriate guidelines for pregnant women and their children.

Substantial evidence assessing the MeHg neurotoxicity on the developing fetus has been obtained in several high fish-eating populations with conflicting results. Some studies have found associations between fish/seafood intake during pregnancy and neurodevelopmental deficits (Grandjean *et al.,* 1997; Oken *et al.,* 2005; Jedrychowski *et al.,* 2007; Lederman *et al.,* 2008; Oken *et al.,* 2008; Freire *et al.,* 2010; Marques *et al.,* 2015; Prpić *et al.,* 2017), whereas others have consistently reported no associations between MeHg exposure and neurological function (Davidson *et al.,* 2008; Strain *et al.,* 2008; Valent *et al.,* 2013; Hsi *et al.,* 2014; Gustin *et al.,* 2017; van Wijngaarden *et al.,* 2017), thereby implicating benefits for neurocognitive development in children, whose mothers consumed fish and seafood during pregnancy (Hibbeln *et al.,* 2019).

Impact of fish-derived nutrients against Hg toxicity

The majority of studies that reported associations between Hg status and adverse health outcomes, including neurological (Grandjean *et al.,* 1997; Oken *et al.,* 2005; Auger *et al.,* 2005; Jedrychowski *et al.,* 2007; Lederman *et al.,* 2008; Philibert *et al.,* 2008; Oken *et al.,* 2008; Freire et al., 2010; Grandjean *et al.,* 2014; Marques *et al.,* 2015; Prpić *et al.,* 2017), immune (Silva et al., 2004; Alves et

al., 2006; Gardner *et al.,* 2010; Nyland *et al.,* 2011; Somers *et al.,* 2015) and cardiovascular dysfunction (Salonen *et al.,* 1995; Guallar et al., 2002; Virtanen *et al.,* 2005) did not adjust their analyses for nutritional factors associated with fish intake. However, including other constituents present in fish (such as n-3 LCPUFA) is critical, as they may act as negative confounders and lead to underestimation of associations between health outcomes and MeHg exposure in fish consumers (Strain *et al.,* 2008; Choi *et al.,* 2014).

The high nutritional value of fish in human diet is supported by multiple mother-child cohorts from the Republic of Seychelles (Strain et al., 2015; van Wijngaarden et al., 2017), Bangladesh (Gustin et al., 2017), Italy (Valent *et al.,* 2013), Taiwan (Hsi *et al.,* 2014) and the UK (Golding et al., 2017) which found no association between MeHg exposure and adverse neurological effects, while demonstrating that health benefits of marine nutrients, such as vitamin E and D, iodine (I), Se, and most importantly n-3 LCPUFA can not only outweigh risks of MeHg neurotoxicity, but also support child neurodevelopment (Strain *et al.,* 2014).

In addition, dietary differences at a population level linked to the nutritional composition of MeHg-containing food sources may partially explain discrepancies in the findings reported in the Faroese (geometric median Hg in maternal hair 4.27 μg/g) and the Seychellois (median Hg in maternal hair 5.9 μg/g) cohorts. Results obtained from the Faroe Islands study found associations between prenatal MeHg exposure and adverse effects on brain function, including deficits in language, attention and memory (Grandjean *et al.,* 2012), whereas findings from the Republic of Seychelles have consistently reported no relationship between MeHg exposure and neurodevelopmental outcomes (Strain *et al.,* 2015), instead showing positive associations between psychomotor score and maternal n-3 LCPUFA status during pregnancy (Strain *et al.,* 2008). Of note is that, in the Faroe Islands, there are two dietary sources of MeHg exposure – fish (eaten as dinner one to three times a week) and pilot whale (eaten sporadically once a month), whilst in the Seychelles MeHg exposure is attributed exclusively to the ocean fish, free from other than MeHg pollutants (e.g dioxins and polychlorinated biphenyls), which are consumed on

a daily basis (on average 12 fish meals a week). The differences in dietary patterns and nutrient profiles among these two cohorts may explain why similarly high Hg status in the fish-consuming populations can vary in reported health outcomes that seem to be dependent on the nutritional content of Hg-containing fish/seafood (ocean fish eaten in Seychelles is a rich n-3 LCPUFA and Se source) and the time pattern of the exposure (periodical vs constant) (Grandjean *et al.,* 2001).

n-3 long chain polyunsaturated fatty acids

Fish and seafood have the highest content of any foods of the n-3 LCPUFA eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Tacon and Metian, 2013). Dietary intake of EPA and DHA has been associated with positive neurodevelopmental and cardiometabolic outcomes (Gribble *et al.,* 2016) and reduced risk of chronic illnesses, such as diabetes, rheumatoid arthritis, systemic lupus erythematosus or allergies (Swanson *et al.,* 2012; Li et al., 2019). Furthermore, fish intake in pregnancy through increasing maternal n-3 LCPUFA status has been linked to benefits for child health, including improved metabolic health (Maslova *et al.,* 2018; Stratakis et al., 2020) and neurodevelopmental outcomes, such as higher developmental scores (Daniels *et al.,* 2004; Davidson *et al.,* 2011) and better infant cognition (Oken *et al.,* 2005), which seems to be lower in mothers who do not consume any seafood/fish (Strain *et al.,* 2014; Golding *et al.,* 2017).

Optimal fish intake, as well as achieving the adequate status of maternal LCPUFA, may have a critical role in impacting on MeHg toxicity. LCPUFA status has been reported to influence the relationships between MeHg and neurodevelopmental outcomes in the child (Strain *et al.,* 2008). In addition, there is accumulating evidence to indicate that the intake of marine foods during pregnancy can bring additional benefits for child development and decrease risk of disease in the future. Large scale investigations, such as the Avon Longitudinal Study of Parents and Children (ALSPAC) in the United Kingdom (Daniels *et al.,* 2004), The Spanish Environment and Childhood Research (INMA) (Llop *et al.,* 2017) and Project Viva conducted in the US (Oken *et al.,* 2005), indicated

positive relationships between maternal fish intake and higher developmental scores in the offspring. In fact, each additional weekly serving of fish increased infant verbal recognition memory (VRM) score, which was highest among children whose mothers consumed more than two fish servings a week during pregnancy and at the same time had hair Hg concentrations ≤ 1.2 μg/g (Oken *et al.,* 2005). Similarly, the Human Early Life Exposome (HELIX) project provided evidence that eating fish one to three times per week during pregnancy, compared to less than once a week, was associated with improved metabolic profile in children and a reduction of pro-inflammatory cytokine (e.g., Interleukin (IL)-1β, IL-6, Tumor necrosis factor (TNF)-α) concentrations, with benefits attributed to maternal intakes of n-3 LCPUFA (Stratakis *et al.,* 2020).

The n-3 LCPUFA exert beneficial effects through the production of eicosanoids and docosanoids from EPA and DHA respectively. Eicosanoids and docosanoids are potent regulators of endothelial function and inflammation (Dennis and Norris, 2015), through their ability to modulate C-reactive protein (CRP), IL-6 and TNF-α (Calder, 2006). Increased consumption of EPA and DHA results in their enhanced incorporation into the cellular membrane displacing the more pro-inflammatory arachidonic acid (AA) over time. This displacement can suppress the production of potent AA-derived pro-inflammatory mediators (e.g., 2-series prostanoids (PGE2) and 4-series leukotrienes LTB4) (Wall *et al.,* 2010) and promote the formation of less inflammatory metabolites, such as the eicosanoids, 3-series prostanoids (PGE3 TXA3), 5-series leukotrienes (LTB5), E-series resolvins (RvE) and pro-resolving lipid mediators, D-series resolvins (RvD), protectins (PD) and maresins (MaR) (Calder, 2015). Therefore, intake of marine n-3 LCPUFA in fish-consuming populations has the potential to mitigate some of the proposed adverse effects of Hg exposure through their ability to modulate immunity via anti-inflammatory and antioxidant activities (Calder, 2008; Gutiérrez *et al.,* 2019).

Selenium

Fish are one of the richest dietary sources of Se, which is an essential trace element and plays a central role in the antioxidant defense of the human body through its incorporation into a broad range of selenoproteins, including glutathione peroxidase (GPx) and thioredoxin reductase (TXNRD) and is critical for maintaining redox homeostasis and preventing oxidative damage (Ralston and Raymond, 2018). Se possesses a high-affinity binding for thiol (-SH) moieties present on many plasma proteins (Yoneda and Suzuki, 1997), being several orders of magnitude higher than other metals such as Hg, which may suggest that Se could have an important role in the prevention of MeHg toxicity (Golzadeh *et al.,* 2020). The potential mitigating role of Se was indicated in several epidemiological studies which attributed the absence of associations between adverse health outcomes and early-life Hg exposure to Se within the fish sources present in the maternal diet (Myers and Davidson, 2000). Considering the evidence to date, it is plausible that increasing Se:Hg molar ratio ≥1 may offer protection against the adverse consequences of MeHg exposures (Ralston, 2008), including oxidative brain damage among high seafood consumers (Grotto *et al.,* 2011; Nyland *et al.,* 2011). Therefore, employing Se:Hg molar ratios into the risk assessments of MeHg exposure and dietary Se intakes has been shown to improve reliability and adequacy of Se health benefit (Se-HBV) index when evaluating consumption of fish and seafoods by vulnerable groups, including pregnant women (Ralston *et al.,* 2016). An observational study conducted in the general population of Taiji in Japan reported that high exposure to Hg through whale meat consumption (mean hair Hg 14.9 μg/g, ranges 0.6–101.9 μg/g) was not significantly correlated with any of the clinical signs of neurological disturbance. Evaluation of whole blood Hg and Se concentrations revealed a significant positive association between Hg and Se, with the Hg:Se molar ratios above one in all participants, thereby suggesting that sufficient Se intake in this population appears to be protective against adverse health effects arising from MeHg exposure (Nakamura *et al.,* 2014).

Hg has high-affinity binding with Se, which through binding to the active site of the selenoproteins (GPx, TXNRD), including selenocysteine (SeCys) residues, may cause their irreversible inhibition and reduce antioxidant defense and increase oxidative damage, an important manifestation of Hg toxicity.

A molar excess of Se is necessary to replenish the inactivated enzymes, which in the case of SeCys must be newly synthesized, as the Hg-SeCys cannot be reused. Therefore, achieving a substantial excess of Se over Hg reflected in an Se:Hg molar ratio > 1 confers protection so long as the Se itself is not at toxic concentrations (Gochfeld and Burger, 2021). Indeed, there is some indication that applying the Se:Hg ratio might be a more efficient metric for stratifying Hg toxicity in fish consumers (Azad *et al.,* 2019) than MeHg concentrations alone, as molar Se:Hg ratio is reported to be negatively associated with Hg content in fish (Polak-Juszczak and Robak, 2015). Nevertheless, current limitations in understanding the interactions between Hg-Se make it difficult to define a reference value for the amount of Se required to provide protection against Hg toxicity. Therefore, it is necessary to undertake further research to confirm if a 1:1 Se:Hg molar ratio would be sufficient to prevent harmful effects of Hg exposure, particularly in consumers of large predatory fish, including whale and shark (Gochfeld and Burger, 2021).

Impact of applied cooking and processing methods on nutritional value of fish

The observed variation associated with risks and benefits of fish consumption, resultant from high nutritional value and presence of toxic MeHg, indicate a role of other factors, such as individual preferences and cooking habits in determining health outcomes reported among customers. Applying different culinary practices which influence the physiochemical properties of fish meat may impact on the content of both nutrients and contaminants, including Hg present in the fish. Although there is no effective method that could eliminate Hg completely from fish, some practices, if applied before consumption, might help to reduce Hg bioavailability (Bradley *et al.,* 2017). Cooking techniques, such as steaming, boiling, or grilling, have been shown *in vitro* to decrease MeHg bioaccessibility by 75%–96% for grouper and 29%–77% for rabbitfish, when compared to the raw fillets (He and Wang, 2011). Similarly, preservation methods such as the use of salt (1M solution of sodium chloride, NaCl) (James *et al.,* 2005) or a 0.1% citric acid solution have been shown to aid Hg removal from shark meat. A reduction of ~ 40% was observed with the use of salt (James *et al.,* 2005) and 30% for citric acid, whereas preserving mackerel with an acidic solution reduced Hg by 75% (Hajeb and

Jinap, 2009). These effects may be as a result of changes in the fish protein structure due to denaturation, which decreases its digestibility and reduces the release of trapped Hg (Afonso *et al*., 2015). Using methods that generate heat, such as frying, when cooking a small fish, such as anchovy and sardine, seems to have a concentrating effect on Hg concentrations, as higher Hg content was reported in the fried fish than in raw (0.051 μg Hg/g vs 0.034 μg Hg/g per total weight in fried vs raw sardine respectively). Of note is that differences observed in Hg content may be an effect of high temperature owing to water loss in the fillet, thereby reducing its overall weight and volume (Perelló *et al.,* 2008; Marmelo *et al.,* 2020).

The way fish is cooked has also been suggested to be an important determinant of the final nutritional content. In particular, all cooking methods have been shown to significantly reduce n-3 LCPUFA content, with frying (deep-frying or pan-frying) using hydrogenated vegetable oils leading to the most pronounced reduction of DHA and EPA in fish fillets (Weber *et al.,* 2008; Gladyshev *et al.,* 2014; Karimian-Khosroshahi *et al.,* 2016). Processing that reduces fat content within the fish, as well as using frying or boiling to prepare fillets, also impacts bioavailability of lipid-soluble nutrients, including vitamin D and E, and reduces their content in the fish meat (Karimian-Khosroshahi *et al.,* 2016).

In general, fish consumption is considered as a primary route of dietary exposure to toxic MeHg in humans, where neurotoxic effects have been shown to be particularly harmful for the developing nervous system. Although, this may raise public health concerns in vulnerable populations of pregnant women and young children, there is growing evidence to support the role of fish-derived nutrients, such as n-3 LCPUFA and Se, in mitigating any adverse effects associated with Hg toxicity in fish consumers. Owing to reported variation among studies investigating the impact of Hg exposure on health outcomes in fish-consuming populations (Jedrychowski *et al.,* 2007; Lederman *et al.,* 2008; *Marques et al.,* 2015; Somers *et al.,* 2015; Prpić *et al.,* 2017), dietary factors, such as fish-derived nutrients (n-3 LCPUFA, Se) and applied fish cooking and processing methods, should also be considered. Consequently,

including a nutritional matrix of marine foods with the meal and overall diet, rather than single nutrients of the food, may help to explain differences in the health outcomes among fish and seafood consumers.

Hg exposure from plant-based foods

A plant-based diet that encompasses the consumption of wholegrains, legumes, vegetables and fruits plays a crucial role in human health, as it provides nutrients important for disease prevention and overall wellbeing (Hever and Cronise, 2017). Nevertheless, Hg accumulation in the environment (soil, water and air) from industrial emissions and direct use of fungicides and fertilizers (Tang *et al.,* 2018) can compromise the nutritional value of crops (Rai *et al.,* 2019) and additionally contribute to Hg exposure in their consumers (Li *et al.,* 2017). Whilst fish is the primary dietary source of MeHg, depending on the pollution source, the dominant type of Hg in plant foods (e.g., rice and green leafy vegetables) has not been clearly identified and, depending on the pollution source (see table 3), it appears to be a combination of all three forms of Hg, including Hg^{2+}/iHg, MeHg and Hg^{0}), with the majority of studies reporting only total Hg content.

Rice (*Oryza sativa L.*), fruit and vegetables are an integral part of a healthy diet, as they are a rich source of dietary fibre, vitamins, minerals and various phytonutrients (e.g. phytoestrogens, anthocyanins, flavanols) with known antioxidant and anti-inflammatory properties (Pem and Jeewon, 2015). Nevertheless, their nutritional value might be compromised (Rai *et al.,* 2019) by pollutant accumulation (Bayissa and Gebeyehu, 2021). Growing crops in the proximity to mining (Sierra *et al.,* 2008) or industrial (e.g. power plants, fluorescent lamp factories, oil wells) sites can increase heavy metal accumulation in edible plants, including Hg (Lenka *et al.,* 1992; Cao *et al.,* 2010; Li *et al.,* 2017) (see table 3). This can be of particular importance for health risk assessments in rice consumers, as co-ingestion of Hg along with other heavy metals, such as arsenic (As), lead (Pb) and cadmium (Cd) can make very difficult to separate out adverse health effects attributed to Hg toxicity from the overall toxicity (Rai *et al.,* 2019).

In addition, crops that require anaerobic conditions, such as rice grown on flooded paddy soils, due to increased activity of microbial communities (e.g. methanogens, sulfate-reducing, iron-reducing bacteria), that has been shown to promote iHg methylation to MeHg, what result in increasing MeHg accumulation in the rice grain by 10–100 times, when compared to other commercial crops, such as corn, cabbage or tobacco upon identical Hg exposure (Li *et al.,* 2010; Wang *et al.,* 2018). During rice plant growth, MeHg, but not iHg, is actively transported to the grains until the end of the ripening period (Kodamatani *et al.,* 2020), whilst iHg remains complexed by phytochelatins in the roots (Feng *et al.,* 2016). This process may be further enhanced by agricultural practices, such as during a flooding period (Zulkafflee *et al.,* 2019).

Whilst Hg in rice is assimilated by the root system, Hg in fruit and vegetables is thought to be of atmospheric origin (Xia *et al.,* 2020), as higher Hg concentrations have been found above ground organs, such as leaves (116.17±14.69 μg/kg) and fruits (29.07±1.45 μg/kg), than in the roots (13.64±1.37 μg/kg) (Li *et al.,* 2017). This difference is of particular concern for leafy vegetables, grown close to regions of industrial pollution, which absorb atmospheric Hg through the broad leaf surfaces and can be more prone to accumulate Hg from dust and rainwater (Li *et al.,* 2017; Yu *et al.,* 2018). Hg absorption via roots is dependent on soil properties (e.g., soil organic matter (SOM), carbon exchange capacity, redox potential, pH) which through increasing Hg bioavailability in the soil content can promote Hg uptake in the plant. For instance, the acidic environment of soil (pH < 6.5) has been found to increase Hg uptake in plants, whereas SOM < 20g/kg seems to lower Hg adsorption capacity in tuber plants, likewise SOM 20–30g/kg in cucurbit vegetables (Yu *et al.,* 2018). Differences in Hg absorption observed between crops from polluted soil indicates a role for genetic variability in Hg accumulation within plant varieties (Rothenberg *et al.,* 2012). In rice, Hg concentrations seem to be higher in long-grain (4.1±1.7 μg/kg), as well as black or red pigmented (4.7±1.2 μg/kg) varieties, when compared to short/medium (2.0±1.8 μg/kg), brown (3.5±2.3 μg/kg) and white (2.6±1.6 μg/kg) rice (Lin and Lin, 2019).

Variation in the Hg content of rice can vary depending on the country of origin (see table 3). For example, the highest Hg concentrations were found in brown rice from Jamaica (0.062 mg/kg), while the lowest were reported in rice from Canada (3.5±2.3 μg/kg) (Antoine *et al.,* 2012; Lin and Lin, 2019). Hg and MeHg concentrations in the rice products on the European market ranged from 0.53 to 11.1 μg/kg (mean 3.04 ± 2.7 μg/kg) for Hg and 0.11 to 6.45 μg/kg (mean 1.91±1.07 μg/kg) for MeHg. Rice grown in regions with elevated Hg emissions might explain why some samples had high Hg content. Of note is that most reports assessing Hg/MeHg content in rice and fruit/vegetables (see table 3) are predominantly obtained from Asian countries, where rapid industrialization has raised concerns about food safety (Xu *et al.,* 2020). Samples obtained from farmlands with known artisanal activities and/or discharge of sewage, such as the Hg-mining area of Wanshan in Guizhou province, as well as Fujian (Longyan; 1.7 μg/kg), Guangxi (Nanning; 20.4 μg/kg) and Zhejiang (Jiaxing; 23.3 μg/kg), have reported Hg concentrations in the crops that exceed up to 20 times the legal limit set by the Chinese National Food Safety Standard (GB 2762–2017) for vegetables and fruits (10 μg/kg) and staple foods (20 μg/kg) (GB 2762–2012) (Xu *et al.,* 2020). This was especially evident in the rice samples which were as high as 23.3 μg/kg in samples from the Jiaxing area (Xu *et al.,* 2020), as well as in vegetables (e.g., tomato, eggplant, pepper, cucumber, and cowpea) grown in the proximity of power plants in China. These vegetables had on average 8.6 times higher Hg concentrations than the allowed values (Li *et al.,* 2017). Similar to Jiaxing area of China, high Hg contamination was also found in tomatoes and cabbages (3.43 mg/kg and 4.23 mg/kg dry weight respectively) obtained from the Ethiopian farmlands of the Mojo industrial district (Li *et al.,* 2017; Gebeyehu and Bayissa, 2020; Xia *et al.,* 2020) (see table 3). Although human activities seemed to be the main Hg source in plant-based foods, the diversity in reported Hg content suggest some influence of season, plant age and time of harvest (Kowalski and Frankowski, 2016). Furthermore, the environment (e.g., weather conditions/climate, aggregate size, mineralogical soil properties) may also have considerable impact on Hg and Se concentrations within these plants and should be considered in future research (Gworek *et al.,* 2020).

The presence of detectable Hg concentrations in plant products available on local markets in countries with known Hg pollution may raise safety concerns and question their suitability for consumption. With that said, for the majority of cases, the Hg concentration of grains and vegetables is far below the legal limits set by local authorities (Cao *et al.,* 2010). A recent evaluation of 43 edible plant crops in China indicated that the majority (>99.5%) of the commercial rice had a Hg concentration well below the limits established by the Chinese authority (GB 2762–2012) (3.44 and 6.11 μg/kg) (Xu *et al.,* 2020) and comparable to the rice obtained from Korean (2.91 ± 0.86 μg/kg) (Eom *et al.,* 2014) and European (3.04 ± 2.07 μg/kg) (Brombach *et al.,* 2017) markets. Similarly, leafy vegetables obtained from The Pearl River Delta in South China, despite reported Hg pollution in the local soils, remained within the safety limit (GB 2762–2012), thereby indicating no health risk to the residents who consume locally grown products (Chang *et al.,* 2014).

Even though detectable Hg concentration in plant foods is low and considered as safe (Xu *et al.,* 2020), there is some evidence to indicate that regular consumption of meals containing rice and leafy greens obtained from areas with reported Hg contamination can increase overall Hg exposure (Feng *et al.,* 2016; Hong *et al.,* 2016; Wells *et al.,* 2020) and exceed the provisional daily tolerable intake (PDI) for Hg in the local consumers (Zhang *et al.,* 2010; Liu *et al.,* 2019; Xu *et al.,* 2020). This may be of particular importance to vulnerable individuals, such as pregnant women and young children (Chang *et al.,* 2014), for whom dietary guidelines encourage fish and vegetable intake as part of a well-balanced diet (Danielewicz *et al.,* 2017).

Health assessments conducted in the Philippines, as the Provincial Health Office response to increased complaints of unusual symptoms (e.g. miscarriages, tooth loss, muscle weakness, paralysis, anemia, tremors, etc.) have found a significantly higher Hg and MeHg content in hair samples in the children and pregnant women/mothers (total Hg and MeHg in hair samples ranged from 0.18 to 13.29 μg/g and 0 to 13.29 μg/g respectively) from the Hg-contaminated mining areas of Barangay Tagburos, compared to the control subjects recruited from Puerto Princesa (total Hg and MeHg in hair samples ranged from

0.16 to 8.28 μg/g and 0.19 to 8.28 μg/g respectively) who consumed locally sourced staple foods, including fish and rice (Maramba *et al.,* 2006). Similarly, combining two dietary Hg sources (fish and vegetables) within one meal, but not when eaten separately, was considered a significant contributor to estimated health risks in children from Dong Li district, which accounted for 45% of the total target hazard quotient (THQ) among all heavy metals (copper (Cu), zinc (Zn), Pb, Cd, and Hg) (Wang *et al.,* 2005). The habitual intake of vegetables and grains with Hg concentrations exceeding the permissible limits has been associated with increased risk of cardiovascular disease (CVD) and neurological complications (Gebeyehu and Bayissa, 2020); nevertheless, the clinical impact of low-level exposure to Hg through plant foods has not been widely investigated. The issue of increased Hg status resultant from food staples contaminated with Hg should warrant attention, especially in vulnerable groups of children, women of childbearing age and nursing mothers, as they are more likely to experience long-term health consequences of MeHg exposure through diet.

Owing to increased health concerns of Hg contamination in staple foods, such as rice (Liu *et al.,* 2019), individuals who rely on locally sourced foods obtained from areas with known Hg pollution are advised to use cooking methods, such as boiling and pressure-cooking in excess volume of water (6:3 water-to-rice ratio), to decrease the Hg content in rice and therefore limit potential adverse effects associated with Hg exposure (Lin *et al.,* 2019). Additionally, applying monitoring measures, especially in those countries with industrial pollution, by tracking changes in Hg concentrations of the commercially available plant products, could help to inform future research and to determine potential risk for local populations.

Beneficial nutrients in plant-based foods against Hg exposure

Plant foods provide many essential nutrients (e.g., dietary fibre, vitamins, minerals, polyphenols) with proposed protective properties against Hg-induced toxicity and which may modify Hg metabolism and absorption in the gastrointestinal tract (Michalak, 2006). Although the mechanisms associated with plant-derived bioactives in modulating MeHg toxicity are limited, evidence obtained

from observational studies demonstrated that intake of fresh seasonal fruits, such as bananas, ingas and oranges, have the potential to modify Hg accessibility from fish when eaten as one meal (Passos *et al.,* 2008). This has been supported by studies conducted in the Brazilian Amazon which have shown that frequent fruit intake of more than 10 pieces of fruit a week can reduce blood Hg concentrations by 14.4% when compared to those who eat three or less (Passos *et al.,* 2008). In women, intake of at least one tropical fruit per day was linked to lower MeHg concentrations, compared to those who did not eat fruit at all, despite high Hg exposure from frequent fish consumption (median of 9.1 μg/g, ranging from 4.0 to 20.0 μg/g) (Passos *et al.,* 2003).

Plant foods may have the ability to reduce Hg bioavailability owing to the presence of bioactives within the plants such as dietary fibres (lignin, pectin and cellulose derivatives), polyphenols (e.g., epicatechin, epigallocatechin gallate, rutin cafeic acid) and organic acids (e.g. oxalate, acetate, malate and citrate, tannic acid). *In vitro* studies that used gastrointestinal digestion models reported that combining polyphenol-rich, plant-based products, such as virgin olive oil or tea infusions, can enhance the formation of insoluble complexes with Hg^{2+} ions which could have the potential to reduce Hg bioaccessibility when consuming fish (Kalogeropoulos *et al.,* 2012; Jadán-Piedra *et al.,* 2016; Girard *et al.,* 2018). Furthermore, antioxidative properties demonstrated by many plant-derived antioxidants have the potential to reduce oxidative damage and restore redox balance (Shanker *et al.,* 2005; Chang *et al.,* 2019) and could help to ameliorate the toxic effects of MeHg exposure. Flavonoids, such as myricetin, myricitrin and fisetin (3, 3, 4, 7-tetrahydroxyflavone), present in fruits, including apples, tomatoes, grapes, and oranges, have been proposed to be efficient in protecting against MeHg-induced mitochondrial dysfunction as well as in reducing free radical formation and lipid peroxidation in the brain (Franco *et al.,* 2010; Jacob and Thangarajan, 2017).

Antioxidant capabilities were also reported in Japonica rice, especially in pigmented varieties, which are rich in the rice-derived polyphenols, cyanidin 3-glucoside and peonidin 3-glucoside, and have previously been reported to exhibit potent antioxidant activities and free radical scavenging *in vitro* (Hu

et al., 2003). These effects appear to be the most pronounced in black rice, as its phenolic content is much higher compared to light-pigmented brown rice (10.0–13.1 μM TE/g and 56.3–345.33 μM TE/g respectively) (Goffman and Bergman, 2004; Goufo and Trindade, 2014). Consequently, consumption of wholegrain purple and red-pigmented rice, in contrast to the white or brown varieties, significantly improved antioxidant status and reduced inflammatory markers in the plasma (CRP, IL-10, IL-6, IL-8 and TNF-α) of consumers (Callcott *et al.,* 2019). Furthermore, combining Hg-containing foods like fish with high-fibre foods is proposed to reduce Hg bioaccessibility within the meals (Marmelo *et al.,* 2020), with the reduction ranging from 15% to 31% when consuming psyllium, to 72 to 84% when wheat is added (Shim *et al.,* 2009). This effect may be dependent on the fibre matrix and its chelating capacity, as insoluble fibres (e.g., cellulose, hemi-cellulose, lignins) tend to be more effective in reducing Hg bioaccessibility compared to soluble fibres (pectin, gums, mucilagnes) (Shim *et al.,* 2009; Dhingra *et al.,* 2012; Hajeb *et al.,* 2014; Jadán-Piedra *et al.,* 2016).

Dietary fibres may also reduce Hg toxicity through their beneficial effect on the gut microbiota composition which affects MeHg metabolism (Rothenberg *et al.,* 2016) and detoxification (Barkay *et al.,* 2003), including the transformation of organic Hg derivatives into less toxic inorganic forms (Duan *et al.,* 2020) or by inducing the expression of antioxidant enzymes (e.g., superoxide dismutase (SOD), catalase (CAT), GSH) (*Kullisaar et al.,* 2002; Ahire *et al.,* 2013). These effects are pronounced for the Peptococcaceae family, which through increasing the demethylation of MeHg has been shown to decrease MeHg levels in intestinal contents of MeHg-exposed rats (Lin *et al.,* 2020). Similarly, commensal colonic bacteria (e.g., Bacteroidetes and Firmicutes), by increasing the production of metabolites with immunomodulatory functions such as short-chain fatty acids (SCFA), particularly butyrate, may help to restore immune homeostasis and enhance toxicological defenses compromised upon Hg exposure (Parada Venegas *et al.,* 2019).

Taken together, it appears that plant-derived bioactives, through decreasing Hg bioaccessibility, reducing inflammation, and restoring redox homeostasis, may have the potential to mitigate effects of

Hg toxicity. Nevertheless, the majority of evidence to support a role for these compounds/nutrients in reducing the negative effects of Hg is derived from *in vitro* and *in vivo* animal studies and thus translation of these findings should be interpreted with caution (Canuel *et al.,* 2006; Chang *et al.,* 2019). There is a need for much more evidence from human studies on the benefits of these foods and derived compounds on Hg toxicity.

Hg exposure from meat and animal products

Meat and its products can be contaminated with Hg when the animal is reared in the areas where there is uncontrolled disposal of Hg-containing waste, mining, smelting and through the use of certain agricultural practices (e.g., use of pesticides and phosphate fertilizers). Hg deposited in the environment, similar to aquatic ecosystems, can be incorporated into terrestrial food webs through plants and then transferred along the food chain into animals (Gworek *et al.,* 2020). Although studies have shown that Hg bioaccumulation in land ecosystems is less pronounced than in aquatic sediments, herbivorous animals, especially livestock and their predators (humans), can attain significant amounts of Hg (Beckers and Rinklebe, 2017) that can be further modified by other factors linked to animal characteristics (e.g., age, sex, musculature, breed) and feeding habits (Rudy, 2009).

Livestock are exposed to Hg through the ingestion of Hg-polluted grass (Pietrzkiewicz *et al.,* 2018), fungicide (source of organic Hg-contaminated grain) (Englender *et al.,* 1980; Davis *et al.,* 1994) or feed with added marine fish (source of MeHg exposure) (Plummer and Bartlett, 1975; Jorhem *et al.,* 1991). This can lead to Hg accumulation in animal products (e.g., meat, eggs, milk, kidneys, liver) dedicated for human consumption (Lindberg *et al.,* 2004; Nkansah and Ansah, 2014; Arianejad *et al.,* 2015; Barej *et al.,* 2015; Pietrzkiewicz *et al.,* 2018; Di Bella *et al.,* 2020; Nawrocka *et al.,* 2020). Nevertheless, Hg concentrations detected in those foods are below the European legal limit (1000 µg/kg) (Nawrocka *et al.,* 2020) for Hg in foodstuffs (EC, 2006) (see table 1), as they range between 9 and 8.7 µg/kg for raw meat and 8.70 to 125 µg/kg for meat products, with raw chicken meat being the

lowest (9 μg/kg) and sausage (125 μg/kg) being the highest in the Hg content (Alturiqi and Albedair, 2012) (see table 4). Although the reasons for reported discrepancies between raw meat and processed products remains unknown (Alturiqi and Albedair, 2012), certain technological processes used in the sausage meat production, such as homogenization and the addition of food additives (e.g., spices or curing compounds), were proposed as potential sources of Hg contamination in commercial meat products (Lukáčová *et al.,* 2014) (see table 4).

Several studies suggest that the frequent consumption of large amounts of Hg-contaminated foods of animal origin may increase MeHg/Hg status (Sell *et al.,* 1975; Bjornberg *et al.,* 2003; Lindberg *et al.,* 2004), however, the evidence for its impact on health is limited to two reports which present conflicting results. A follow-up study on family members including pregnant women, who experienced acute MeHg intoxication from inadvertently feeding their hogs seed grain that was contaminated with an MeHg-containing fungicide, indicated 22 years after that exposure to MeHg resulted in severe neurological dysfunction (e.g. cortical blindness or constricted visual fields, diminished hand proprioception, choreoathetosis and attentional deficits) and histological damage of brain structures in the children of the mothers who consumed the Hg-contaminated pork meat over a three-month period during pregnancy (Davis *et al.,* 1994). Another study, despite high levels of Hg exposure (Hg blood concentrations ranged from 0.009 to 0.0202 μg/L), reported no adverse outcomes in family members who consumed eggs obtained from chicken flock which were accidentally fed barley seed grain treated with mercurial fungicide (Englender *et al.,* 1980).

Although the presence of Hg in animal products may raise concerns about the quality and safety of those foods, these Hg concentrations do not exceed safety limits and they are considered safe for consumption (Beckers and Rinklebe, 2017; Pietrzkiewicz *et al.,* 2018; Nawrocka *et al.,* 2020). To ensure that these levels are maintained long term, comprehensive control programs, which include veterinary inspections, risk assessments and nationwide epidemiological studies, would bring benefits

to animal welfare and help to prevent potential exposure to heavy metals among consumers (Nawrocka *et al.,* 2020).

Conclusions

Dietary intake, after occupational exposure, is one of the most common routes of Hg exposure in humans, with fish and shellfish being dominant sources of MeHg in the diet. Other foods, including those of plant and animal origin (e.g. rice, leafy green vegetables, game meat) can also contain amounts of iHg/MeHg, especially if obtained from regions where Hg-pollution is a concern, such as in China and Asian countries.

Evidence from studies investigating the health effects of consuming foods contaminated with Hg remain inconsistent and are dominated by a large number of epidemiological studies which reported no associations between consumption of MeHg polluted fish and adverse health outcomes, including in pregnant women and in children. Despite limited number of studies investigating effect of Hg-polluted foods intake on consumer Hg status, there is some indication, that similarly to fish, habitual intake of Hg-contaminated vegetables, rice or meat can increase Hg status (see tables 2, 3,4). For example, in Philippines, individuals who consumed rice from polluted site of abandoned Hg mines (total Hg mean in rice 0.00306 μg Hg/g) had total Hg concentrations determined in hair ranging from 0.18-13.29 μg Hg/g (Maramba *et al*., 2006). Similar observations were noted in the native residents of Kratie in Cambodia, who consumed pork/beef sourced from the areas polluted by iHg-containing wastes (total Hg mean in pork was 1.58 ng/g; and in beef was 21.88 ng/g), had Hg status ranging from 0.54 to190 µg Hg/g in hair (Cheng *et al*., 2013). Although these reported increase in Hg status appears to be low when compared to Hg status in fish consuming populations (see table 3), it should not be ignored. Therefore, there is emerging need for further studies, which would monitor Hg concentrations in both plant and animal products obtained from areas where Hg pollution is a concern. In parallel to that, the Hg status should be also assessed in the residents who are based on locally sourced foods.

Significant differences in the nutritional contents of marine, plant and meat products does not allow us to generalize findings obtained from individuals exposed to Hg from high consumption particular products. Therefore, when evaluating the health risks against benefits of regular intake of Hg-containing foods, the whole nutritional matrix of food as well as the overall dietary composition should be considered, owing to the fact that interactions between nutrients as well as applied technological processing approaches (e.g. homogenization) or cooking methods (boiling, frying, curing, marinating, canning) could influence Hg bioaccessibility within the food and whole meal and/or ameliorate the toxic effects of Hg.

Health risk assessment based on Hg content reported in commercially available/worldwide consumed fish/seafood, plant and meat products from Europe, China and USA (see tables 5 and 6) indicate that consumption of Hg-containing products within the recommended portion sizes and their number eaten and frequency within the week (Cámara *et al.,* 2021) may exceed PTWI for Hg established by the World Health Organization (1 μg of Hg/kg bw) (JECFA, 2003) in children between two and ten years old, as well as in certain adult populations, in particular those of Asian origin. Consuming fruits and vegetables obtained from areas with known industrial Hg pollution (China) (Li *et al.,* 2017; Xu *et al.,* 2020) has been shown to exceed Hg PTWI in two and four year old children, similar to predatory fish (Mackerel, Swordfish, Whitefish, Cod – US and China only; Halibut – US only; Monkfish, Grass carp and canned/fresh Tuna Bluefin/Skipjack products). In adults, owing to the differences in body weight, Asian, compared to European and American populations, seem to have a higher probability of exceeding PTWI of Hg-containing products (see table 5).

In addition, consuming Hg-containing foods in combination within the one meal with the assumption that it is consumed on a regular basis can exceed PTWI in certain consumer groups. Therefore, a lunch meal consisting of rice/potatoes and salad (lettuce, tomato and peppers) with a portion of fish (140g for adults and 70g for children) or meat (70g for adults and 35.5g for children) if eaten twice a week has

been shown to exceed PTWI in Asian adults as well as in two, four and eight year old children, with potato-based dishes contributing to higher Hg intakes within the meal (see table 5).

Furthermore, it would be worth considering other ways of mitigating Hg exposure which may be applied in the countries where Hg pollution is a concern. An appropriate information campaign and guidelines could be developed to educate consumers about the importance of choosing appropriate cooking methods in order to minimize potential Hg exposure or eating foods in the right combinations to decrease absorption of Hg. Another important action may involve other stakeholders, including food producers, distributors and legal authorities, which through applying adequate monitoring of Hg in marine, animal and plant-food sources can provide reliable information on potential exposure risk of this element to livestock and humans. In this case, regulatory guidelines and approved threshold limits may offer the best means for the effective avoidance of Hg poisoning and intoxication.

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Table 1. Comparison of permittable Hg levels in the food products in different regions.

Country/Region	Food product	Hg safety limit	Reference
EPA/FDA	Drinking water	0.002 µg Hg/mL	ATSDR, 1999
	Food products	1 µg Hg/g	
	Animal feed	1 µg Hg/g	
	Fish	1 µg MeHg/g	
European Union	Fishery products, fish and crustaceans	0.5 µg Hg/g	EC, 2006
	Fish (anglerfish, Atlantic catfish, bonito, eel, emperor, grenadier, halibut, marlin, megrim, mullet, pike, cod, dogfish, rays, redfish, sail fish, scabbard fish, seabream, shark, butterfish, sturgeon, swordfish, tuna)	1 µg Hg/g	
	Canned foods beverages (fruit and vegetable juices)	1 µg Hg/g	
	Canned baby foods and processed cereal-based foods for infants and young children	0.5 µg Hg/g	
	Canned infant formulae and follow-on formulae (infant milk and follow-on milk)	0.5 µg Hg/g	
	Canned dietary foods for special medical purposes (intended for infants)	0.5 µg Hg/g	
China	Grains (rice, wheat)	0.02 µg Hg/g	National Health Commission of People's Republic of China, 2017
	Vegetables	0.01 µg Hg/g	
	Fungi	0.1 µg Hg/g	
	Eggs and meat	0.05 µg Hg/g	

	Milk	0.01 µg Hg/g	
	Predatory fish and its products	1 µg MeHg/g	
	Fish (other than predatory) and its products	0.5 µg MeHg/g	
Canada	Drinking water	0.001 µg Hg/mL	Health Canada, 2007
	Fish	0.5 µg MeHg/g	
	Salt and food grade	0.1 µg Hg/g	
Poland	Meat	0.02 µg Hg/g	Pietrzkiewicz *et al.,* 2018
	Liver	0.05 µg Hg/g	
	Predatory fish and its products	1 µg MeHg/g	
	Fish (other than predatory) and its products	0.5 µg MeHg/g	
India	Drinking water	0.01 µg Hg/mL	
	Fish	0.5 µg Hg/g	Bhawan and Nagar, 2009
	Food products	1 µg Hg/g	
		0.25 µg MeHg/g	
Japan	Fish and shellfish (other than predatory fish)	0.4 µg Hg/g	The Food Safety Commission, Japan The
	Predatory Fish	0.3 µg MeHg/g	Contaminant Expert Committee, 2005
Thailand	Seafood	0.5 µg Hg/g	Thailand Ministry of Public Health, 1986

	Food products	0.02 µg Hg/g	Zarcinas *et al.,* 2004
New Zealand	Seafood	0.5 µg Hg/g	Food Standards Australia New Zealand, 2005
	Food products	< 0.002 µg Hg/g	
	Fish	0.5 µg MeHg/g	

Table 2. Frequency of fish consumption, Hg concentration in fish and reported Hg status in the fish-consuming populations.

Population	Frequency of fish intake	Hg concentration in fish	Hg status in consumers	Reference
Barreiras population, Tapajós, Brazil	0-20 fish meals a week	THg in predatory fish - Mean: 0.66 µg Hg/g; Range: 0.30 - 0.98 µg/g	THg in men (range): 2.07–24.93 µg/g	Faial *et al.,* 2015
			THg in women (range): 4.84–27.02 µg/g	
		THg in non-predatory fish: 0.09 µg/g; Range: 0.02 - 0.44 µg/g	MeHg in men (mean): 11.68 µg/g	
			MeHg in men (range): 1.49–19.57 µg/g	
			MeHg in women (mean): 10.38 µg/g	
			MeHg in women (range): 3.73 - 22.35 µg/g	
Sonora, Mexico	307g of fish a day	THg (geomatric mean): 0.15 ± 19 µg/g; Range: <LOD - 0.029-0.390 µg/g	N/A	García-Hernández et al., 2018
Slovak Republic	172.1g fish a week	THg (mean): 1.17 ± 1.23 µg/g; Range: 0.089-6.552 µg/g	THg in cord blood (mean): 0.949 ± 0.683 µg/L	Kimáková et al., 2018
			THg in cord blood (range): 0.200–5.443 µg/L	Ursinyova *et al.,* 2019

			MeHg in cord blood (mean): 0.504 ± 0.551 µg/L	
			THg in cord blood (range): < LOD; 0.053-5.076 µg/L	
Chinese, Indian, Pakistan, Bangladesh & Sri Lankans Newcomers in Canada	Consume fish on monthly basis	THg (range): 0.04 – 0.47 µg/g	THg (geomatric mean): 1.05 µg/L	Wiseman *et al.*, 2019
New Zealand	3 or more times a week	THg (mean): 0.02-2.48 µg/g	Maternal hair Hg (median): 0.43 µg/g	Karatela *et al.,* 2019
		MeHg (mean): 0.04-1.97 µg/g	Children hair Hg (median): 0.32 µg/g	Love *et al.*, 2003
Tapajós River, Brazilian Amazon	7.4 fish meals a week	N/A	THg (mean): 38.6± 21.7 µg/L	Passos *et al.,* 2007
			MeHg (mean): 33.6± 19.4 µg/L	
Manaus population, Brazilian Amazonia	Consume fish on daily basis	N/A	Hair Hg (mean): 35.4± 20.8 µg/g	Silva *et al.,* 2004; Alves *et al.,* 2006; de Silva and de Oliviera Lima, 2020
New York, USA	2.4 cups of fish a week	THg (mean ranges): 0.013-1.123 µg/g	THg (mean): 4.58 µg/L	Monaestro *et al.,* 2017
				FDA, 2012
Florida, USA		THg (mean ranges): 0.013-1.123 µg/g	Hg in hair (mean): 1.53 ± 1.89 µg/g	Schaefer *et al.,* 2014

	Between 3 and 1 fish meal(s) a week			FDA, 2012
Oklahoma, USA	58g of fish a day	Freshwater fish (Grand Lake): 0.024-0.276 µg/g	Hg in hair (mean): 0.27 µg/g	Dong *et al.,* 2015
First Nations, Canada	38g of fish/day	THg (mean ranges): 8.6e-5 ±0.048 (whitefish) - 0.00063 ±0.81 (pike) µg/g	THg in hair (mean): 0.27 µg/g	Juric *et al.,* 2017
		MeHg (mean ranges): 3.9e-5 ±0.022 (whitefish) - 0.0003 ±0.28 (pike) µg/g	THg in hair (range): 0.03 -13.54 µg/g	
USA	Between 3 and 1 fish meal(s) a week	THg (mean ranges): 0.013-1.123 µg/g	Children hair Hg (geometric mean): 0.12± 0.01 µg/g	McDowell *et al.,* 2004
			Maternal hair Hg (geometric mean): 0.20±0.02 µg/g	FDA, 2012
Pearl River Delta, South China	52-341 g of fish a day	THg (mean): 59.0 ± 46.4 ng/g; range: 2.81–208.5 ng/g	THg in hair (mean): 1.08 ± 0.94 µg/g	Shao *et al.,* 2013
		MeHg (mean): 48.7 ± 38.8 ng/g; range: 1.63–287 ng/g	MeHg in hair (mean): 0.58 ± 0.59 µg/g	
Japan	≥ 1 fatty fish meal/week: 76.8% (Hair Hg<1ppm) and	THg (mean): 0.148 µg/g	Hg in hair (geomatric mean): 0.87 µg/g	Kusanagi *et al.,* 2018

23.3% (Hair Hg≥1ppm)		
< 1 fatty fish meal/week: 52.9% (Hair Hg < 1ppm) and 47.1% (Hair Hg > 1ppm)		Nakagawa *et al.,* 1997
≥ 1 non-fatty fish meal/week: 57.1% (Hair Hg < 1ppm) and 42.9% (Hair Hg ≥ 1ppm)		—
< 1 non-fatty fish meal/week: 60.5% (Hair Hg < 1ppm) and 39.5% (Hair Hg > 1ppm		—

Table 3. Regional differences in total Hg and MeHg concentrations in plant foods and reported consumers Hg status.

Country	Plant food	Hg concentration	Reported pollution	Hg status in consumers	Reference
China	Rice	THg (mean): 4.03 ± 2.37 μg/kg; Range: 0.638–31.7 μg/kg	Commercial product	N/A	Xu *et al.,* 2020
		MeHg (mean): 1.40 ± 1.21 μg/kg; Range: 0.0200–18.6 μg/kg			
	Amaranth	Hg (mean): 46.40 ± 2.33 μg/kg	Coal fired power plants		Li *et al.,* 2017
		Hg (mean): 0.28 ± 0.21 μg/kg	Control site		
	Tomato	Hg (mean): 71.80 ± 11.95 μg/kg	Coal fired power plants		
		Hg (mean): 0.73 ± 0.36 μg/kg	Control site		
	Lettuce	Hg (mean): 39.04 ± 4.41 μg/kg	Coal fired power plants		
		Hg (mean): 0.35 ± 0.10 μg/kg	Control site		
	Eggplant	Hg (mean): 42.37 ± 4.24 μg/kg	Coal fired power plants		
		Hg (mean): 0.43 ± 0.39 μg/kg	Control site		

	Pepper	Hg (mean): 49.66 ± 1.40 µg/kg	Coal fired power plants		
		Hg (mean): 0.93 ± 0.84 µg/kg	Control site		
	Cucumber	Hg (mean): 38.45 ± 1.40 µg/kg	Coal fired power plants		
		Hg (mean): 0.87 ± 0.24 µg/kg	Control site		
	Cowpea	Hg (mean): 56.31 ± 4.03 µg/kg	Coal fired power plants		
		Hg (mean): 0.93 ± 0.13 µg/kg	Control site		
	Maize	Hg (mean): 21.02 ± 1.98 µg/kg	Coal fired power plants		
		Hg (mean): 0.72 ± 0.19 µg/kg	Control site		
	Water spinach	Hg (mean): 86.69 ± 2.16 µg/kg	Coal fired power plants		
		Hg (mean): 0.85 ± 0.22 µg/kg	Control site		
Wanshan, China	Rice	Hg (mean): 65.49±17.34 µg/kg	Hg mining area	N/A	Xia *et al.,* 2020
	Corn	Hg (mean): 15.09±12.60 µg/kg			
	Soybean	Hg (mean): 69.20±12.95 µg/kg			

	Potato	Hg (mean): 4.09±3.06 μg/kg			
	Peanut	Hg (mean): 42.33±26.08 μg/kg			
	Vegetables	Hg (mean): 48.37±22.88 μg/kg			
	Fruits	Hg (mean): 43.77±28.30 μg/kg			
	Strawberry	Hg (mean): 5.27±2.18 μg/kg			
Pearl River Delta, South China	Vegetables	THg (mean): 1.32 ± 0.57 ng/g	N/A	THg in hair (mean): 1.08 ± 0.94 μg/g	Shao *et al.,* 2013
		MeHg (mean): 0.03 ± 0.01 ng/g		MeHg in hair (mean): 0.58 ± 0.59 μg/g	
Northern Jiangsu, Northern China	Rice	MeHg (mean): 2.3 ± 0.8 ng/g; Range: 0.9–3.3 ng/g	No local Hg point sources	N/A	Gong *et al.,* 2018
Northern-eastern China	Rice	MeHg (mean): 38.9 ± 9.5 ng/g; Range: 25.1–56.3 ng/g	No local Hg point sources	N/A	Gong *et al.,* 2018
Eastern China	Rice	THg (mean): 12.9 ± 7.14 μg/kg; range: 1.30–41.2 μg/kg	Compact fluorescent lamp (CFL) industries	N/A	Liang *et al.,* 2015
		MeHg (mean): 6.01 ± 3.65 μg/kg; range: 0.09–21.5 μg/kg			
India	Rice	THg (mean): 1.88 ± 0.180 μg/kg; Range: 1.73– 2.16 μg/kg	N/A	N/A	Xu *et al.,* 2020

		MeHg (mean): 0.680 ± 0.163 µg/kg; Range: 0.423–0.889 µg/kg			
	Amaranth	THg (mean): 4.33 µg/g	Hg pollution in the	N/A	Lenka *et al.,* 1992
	Cabbage	THg (mean): 9.33 µg/g	from chloralkali		
	Chilli	THg (mean): 2.1 µg/g	plant		
Ethiopia	Cabbage	Hg (mean): 4.23± 0.28 µg/g	Industrial area	N/A	Gebeyehu and Bayissa, 2020
	Tomato	Hg (mean): 3.43 ± 0.05 µg/g	(Mojo) with leather and textile factories, plastic factory and edible oils		
Pakistan	Rice	THg (mean): 2.33 ± 0.455 µg/kg; Range: 1.63 – 2.85 µg/kg	N/A	N/A	Xu *et al.,* 2020
		MeHg (mean): 7.38 ± 0.261 µg/kg; Range: 3.38 – 9.88 µg/kg			
	Cabbage	Hg (mean): 0.003 ±0.00042 µg/g	Land contamination		Abbas *et al.,* 2010
	Chilli	Hg (mean): 0.010 ±0.00113 µg/g	due to human		
	Tomato	Hg (mean): 0.005 ±0.00072 µg/g	activities		
	Cucumber	Hg (mean): 0.0056 ±0.0004 µg/g			

	Onion	Hg (mean): 0.009 ±0.00053 µg/g			
	Potato	Hg (mean): 0.001 ±0.00010 µg/g			
	Radish	Hg (mean): 0.005 ±0.00022 µg/g			
	Turnip	Hg (mean): 0.001 ±0.00016 µg/g			
	Pumpkin	Hg (mean): 0.0100±0.00075 µg/g			
	French beans	Hg (mean): 0.008±0.00102 µg/g			
	Spinach	Hg (mean): 0.009±0.00021 µg/g			
	Cauliflower	Hg (mean): 0.009 ±0.00103 µg/g			
Tanzania	Rice	Hg (rage): 0.011-0.031 µg/g	Contamination of local soils and rivers due to artisanal gold mining activities	N/A	Taylor *et al.,* 2005
	Cabbage	Hg (range): <0.004 µg/g			
	Tomato	Hg (range): <0.004 µg/g			
	Yam	Hg (range): <LOD-0.092 µg/g			
	White beans	Hg (range): <0.004 µg/g			
	Green beans	Hg (range): <0.004 µg/g			
	Maize	Hg (range): <0.004 µg/g			
	Onion	Hg: <0.004 µg/g			
Kratie, Cambodia	Rice	THg (mean): 12.7 ng/g; Range: 9.90–16.7 ng/g		Hair Hg (mean): 3.7 µg/g	Cheng *et al.,* 2013

	MeHg (mean): 1.54 ng/g; Range: 1.06–2.31 ng/g	Disposal of industrial iHg-containing wastes	
Cabbage	THg (mean): 0.40 ng/g; Range: 0.35–0.47 ng/g		
	MeHg (mean): 0.01 ng/g; Range: 0.01–0.01 ng/g		Hair Hg (range): 0.54–190 μg/g
Chinese radish	THg (mean): 0.19 ng/g; Range: 0.16–0.20 ng/g		
	MeHg (mean): 0.01 ng/g; Range: <LOD – 0.01 ng/g		
Long bean	THg (mean): 0.52 ng/g; Range: 0.32–0.87 ng/g		
	MeHg (mean): 0.05 ng/g; Range: 0.02–0.06 ng/g		
Wintermelon	THg (mean): 0.62 ng/g; Range: 0.32–0.87 ng/g		
	MeHg (mean): 0.01 ng/g; Range: 0.01–0.01 ng/g		

Canada	Fruit and fruit products	Hg (mean): 0.16 ng/g; range: 0.04-0.70 ng/g	Canadian cities - Whitehorse and Ottawa	N/A	Dabeka *et al.,* 2003
	Cereal and cereal products	Hg (mean): 0.34 ng/g; range: 0.09-1.8 ng/g			
	Vegetables and vegetable products	Hg (mean): 0.68 ng/g; range: 0.04-16 ng/g			
Spain	Rice	THg (mean): 2.30 ± 0.009 µg/kg; Range: 2.29 – 2.30 µg/kg	N/A	N/A	Xu *et al.,* 2020
		MeHg (mean): 6.80 ± 0.207 µg/kg; Range: 5.34 – 8.27 µg/kg			
	Lettuce	Hg: <0.008 µg/g	Air pollution in urban and metropolitan areas		Ercilla-Montserrat *et al.,* 2018
Japan	Rice	THg (mean): 3.74 ± 1.36 µg/kg; Range: 1.88 – 6.02 µg/kg		Hair Hg: 1.29 - 11.86µg/g	Xu *et al.,* 2020
		MeHg (mean): 1.21 ± 0.749 µg/kg; Range: 4.09 – 2.29 µg/kg			

	Chilli	MeHg (range): 0.003-0.047 mg/kg		N/A	Morishita *et al.,* 1982
Indonesia	Rice	MeHg in seed (mean): 57.7 ± 42.9 ng/g; range: 10.6-115 ng/g	Region with high Hg-mining activity	THg in hair (mean): 7.72± 11 610 µg/g	Krisnayanti *et al.,* 2012
				THg in hair (range): 0.805–52.5 µg/g	
		MeHg in hull (mean): 28.6 ± 25.3 ng/g; range: 4.33-64.9 ng/g		MeHg in hair (mean): 1.004± 605 µg/g	
				MeHg in hair (range): 0.356-2.550 µg/g	
USA	Rice white	THg (range): 2.84 - 2.00 ng/g	N/A	N/A	Palmieri *et al.,* 2020
	Rice brown	THg (range): 3.71 -1.26 ng/g			
Italy	Rice	THg (mean): 4.29 ± 0.200 µg/kg; Range: 4.09 – 4.49 µg/kg	N/A	N/A	Xu *et al.,* 2020
		MeHg (mean): 1.90 ± 0.600 µg/kg; Range: 1.40 – 2.57 µg/kg			
Russia	Rice	THg (mean): 2.59 ± 1.14 µg/kg; Range: 1.48 – 4.67 µg/kg	N/A	N/A	Xu *et al.,* 2020

		MeHg (mean): 5.84 ± 0.360 µg/kg; Range: 1.84–1.10 µg/kg			
Vietnam	Rice	THg (mean): 2.03 ± 0.780 µg/kg; Range: 1.88 – 6.02 µg/kg	N/A	N/A	Xu *et al.,* 2020
		MeHg (mean): 1.21 ± 0.749 µg/kg; Range: 4.09 – 2.29 µg/kg			
Thailand	Rice	THg (mean): 3.44 ± 1.59 µg/kg; Range: 1.74 – 5.81 µg/kg	N/A	N/A	Xu *et al.,* 2020
		MeHg (mean): 0.820 ± 0.491 µg/kg; Range: 0.0690–1.47 µg/kg			
Philippines	Rice	THg (mean): 0.00306 µg/g	Abandoned Hg mines	THg in hair (range): 0.18-13.29 µg/g	Maramba *et al.,* 2006
		THg (mean): 0.00361 µg/g	Control site	MeHg in hair (range): 0-13.29 µg/g	
Poland	Wheat grain	Hg (mean): 2.8 ± 2.5 µg/kg; Range: <0.1–13 µg/kg	No pollution reported	N/A	Jedrzejczak *et al.,* 2002
	Rye grain	Hg (mean): 2.0 ± 2.0 µg/kg; Range: <0.1–14 µg/kg			

Vegetables	Hg (mean): 0.5 ± 0.4 µg/kg; Range: <0.1–2.4 µg/kg
Carrot	Hg (mean): 0.6 ± 0.4 µg/kg; Range: <0.1–1.3 µg/kg
Cucumber	Hg (mean): 0.5 ± 0.3 µg/kg; Range: <0.1–2.4 µg/kg
Cabbage	Hg (mean): 0.5 ± 0.4 µg/kg; Range: <0.1–1.5 µg/kg
Cauliflower	Hg (mean): 0.5 ± 0.3 µg/kg; Range: <0.1–0.9 µg/kg
Tomato	Hg (mean): 0.3 ± 0.2 µg/kg; Range: <0.1–0.5 µg/kg
Potato	Hg (mean): 0.6 ± 0.4 µg/kg; Range: <0.1–1.2 µg/kg
Fruit	Hg (mean): 1.1 ± 0.9 µg/kg; Range: <0.1–5.1 µg/kg
Strawberry	Hg (mean): 1.1 ± 0.8 µg/kg; Range: <0.1–3.5 µg/kg

Table 4. Regional differences in total Hg and MeHg concentrations in meat and meat products with reported customer Hg status.

Region	Animal product	Hg concentration	Reported pollution	Hg status in consumers	Reference
Pearl River Delta, South China	Meat	THg (mean): 4.85 ± 1.55 ng/g	No pollution reported	THg in hair (mean): 1.08 ± 0.94 µg/g	Shao *et al.,* 2013
		MeHg (mean): 2.30 ± 1.02 ng/g		MeHg in hair (mean): 0.58 ± 0.59 µg/g	
Wanshan, China	Meat	THg (mean): 220 µg/kg	Hg mining area	N/A	Feng *et al.,* 2008
		MeHg (mean): 0.85 µg/kg			
	Polutry	THg (mean): 160 µg/kg		N/A	Ji *et al.,* 2006
		MeHg (mean): 2.4 µg/kg			
Kratie, Cambodia	Pork	THg (mean): 1.58 ng/g; range: 1.56–1.62 ng/g	Disposal of industrial iHg-containing wastes	Hair Hg (mean): 3.7 µg/g	Cheng *et al.,* 2013
		MeHg (mean): 1.09 ng/g; range: 0.44–1.42 ng/g		Hair Hg (range): 0.54–190 µg/g	

	Beef	THg (mean): 21.88 ng/g; range: 14.2–35.9 ng/g			
		MeHg (mean): 2.64 ng/g; range: 4.06-6.80 ng/g			
Canada	Meat and meat products	Hg (mean): 0.12 ng/g; range: 0.29-2.3 ng/g	Canadian cities - Whitehorse and Ottawa	N/A	Dabeka *et al.,* 2003
	Poultry and poultry products	Hg (mean): 1.4 ng/g; range: 0.39-1.8 ng/g			
Slovak Republic	Pork	Hg (mean): 1.494 ± 0.511 µg/kg	Technological processes related to meat product (salami) production, use of food additives	N/A	Lukáčová *et al.,* 2014
	Beef	Hg (mean): 2.751 ± 1.095 µg/kg			
	Pork bacon	Hg (mean): 1.364 ± 0.262 µg/kg			
	Salami Malokarpatska	Hg (mean): 9.295 ± 2.367 µg/kg			

Ghana	Pork	Hg (mean): 0.012 ± 0.1195 µg/g	Animal feed and drinking water	N/A	Nkansah and Ansah, 2014
	Beef	Hg (mean): 0.052 ± 0.010 µg/g			
	Mutton	Hg (mean): 0.015 ± 0.305 µg/g			
	Chevon	Hg (mean): 0.034 ± 0.052 µg/g			
	Deer	Hg (mean): 0.051 ± 0.005 µg/g			
	Grasscutter	Hg (mean): 0.071 ± 0.046 µg/g			
Poland	Beef	Hg (mean): 0.8 ± 1.2 µg/kg; range: <LOD-18.1 µg/kg	No pollution reported	N/A	Nawrocka *et al.,* 2020
	Pork	Hg (mean): 0.8 ± 1.4 µg/kg; range: <LOD-30.2 µg/kg			

	Poultry	Hg (mean): 0.6 ± 0.7 µg/kg; range: <LOD-10.0 µg/kg			
	Wild boar	Hg (mean): 5.6 ± 10.2 µg/kg; range: <LOD-215 µg/kg			
	Roe deer	Hg (mean): 1.0 ± 2.1 µg/kg; range: <LOD-28.0 µg/kg			
Saudi Arabia	Sausage meat	Hg (mean): 0.125 ± 0.013 µg/g	Major industrial and urban cities (Tabouk, Riyadh, Damamm and Jazan)	N/A	Alturiqi and Albedair, 2012
	Pastrami	Hg (mean): 0.097 ± 0.009 µg/g			
	Beef lain	Hg (mean): 0.087 ± 0.004 µg/g			
	Luncheon	Hg (mean): 0.112 ± 0.011 µg/g			
	Chicken	Hg (mean ranges): 0.009-0.015 µg/g			

Veal	Hg (mean ranges): 0.032-0.087 µg/g
Sheep	Hg (mean ranges): 0.011-0.027 µg/g
Camel	Hg (mean ranges): 0.024-0.054 µg/g

Table 5. Estimate of weekly intakes of Hg from consumption of Hg-containing foods in Europe, USA and Asia according to dietary guidelines in adults and children (2-10 years of age).

Food	Country of origin	Hg source	Reference	Hg in food (µg/g)	Portion size (g)	Portions eaten per week	Hg intake per week	% PTWI for EU adult (70kg)	% PTWI for US adult (80kg)	% PTWI for Asian adult (58kg)	% PTWI for child (2 years, 12kg)	% PTWI for child (4 years, 16kg)	% PTWI for child (8 years, 26kg)	% PTWI for child (10 years, 36kg)
Rice (total)	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.08	75	7	40.95	58.50	51.19	70.60	170.63	127.97	78.75	56.88
Rice white	USA	N/A	Palmieri *et al.,* 2020	0.00	75	7	1.27	1.82	1.59	2.19	5.29	3.97	2.44	1.76
	China	N/A	Palmieri *et al.,* 2020	0.00	75	7	1.27	1.81	1.58	2.18	5.27	3.95	2.43	1.76
Brown rice	USA	N/A	Palmieri *et al.,* 2020	0.00	75	7	1.23	1.76	1.54	2.13	5.14	3.86	2.37	1.71

Vegetables (total)	Poland	No pollution reported	Jędrzejczak *et al.,* 2002	0.00	75	35	1.31	1.88	1.64	2.26	5.47	4.10	2.52	1.82
	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.13	75	35	341.25	487.50	426.56	588.36	1421.88	1066.41	656.25	473.96
Fruits (total)	Poland	No pollution reported	Jędrzejczak *et al.,* 2002	0.00	75	28	2.31	3.30	2.89	3.98	9.63	7.22	4.44	3.21
	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.04	75	28	91.92	131.31	114.90	158.48	382.99	287.24	176.76	127.66
Potato	Poland	No pollution reported	Jędrzejczak *et al.,* 2002	0.00	170	7	0.71	1.02	0.89	1.23	2.98	2.23	1.37	0.99
	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.00	170	7	4.87	6.95	6.08	8.39	20.28	15.21	9.36	6.76

Cabbage	Poland	No pollution reported	Jędrzejczak *et al.,* 2002	0.00	89	7	0.31	0.45	0.39	0.54	1.30	0.97	0.60	0.43
Tomato	Poland	No pollution reported	Jędrzejczak *et al.,* 2002	0.00	180	7	0.38	0.54	0.47	0.65	1.58	1.18	0.73	0.53
	China	Hg mining area	Li *et al.,* 2017	0.03	180	7	36.63	52.33	45.79	63.15	152.62	114.46	70.44	50.87
Cucumber	Poland	No pollution reported	Jędrzejczak *et al.,* 2002	0.00	52	7	0.18	0.26	0.23	0.31	0.76	0.57	0.35	0.25
	China	Coal-fired power plants	Li *et al.,* 2017	0.04	52	7	14.00	19.99	17.49	24.13	58.32	43.74	26.92	19.44
Lettuce	China	Coal-fired	Li *et al.,* 2017	0.04	150	7	40.99	58.56	51.24	70.68	170.80	128.10	78.83	56.93

		power plants											
	China	No pollution reported	Li *et al.,* 2017	0.00	150	7	0.37	0.53	0.46	0.63	1.53	1.15	0.71	0.51
Pepper	China	Coal-fired power plants	Li *et al.,* 2017	0.05	80	7	27.81	39.73	34.76	47.95	115.87	86.91	53.48	38.62
	China	No pollution reported	Li *et al.,* 2017	0.00	80	7	0.52	0.74	0.65	0.90	2.17	1.63	1.00	0.72
Meat (total)	China	No pollution reported	Shao *et al.,* 2013	0.00	90	2	0.87	1.25	1.09	1.51	3.64	2.73	1.68	1.21
	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.22	90	2	39.60	56.57	49.50	68.28	165.00	123.75	76.15	55.00

Beef	Poland	No pollution reported	Nawrocka *et al.,* 2020	0.01	90	2	1.44	2.06	1.80	2.48	6.00	4.50	2.77	2.00
Pork	Poland	No pollution reported	Nawrocka *et al.,* 2020	0.01	90	2	1.44	2.06	1.80	2.48	6.00	4.50	2.77	2.00
Poultry	Poland	No pollution reported	Nawrocka *et al.,* 2020	0.01	90	2	1.08	1.54	1.35	1.86	4.50	3.38	2.08	1.50
	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.16	90	2	28.80	41.14	36.00	49.66	120.00	90.00	55.38	40.00
Fish (total)	Wanshan area, China	Hg mining area	Xu *et al.,* 2020	0.29	140	2	81.20	116.00	101.50	140.00	338.33	253.75	156.15	112.78
Seabass	USA	No pollution reported	FDA, 2012	0.17	140	2	46.76	66.80	58.45	80.62	194.83	146.13	89.92	64.94

	EU	No pollution reported	Barone *et al.,* 2021	0.13	140	2	36.40	52.00	45.50	62.76	151.67	113.75	70.00	50.56
	Guangdong Province, South China	No pollution reported	Li *et al.,* 2012	0.04	140	2	12.52	17.88	15.65	21.58	52.15	39.11	24.07	17.38
	Hong-Kong	No pollution reported	Chung *et al.,* 2008	0.04	140	2	10.64	15.20	13.30	18.34	44.33	33.25	20.46	14.78
Mackerel Chub	USA	No pollution reported	FDA, 2012	0.09	140	2	24.64	35.20	30.80	42.48	102.67	77.00	47.38	34.22
	EU	No pollution reported	Barone *et al.,* 2021	0.21	140	2	58.80	84.00	73.50	101.38	245.00	183.75	113.08	81.67
	Hong Kong	No pollution reported	Chung *et al.,* 2008	0.21	140	2	59.64	85.20	74.55	102.83	248.50	186.38	114.69	82.83

Swordfish	Italy	No pollution reported	Pastorelli *et al.,* 2012	0.53	140	2	148.12	211.60	185.15	255.38	617.17	462.88	284.85	205.72
	USA	No pollution reported	FDA, 2012	1.12	140	2	314.44	449.20	393.05	542.14	1310.17	982.63	604.69	436.72
Whitefish	USA	No pollution reported	FDA, 2012	0.09	140	2	24.92	35.60	31.15	42.97	103.83	77.88	47.92	34.61
Cod	Poland	No pollution reported	Kuras *et al.,* 2017	0.05	140	2	13.72	19.60	17.15	23.66	57.17	42.88	26.38	19.06
	Guangdong Province, South China	No pollution reported	Li *et al.,* 2012	0.11	140	2	29.68	42.40	37.10	51.17	123.67	92.75	57.08	41.22
	USA	No pollution reported	FDA, 2012	0.11	140	2	31.08	44.40	38.85	53.59	129.50	97.13	59.77	43.17

Haddock	USA	No pollution reported	FDA, 2012	0.06	140	2	15.40	22.00	19.25	26.55	64.17	48.13	29.62	21.39
Hake	Bosnia and Herzegovina (Spain)	Increased content of heavy metal in fish from Neretva River	Djedjibegovic *et al.,* 2020	0.02	140	2	6.44	9.20	8.05	11.10	26.83	20.13	12.38	8.94
	Poland	No pollution reported	Kuras *et al.,* 2017	0.11	140.00	2	0.11	0.11	0.13	0.18	0.44	0.33	0.20	0.15
	USA	No pollution reported	FDA, 2012	0.08	140	2	22.12	31.60	27.65	38.14	92.17	69.13	42.54	30.72
Halibut	Hong Kong	No pollution reported	Chung *et al.,* 2008	0.05	140	2	13.44	19.20	16.80	23.17	56.00	42.00	25.85	18.67

	USA	No pollution reported	FDA, 2012	0.24	140	2	67.48	96.40	84.35	116.34	281.17	210.88	129.77	93.72
Herring	USA	No pollution reported	FDA, 2012	0.08	140	2	21.84	31.20	27.30	37.66	91.00	68.25	42.00	30.33
Mackerel King	Hong Kong	No pollution reported	Chung *et al.,* 2008	0.73	140	2	204.40	292.00	255.50	352.41	851.67	638.75	393.08	283.89
	USA	No pollution reported	FDA, 2012	0.73	140	2	204.40	292.00	255.50	352.41	851.67	638.75	393.08	283.89
Mackerel Atlantic	Bosnia and Herzegovina (Morocco)	Increased content of heavy metals in fish from Neretva River	Djedjibegovic *et al.,* 2020	0.19	140	2	53.76	76.80	67.20	92.69	224.00	168.00	103.38	74.67

	USA	No pollution reported	FDA, 2012	0.05	140	2	14.00	20.00	17.50	24.14	58.33	43.75	26.92	19.44
Monkfish	USA	No pollution reported	FDA, 2012	0.16	140	2	45.08	64.40	56.35	77.72	187.83	140.88	86.69	62.61
Salmon	Hong Kong	No pollution reported	Chung *et al.,* 2008	0.03	140	2	9.52	13.60	11.90	16.41	39.67	29.75	18.31	13.22
	USA	No pollution reported	FDA, 2012	0.02	140	2	6.16	8.80	7.70	10.62	25.67	19.25	11.85	8.56
Tuna fresh skipjack/ steak	Hong Kong	No pollution reported	Chung *et al.,* 2008	0.14	140	2	40.04	57.20	50.05	69.03	166.83	125.13	77.00	55.61
	USA	No pollution reported	FDA, 2012	0.14	140	2	40.32	57.60	50.40	69.52	168.00	126.00	77.54	56.00

Blue mussel	Italy	No pollution reported	Pastorelli *et al.,* 2012	0.02	140	2	5.60	8.00	7.00	9.66	23.33	17.50	10.77	7.78
	Bosnia and Herzegovina (Spain)	Increased content of heavy metals in fish from Neretva River	Djedjibegovic *et al.,* 2020	0.04	140	2	12.32	17.60	15.40	21.24	51.33	38.50	23.69	17.11
Shrimp (deep-water rose)	Italy	No pollution reported	Pastorelli *et al.,* 2012	0.05	140	2	14.00	20.00	17.50	24.14	58.33	43.75	26.92	19.44
Shrimp	USA	No pollution reported	FDA, 2012	0.01	140	2	2.52	3.60	3.15	4.34	10.50	7.88	4.85	3.50
Shrimp (tiger)	Bosnia and Herzegovina (China)	Increased content of heavy	Djedjibegovic *et al.,* 2020	0.06	140	2	16.24	23.20	20.30	28.00	67.67	50.75	31.23	22.56

		metals in fish from Neretva River												
Pollock	USA	No pollution reported	FDA, 2012	0.03	140	2	8.68	12.40	10.85	14.97	36.17	27.13	16.69	12.06
	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.02	140	2	5.04	7.20	6.30	8.69	21.00	15.75	9.69	7.00
Atlantic bluefin tuna	Italy	No pollution reported	Pastorelli *et al.,* 2012	0.32	140	2	88.48	126.40	110.60	152.55	368.67	276.50	170.15	122.89
Crab	Guangdong Province, South China	No pollution reported	Li *et al.,* 2012	0.03	140	2	9.13	13.04	11.41	15.74	38.03	28.53	17.55	12.68
	USA	No pollution reported	FDA, 2012	0.07	140	2	18.20	26.00	22.75	31.38	75.83	56.88	35.00	25.28

Grass carp	Guangdong Province, South China	No pollution reported	Li *et al.,* 2012	0.01	140	2	2.32	3.31	2.89	3.99	9.65	7.24	4.45	3.22
	USA	No pollution reported	FDA, 2012	0.11	140	2	30.80	44.00	38.50	53.10	128.33	96.25	59.23	42.78
Tilapia	Guangdong Province, South China	No pollution reported	Li *et al.,* 2012	0.01	140	2	2.19	3.12	2.73	3.77	9.11	6.83	4.21	3.04
	USA	No pollution reported	FDA, 2012	0.01	140	2	3.64	5.20	4.55	6.28	15.17	11.38	7.00	5.06
Mullet	USA	No pollution reported	FDA, 2012	0.05	140	2	14.00	20.00	17.50	24.14	58.33	43.75	26.92	19.44
	Guangdong Province, South China	No pollution reported	Li *et al.,* 2012	0.01	140	2	2.23	3.19	2.79	3.85	9.30	6.97	4.29	3.10

Sausage meat	Saudi Arabia	Major industrial and urban cities	Alturiqi and Albedair, 2012	0.13	70	2	17.50	25.00	21.88	30.17	72.92	54.69	33.65	24.31
Pastrami	Saudi Arabia	Major industrial and urban cities	Alturiqi and Albedair, 2012	0.10	70	2	13.58	19.40	16.98	23.41	56.58	42.44	26.12	18.86
Beef lain	Saudi Arabia	Major industrial and urban cities	Alturiqi and Albedair, 2012	0.09	70	2	12.18	17.40	15.23	21.00	50.75	38.06	23.42	16.92
Luncheon	Saudi Arabia	Major industrial and	Alturiqi and Albedair, 2012	0.11	70	2	15.68	22.40	19.60	27.03	65.33	49.00	30.15	21.78

		urban cities												
Canned Sardines	USA	No pollution reported	FDA, 2012	0.01	140	2	3.64	5.20	4.55	6.28	15.17	11.38	7.00	5.06
Canned Tuna (light)	USA	No pollution reported	FDA, 2012	0.13	140	2	35.28	50.40	44.10	60.83	147.00	110.25	67.85	49.00
Canned Skipjack Tuna	Hong Kong	No pollution reported	Chung *et al.,* 2008	0.16	140	2	45.64	65.20	57.05	78.69	190.17	142.63	87.77	63.39
Canned Bluefin tuna	Bosnia and Herzegovina (Thailand)	Increased content of heavy metals in fish from Neretva River	Djedjibegovic *et al.,* 2020	0.06	140	2	17.36	24.80	21.70	29.93	72.33	54.25	33.38	24.11

Canned Tuna Bonito	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.08	140	2	22.37	31.96	27.97	38.57	93.22	69.91	43.02	31.07
Canned Salmon	USA	No pollution reported	FDA, 2012	0.01	140	2	3.92	5.60	4.90	6.76	16.33	12.25	7.54	5.44
Fried cod filet	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.06	140	2	16.80	24.00	21.00	28.97	70.00	52.50	32.31	23.33
Fried Pollock	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.02	140	2	6.44	9.20	8.05	11.10	26.83	20.13	12.38	8.94
Pollock fish sticks	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.13	140	2	35.56	50.80	44.45	61.31	148.17	111.13	68.38	49.39
Herring salad	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.09	140	2	24.19	34.56	30.24	41.71	100.80	75.60	46.52	33.60

Octopus, frozen	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.09	140	2	25.62	36.60	32.03	44.17	106.75	80.06	49.27	35.58
Cod fish sticks	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.12	140	2	33.60	48.00	42.00	57.93	140.00	105.00	64.62	46.67
Tuna (Bonito) paste	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.13	140	2	36.40	52.00	45.50	62.76	151.67	113.75	70.00	50.56
Herring sauce	Poland	No pollution reported	Pawlaczyk *et al.*, 2020	0.1	140	2	28.00	40.00	35.00	48.28	116.67	87.50	53.85	38.89

Table 6. Estimated PTWI values for meals composed with Hg-containing products for adults and children in Europe, USA and Asia.

Meal components		Meal eaten once a week							Meal eaten twice a week						
		% PTWI EU adult (70kg)	% PTWI US adult (80kg)	% PTWI Asian adult (58kg)	% PTWI child (2 years, 12kg)	% PTWI child (4 years, 16kg)	% PTWI child (8 years, 26kg)	% PTWI child (10 years, 36kg)	% PTWI EU adult (70kg)	% PTWI US adult (80kg)	% PTWI Asian adult (58kg)	% PTWI child (2 years, 12kg)	% PTWI child (4 years, 16kg)	% PTWI child (8 years, 26kg)	% PTWI child (10 years, 36kg)
Rice + Salad (lettuce, tomato, pepper)	Seabass, US	42.03	36.77	50.72	122.50	91.87	56.54	40.83	84.06	73.55	101.45	245.00	183.75	113.08	81.67
	Seabass, EU	34.63	30.30	41.79	100.92	75.69	46.58	33.64	69.26	60.60	83.59	201.83	151.37	93.15	67.28
	Seabass, China	17.57	15.37	21.20	51.16	38.37	23.61	17.05	35.14	30.74	42.41	102.31	76.74	47.22	34.10
	Mackerel Chub, US	26.23	22.95	31.65	76.42	57.31	35.27	25.47	52.46	45.90	63.31	152.83	114.62	70.54	50.94
	Mackerel Chub, EU	50.63	44.30	61.10	147.58	110.69	68.11	49.19	101.26	88.60	122.21	295.16	221.37	136.23	98.39
	Mackerel Chub, Hong Kong	51.23	44.82	61.83	149.33	112.00	68.92	49.78	102.46	89.65	123.65	298.66	224.00	137.85	99.55
	Swordfish, Italy	114.43	100.12	138.10	333.67	250.25	154.00	111.22	228.86	200.25	276.21	667.33	500.50	308.00	222.44
	Swordfish, US	233.23	204.07	281.48	680.17	510.12	313.92	226.72	466.46	408.15	562.96	1360.33	1020.25	627.85	453.44
	Whitefish, US	26.43	23.12	31.90	77.00	57.75	35.54	25.67	52.86	46.25	63.79	154.00	115.50	71.08	51.33

Cod, Poland	18.43	16.12	22.24	53.67	40.25	24.77	17.89	36.86	32.25	44.48	107.33	80.50	49.54	35.78
Cod, China	29.83	26.10	36.00	86.92	65.19	40.11	28.97	59.66	52.20	72.00	173.83	130.37	80.23	57.94
Cod, US	30.83	26.97	37.21	89.83	67.37	41.46	29.94	61.66	53.95	74.41	179.66	134.75	82.92	59.89
Haddock, US	19.63	17.17	23.69	57.17	42.87	26.38	19.06	39.26	34.35	47.38	114.33	85.75	52.77	38.11
Hake, Spain	13.23	11.57	15.97	38.50	28.87	17.77	12.83	26.46	23.15	31.93	77.00	57.75	35.54	25.67
Hake, US	24.43	21.37	29.48	71.17	53.37	32.85	23.72	48.86	42.75	58.96	142.33	106.75	65.69	47.44
Halibut, US	18.23	15.95	22.00	53.08	39.81	24.50	17.69	36.46	31.90	44.00	106.16	79.62	49.00	35.39
Halibut, Hong Kong	56.83	49.72	68.59	165.67	124.25	76.46	55.22	113.66	99.45	137.17	331.33	248.50	152.92	110.44
Herring, US	24.23	21.20	29.24	70.58	52.94	32.58	23.53	48.46	42.40	58.48	141.16	105.87	65.15	47.05
Mackerel Atlantic, Morocco	47.03	41.15	56.76	137.08	102.81	63.27	45.69	94.06	82.30	113.52	274.16	205.62	126.54	91.39
Mackerel Atlantic, US	18.63	16.30	22.48	54.25	40.69	25.04	18.08	37.26	32.60	44.96	108.50	81.37	50.08	36.17
Salmon, US	13.03	11.40	15.72	37.92	28.44	17.50	12.64	26.06	22.80	31.45	75.83	56.87	35.00	25.28
Salmon, Hong Kong	15.43	13.50	18.62	44.92	33.69	20.73	14.97	30.86	27.00	37.24	89.83	67.37	41.46	29.94
Tuna Skipjack fresh steak, US	13.03	11.40	15.72	37.92	28.44	17.50	12.64	26.06	22.80	31.45	75.83	56.87	35.00	25.28

	Tuna Skipjack fresh steak, Hong Kong	37.23	32.57	44.93	108.50	81.37	50.08	36.17	74.46	65.15	89.86	217.00	162.75	100.15	72.33
	Pollock, US	14.83	12.97	17.90	43.17	32.37	19.92	14.39	29.66	25.95	35.79	86.33	64.75	39.85	28.78
	Pollock, Poland	12.23	10.70	14.76	35.58	26.69	16.42	11.86	24.46	21.40	29.52	71.16	53.37	32.85	23.72
	Atlantic bluefin tuna fresh steak, Italy	71.83	62.85	86.69	209.42	157.06	96.65	69.81	143.66	125.70	173.38	418.83	314.12	193.31	139.61
	Crab, China	15.15	13.25	18.28	44.10	33.07	20.35	14.70	30.30	26.51	36.56	88.20	66.15	40.71	29.40
	Crab, US	21.63	18.92	26.10	63.00	47.25	29.08	21.00	43.26	37.85	52.21	126.00	94.50	58.15	42.00
	Carp, China	10.28	9.00	12.41	29.91	22.43	13.80	9.97	20.56	17.99	24.82	59.81	44.86	27.61	19.94
	Carp, US	30.63	26.80	36.97	89.25	66.94	41.19	29.75	61.26	53.60	73.93	178.50	133.87	82.38	59.50
	Tilapia, China	10.19	8.92	12.30	29.64	22.23	13.68	9.88	20.38	17.83	24.60	59.28	44.46	27.36	19.76
	Tilapia, US	11.23	9.82	13.55	32.67	24.50	15.08	10.89	22.46	19.65	27.10	65.33	49.00	30.15	21.78
	Mullet, US	18.63	16.30	22.48	54.25	40.69	25.04	18.08	37.26	32.60	44.96	108.50	81.37	50.08	36.17
	Mullet, China	10.22	8.94	12.34	29.73	22.30	13.72	9.91	20.44	17.89	24.67	59.46	44.60	27.44	19.82
Rice +	Beef, Poland	9.66	8.45	11.65	28.08	21.06	12.96	9.36	19.31	16.90	23.31	56.16	42.12	25.92	18.72
Salad (lettuce,	Pork, Poland	9.66	8.45	11.65	28.08	21.06	12.96	9.36	19.31	16.90	23.31	56.16	42.12	25.92	18.72
tomato, pepper)	Poultry, Poland	9.40	8.22	11.34	27.33	20.50	12.61	9.11	18.80	16.45	22.69	54.66	41.00	25.23	18.22
Rice +	Canned Sardines	11.23	9.82	13.55	32.67	24.50	15.08	10.89	22.46	19.65	27.10	65.33	49.00	30.15	21.78

Salad (lettuce, tomato, pepper)	Canned Tuna (light)	33.83	29.60	40.83	98.58	73.94	45.50	32.86	67.66	59.20	81.65	197.16	147.87	91.00	65.72
	Canned Skipjack Tuna	41.23	36.07	49.76	120.17	90.12	55.46	40.06	82.46	72.15	99.52	240.33	180.25	110.92	80.11
	Canned Bluefin tuna	21.03	18.40	25.38	61.25	45.94	28.27	20.42	42.06	36.80	50.76	122.50	91.87	56.54	40.83
	Canned Tuna Bonito	24.61	21.53	29.70	71.69	53.77	33.09	23.90	49.22	43.06	59.40	143.38	107.54	66.18	47.79
	Canned Salmon	11.43	10.00	13.79	33.25	24.94	15.35	11.08	22.86	20.00	27.59	66.50	49.87	30.69	22.17
	Fried cod filet	20.63	18.05	24.90	60.08	45.06	27.73	20.03	41.26	36.10	49.79	120.16	90.12	55.46	40.05
	Fried Pollock	13.23	11.57	15.97	38.50	28.87	17.77	12.83	26.46	23.15	31.93	77.00	57.75	35.54	25.67
	Pollock fish sticks	34.03	29.77	41.07	99.17	74.37	45.77	33.06	68.06	59.55	82.14	198.33	148.75	91.54	66.11
	Herring salad	28.63	25.05	34.55	83.42	62.56	38.50	27.81	57.26	50.10	69.10	166.83	125.12	77.00	55.61
	Cod fish sticks	32.23	28.20	38.90	93.92	70.44	43.35	31.31	64.46	56.40	77.79	187.83	140.87	86.69	62.61
	Octopus, frozen	26.93	23.56	32.50	78.46	58.84	36.21	26.15	53.86	47.12	65.00	156.91	117.69	72.42	52.30
Rice + Salad (lettuce, tomato, pepper)	Sausage meat	21.13	18.49	25.50	61.54	46.16	28.40	20.51	42.26	36.97	51.00	123.08	92.31	56.81	41.03
	Pastrami	18.33	16.04	22.12	53.37	40.03	24.63	17.79	36.66	32.07	44.24	106.75	80.06	49.27	35.58
	Beef lain	17.33	15.16	20.91	50.46	37.84	23.29	16.82	34.66	30.32	41.83	100.91	75.69	46.58	33.64
	Luncheon	19.83	17.35	23.93	57.75	43.31	26.65	19.25	39.66	34.70	47.86	115.50	86.62	53.31	38.50
Potatoes +	Seabass, US	76.16	66.64	91.92	222.14	166.60	102.53	74.05	152.32	133.28	183.84	444.28	333.21	205.05	148.09
	Seabass, EU	60.37	52.82	72.86	176.08	132.06	81.27	58.69	120.74	105.65	145.72	352.15	264.11	162.53	117.38

Salad (lettuce, tomato, pepper)	Seabass, China	17.88	15.65	21.58	52.15	39.11	24.07	17.38	35.76	31.29	43.16	104.30	78.23	48.14	34.77
	Mackerel Chub, US	35.20	30.80	42.48	102.67	77.00	47.38	34.22	70.40	61.60	84.97	205.33	154.00	94.77	68.44
	Mackerel Chub, EU	84.00	73.50	101.38	245.00	183.75	113.08	81.67	168.00	147.00	202.76	490.00	367.50	226.15	163.33
	Mackerel Chub, Hong Kong	85.20	74.55	102.83	248.50	186.38	114.69	82.83	170.40	149.10	205.66	497.00	372.75	229.38	165.67
	Swordfish, Italy	211.60	185.15	255.38	617.17	462.88	284.85	205.72	423.20	370.30	510.76	1234.33	925.75	569.69	411.44
	Swordfish, US	516.00	451.50	622.76	1505.00	1128.75	694.62	501.67	1032.00	903.00	1245.52	3010.00	2257.50	1389.23	1003.33
	Whitefish, US	154.40	135.10	186.34	450.33	337.75	207.85	150.11	308.80	270.20	372.69	900.67	675.50	415.69	300.22
	Cod, Poland	89.48	78.30	107.99	260.98	195.74	120.45	86.99	178.96	156.59	215.99	521.97	391.48	240.91	173.99
	Cod, China	95.48	83.55	115.23	278.48	208.86	128.53	92.83	190.96	167.09	230.47	556.97	417.73	257.06	185.66
	Cod, US	163.60	143.15	197.45	477.17	357.88	220.23	159.06	327.20	286.30	394.90	954.33	715.75	440.46	318.11
	Haddock, US	191.20	167.30	230.76	557.67	418.25	257.38	185.89	382.40	334.60	461.52	1115.33	836.50	514.77	371.78
	Hake, Spain	306.00	267.75	369.31	892.50	669.38	411.92	297.50	612.00	535.50	738.62	1785.00	1338.75	823.85	595.00
	Hake, US	692.40	605.85	835.66	2019.50	1514.63	932.08	673.17	1384.80	1211.70	1671.31	4039.00	3029.25	1864.15	1346.33
	Halibut, US	504.00	441.00	608.28	1470.00	1102.50	678.46	490.00	1008.00	882.00	1216.55	2940.00	2205.00	1356.92	980.00
	Halibut, Hong Kong	151.60	132.65	182.97	442.17	331.63	204.08	147.39	303.20	265.30	365.93	884.33	663.25	408.15	294.78
	Herring, US	93.20	81.55	112.48	271.83	203.88	125.46	90.61	186.40	163.10	224.97	543.67	407.75	250.92	181.22

Mackerel Atlantic, Morocco	163.60	143.15	197.45	477.17	357.88	220.23	159.06	327.20	286.30	394.90	954.33	715.75	440.46	318.11
Mackerel Atlantic, US	86.40	75.60	104.28	252.00	189.00	116.31	84.00	172.80	151.20	208.55	504.00	378.00	232.62	168.00
Salmon, US	40.00	35.00	48.28	116.67	87.50	53.85	38.89	80.00	70.00	96.55	233.33	175.00	107.69	77.78
Salmon, Hong Kong	54.40	47.60	65.66	158.67	119.00	73.23	52.89	108.80	95.20	131.31	317.33	238.00	146.46	105.78
Tuna Skipjack fresh steak, US	59.60	52.15	71.93	173.83	130.38	80.23	57.94	119.20	104.30	143.86	347.67	260.75	160.46	115.89
Tuna Skipjack fresh steak, Hong Kong	172.80	151.20	208.55	504.00	378.00	232.62	168.00	345.60	302.40	417.10	1008.00	756.00	465.23	336.00
Pollock, US	140.00	122.50	168.97	408.33	306.25	188.46	136.11	280.00	245.00	337.93	816.67	612.50	376.92	272.22
Pollock, Poland	115.20	100.80	139.03	336.00	252.00	155.08	112.00	230.40	201.60	278.07	672.00	504.00	310.15	224.00
Atlantic bluefin tuna fresh steak, Italy	223.20	195.30	269.38	651.00	488.25	300.46	217.00	446.40	390.60	538.76	1302.00	976.50	600.92	434.00
Crab, China	41.84	36.61	50.50	122.03	91.53	56.32	40.68	83.68	73.22	100.99	244.07	183.05	112.65	81.36
Crab, US	48.40	42.35	58.41	141.17	105.88	65.15	47.06	96.80	84.70	116.83	282.33	211.75	130.31	94.11
Carp, China	25.71	22.49	31.03	74.98	56.24	34.61	24.99	51.42	44.99	62.05	149.96	112.47	69.21	49.99
Carp, US	110.00	96.25	132.76	320.83	240.63	148.08	106.94	220.00	192.50	265.52	641.67	481.25	296.15	213.89

	Tilapia, China	72.72	63.63	87.77	212.11	159.08	97.90	70.70	145.45	127.27	175.54	424.22	318.17	195.80	141.41
	Tilapia, US	24.80	21.70	29.93	72.33	54.25	33.38	24.11	49.60	43.40	59.86	144.67	108.50	66.77	48.22
	Mullet, US	153.60	134.40	185.38	448.00	336.00	206.77	149.33	307.20	268.80	370.76	896.00	672.00	413.54	298.67
	Mullet, China	142.63	124.80	172.14	416.00	312.00	192.00	138.67	285.26	249.60	344.27	832.00	624.00	384.00	277.33
Potatoes +	Beef, Poland	31.37	27.44	37.85	91.48	68.61	42.22	30.49	62.73	54.89	75.71	182.96	137.22	84.44	60.99
Salad (lettuce,	Pork, Poland	49.37	43.19	59.58	143.98	107.99	66.45	47.99	98.73	86.39	119.16	287.96	215.97	132.91	95.99
tomato, pepper)	Poultry, Poland	48.67	42.58	58.74	141.95	106.46	65.51	47.32	97.33	85.17	117.47	283.89	212.92	131.03	94.63
Potatoes +	Canned Sardines	9.31	8.15	11.24	27.17	20.38	12.54	9.06	18.63	16.30	22.48	54.33	40.75	25.08	18.11
Salad (lettuce,	Canned Tuna (light)	54.00	47.25	65.17	157.50	118.13	72.69	52.50	108.00	94.50	130.34	315.00	236.25	145.38	105.00
tomato, pepper)	Canned Skipjack Tuna	66.74	58.40	80.55	194.67	146.00	89.85	64.89	133.49	116.80	161.10	389.33	292.00	179.69	129.78
	Canned Bluefin tuna	49.80	43.58	60.10	145.25	108.94	67.04	48.42	99.60	87.15	120.21	290.50	217.88	134.08	96.83
	Canned Tuna Bonito	76.36	66.82	92.16	222.72	167.04	102.79	74.24	152.72	133.63	184.32	445.43	334.08	205.58	148.48
	Canned Salmon	42.40	37.10	51.17	123.67	92.75	57.08	41.22	84.80	74.20	102.34	247.33	185.50	114.15	82.44
	Fried cod filet	63.80	55.83	77.00	186.08	139.56	85.88	62.03	127.60	111.65	154.00	372.17	279.13	171.77	124.06
	Fried Pollock	36.80	32.20	44.41	107.33	80.50	49.54	35.78	73.60	64.40	88.83	214.67	161.00	99.08	71.56
	Pollock fish sticks	106.40	93.10	128.41	310.33	232.75	143.23	103.44	212.80	186.20	256.83	620.67	465.50	286.46	206.89
	Herring salad	155.60	136.15	187.79	453.83	340.38	209.46	151.28	311.20	272.30	375.59	907.67	680.75	418.92	302.56

	Cod fish sticks	137.20	120.05	165.59	400.17	300.13	184.69	133.39	274.40	240.10	331.17	800.33	600.25	369.38	266.78
	Octopus, frozen	93.36	81.69	112.68	272.30	204.23	125.68	90.77	186.72	163.38	225.35	544.60	408.45	251.35	181.53
Potatoes +	Sausage meat	50.20	43.93	60.59	146.42	109.81	67.58	48.81	100.40	87.85	121.17	292.83	219.63	135.15	97.61
Salad (lettuce,	Pastrami	42.59	37.26	51.40	124.22	93.16	57.33	41.41	85.18	74.53	102.80	248.43	186.32	114.66	82.81
tomato, pepper)	Beef lain	20.59	18.01	24.85	60.05	45.04	27.71	20.02	41.18	36.03	49.70	120.10	90.07	55.43	40.03
	Luncheon	24.46	21.40	29.52	71.33	53.50	32.92	23.78	48.91	42.80	59.03	142.67	107.00	65.85	47.56

CHAPTER 3:

Hair mercury is not associated with cytokines of the Th17 axis in fish-consuming young adults from the Republic of Seychelles.

Abstract

Background: Fish consumption is the primary route of exposure to methylmercury (MeHg) and is also a rich source of beneficial nutrients including n-3 long chain polyunsaturated fatty acids (LCPUFA). Exposure to mercury (Hg) has been proposed as a contributing factor in the development of autoimmunity whilst n-3 LCPUFA is known to reduce inflammation and ameliorate autoimmune disease. Inflammatory cytokines associated with a Th17 response contribute to autoimmune pathogenesis; however, the impact of MeHg on Th17 status remains unexplored.

Objectives: To examine the association between MeHg exposure and serum Th17 cytokine status in a high fish-eating population.

Methods: In total, 440 young adults, recruited as part of the Seychelles Child Development Study, were included in this study. Hair Hg was determined using cold vapor atomic absorption spectroscopy while LCPUFA were quantified using gas chromatography-mass spectrometry (GC-MS). Cytokines (IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-31, IL-33) associated with Th17 axis were quantified in the serum, by using a multiplex electrochemiluminescence immunoassay. Multivariable regression investigated associations between hair Hg status and cytokines with and without adjustment for LCPUFA and other covariates.

Results: There was no association between hair Hg and any of Th17-associated cytokines in models unadjusted as well as adjusted for total n-3 LCPUFA, total n-6 LCPUFA and n-6:n-3 LCPUFA ratio.

Conclusions:

In high fish consuming young adults, hair Hg status was not associated with Th17 cytokines. Further research is needed to see if these observations are relevant to autoimmune predisposed individuals.

Keywords: Th17 cytokines; Autoimmunity; IL-17; Mercury; Fish consumption; Diet

Introduction

Autoimmunity results when the human body develops an inappropriate immune response against self-antigens. A loss of tolerance to self-antigen primes the immune system to develop autoreactive T cells and autoantibodies which subsequently can elicit an autoimmune response resulting in chronic inflammation and disease progression over time. The progressive development of autoimmune pathology can result in tissue/organ damage and increased mortality (Martin *et al.,* 2014; Rosenblum *et al.,* 2015). Clinical heterogeneity and the polygenic nature of autoimmune disease imply multifactorial contributions of both genetic and environmental factors in the induction, progression and severity of the pathology (Rosenblum *et al.,* 2015). One proposed environmental stimulus of autoimmune disease is mercury (Hg). Humans are primarily exposed to methylmercury (MeHg) through the consumption of fish, as all fish contain MeHg which bioaccumulates in the aquatic food chain (Khan and Wang, 2020).

Evidence from animal studies supports a role for Hg, both inorganic and organic, in the development and exacerbation of autoimmunity; nevertheless, the evidence from humans remains limited (Crowe *et al.,* 2017). Most of the observations in fish-eating populations have reported no association between Hg exposure and markers of autoimmunity (Monastero *et al.,* 2017; Nyland *et al.,* 2011b), and provide indications that nutrients present in fish, such as the anti-inflammatory n-3 LCPUFA (Stratakis *et al.,* 2020) may play a role in restoring a tolerogenic phenotype (Lee, 2018) to mitigate MeHg-mediated toxicity (Nøstbakken *et al.,* 2012; McSorley *et al.,* 2018). Some studies, however, have suggested a potential link between Hg exposure from fish consumption and risk of autoimmune dysfunction, with a reported higher prevalence of antinuclear antibodies (ANA), anti-nucleolar antibodies (ANoA) (Silva *et al.,* 2004; Nyland *et al.,* 2011a; Somers *et al.,* 2015; McSorley *et al.,* 2020) and serum IL-17 concentrations (Nyland *et al.,* 2011a). Previous research conducted in the cohort of high fish eating 19-year-old adults within the Seychelles (McSorley *et al.,* 2020) reported hair Hg status to be associated with an increased probability of having detectable concentrations of ANA after adjustment for n-6:n-3 LCPUFA ratio. Whilst the presence of ANA is indicative of an autoimmune response, they are not

diagnostic of autoimmunity, as measurable amounts of ANA are known to be detected in healthy individuals who never develop autoimmune disease.

There is a need to explore the impact of Hg exposure on a broad range of indices that are associated with autoimmunity to provide a more informed insight into the potential impact of Hg exposure on autoimmune pathogenesis and immune function in humans. The Th17 immune response is associated with autoimmunity with evidence to implicate a deleterious role for an enhanced Th17 response in the pathogenesis of a range of autoimmune conditions including psoriasis, rheumatoid arthritis (RA) and multiple sclerosis (MS) (McGinley *et al.,* 2020). Studies to date have demonstrated that cytokines of the Th17 axis, including IL-17A, IL-17E, IL-22, are associated with increased disease activity and severity in individuals diagnosed with Systemic Lupus Erythematosus (SLE) (Cheng *et al.,* 2009; Chen *et al.,* 2010; Robak *et al.,* 2013; El-Gazzar *et al.,* 2017; Selvaraja *et al.,* 2019); while concentrations of IL-17 have been shown to be correlated with IL-6, IL-21, IL-22, and IL-23 as well as other clinical markers of autoimmunity, including ANA and rheumatoid factor (Miletic *et al.,* 2012; Hu *et al.,* 2013). Furthermore, IL-17, along with other cytokines of Th17 axis, have been linked to the release of alarmin IL-33 upon cell necrosis and/or as the result of unresolved tissue damage (Liew *et al.,* 2016). Increased concentrations of IL-33 have also been found in patients with autoimmune disease (Pei *et al.,* 2014).

Owing to great importance of fish in the diet, being rich in health-promoting nutrients, including n-3 LCPUFA, however also the primary source of MeHg exposure as consumption of any fish can increase hair MeHg hair (Myers *et al.,* 2009), this study aims to investigate the association between hair Hg and the concentrations of cytokines associated with the Th17 axis in a cohort of high fish eating 19-year-old Seychellois adults. Furthermore, we will investigate if an individual's n-3 LCPUFA profile will impact this relationship. It is hypothesized that hair Hg will be associated with cytokines of the Th17 axis and that higher total n-3 LCPUFA concentrations will modify this association.

Methods

Study population

The participants in the current study formed part of an ongoing multi-cohort longitudinal observational study, the Seychelles Child Development Study (SCDS), which is primarily focused on investigating associations between pre- and post-natal MeHg exposure from frequent consumption of ocean fish with health outcomes, including neurodevelopmental and immune outcomes in the local residents of Mahé, the main island of the Republic of Seychelles (Davidson *et al.,* 1998). The SCDS was created in the response to public health concerns of health risks associated with MeHg exposure from ingestion of Hg-contaminated fish, following the Minamata outbreak in Japan (Harada, 1995), with the primary aim to evaluate associations between prenatal MeHg exposure from diet rich and potential adverse health outcomes. The MeHg exposure in Seychellois population is obtained through daily consumption of ocean fish, with MeHg content similar to commercially available fish worldwide (Davidson *et al.,* 2011). The initial recruitment of the Main Cohort started in 1989-1990 and included 779 child-mother, 6 months postpartum, who agreed to provide a hair sample. Since then, mothers and their children have been subsequently followed up throughout the study duration with the purpose of comprehensive health assessments at various timepoints.

In the current study, data were obtained from the individuals at 19 years of age, who are the children of the mothers (n=779), who were recruited as part of the 'Main SCDS cohort' (Davidson *et al.,* 1998). After subsequent exclusions due to specific reasons, including maternal illness during pregnancy, insufficient sample material (e.g. hair, blood sample), pregnancy-specific (e.g. twin births, prematurity, severe perinatal illness, closed head trauma with loss of consciousness, encephalitis or meningitis) and postnatal conditions (e.g. epilepsy, head trauma or meningitis), a total of 510 participants provided information on anthropometric measures (age/date of birth, weight, height), maternal socioeconomic status and completed fish use questionnaire. Further exclusions resulted from insufficient stored serum samples for analysis (n=174), or missing data on hair Hg (n=70) or LCPUFA concentration (n=4) or

other information related to medical issues or owing to emigration (n=20). A final number of 440 participants were included in the analysis within this paper (see figure 1).

This study was reviewed and approved by the Seychelles Ethics Board and the Research Subjects Review Board at the University of Rochester. All participants provided informed consent. Details of recruitment and demographic characteristics of the study participants have been previously reported (McSorley *et al.,* 2020).

Blood sample collection

Non-fasting blood samples were collected via antecubital venipuncture technique from the participants at the age ~19 y. Serum aliquots were obtained, shipped to and stored at −80°C at Ulster University until analysis.

Cytokines of Th17 axis and IL-33 analysis

Serum samples were analyzed for cytokine concentration using the multiplex electrochemiluminescence-based immunoassay, U-PLEX TH-17 multiplex assay (Combo 1 (hu) SECTOR (K15075K-2) (Meso Scale Discovery (MSD), Gaithersburg, MD, USA) according to the manufacturer's instructions. The assay included quantification of serum concentrations of: IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-31, IL-33. These cytokines were selected based on previous studies, which indicated that cytokines of Th-17 axis, together with alarmin cytokine IL-33, are promising biomarkers of autoimmunity (Silva *et al.,* 2004; Gardner *et al.,* 2010a; Nyland *et al.,* 2011a; Somers *et al.,* 2015; McSorley *et al.,* 2020). MSD plates were analyzed using the MS2400 imager. Results were presented as mean (± SD) in pg/mL. The inter assay coefficient of variance (%CV) for cytokines were IL-17A (17.90%), IL-17E (18.87%), IL-17F (11.34%), IL-21 (23.72%), IL-22 (12.08%), IL-23 (13.41%), IL-27 (8.55%), IL-31 (16.23%) and IL-33 (16.78%). The lower limit of detection (LLOD) for each cytokine were 1.72 pg/ml for IL-17A, 0.28 pg/ml for IL-17E, 113 pg/ml for IL-17F, 0.75 pg/ml for IL-21, 0.06 pg/ml for IL-22, 0.99 pg/ml for IL-23, 8.2 pg/ml for IL-27, 5.5 pg/ml

for IL-31 and 0.53 mg/ml for IL-33.The detection rates above the LLOD were IL-17A (39, 8.86%); IL-17E (104, 23.64%); IL-17F (159, 36.14%); IL-21 (409, 92.95%); IL-22 (437, 99.32%); IL-23 (374, 85.00%); IL-27 (439, 99.77%); IL-31 (229, 52.05%) and IL-33 (343, 77.95%). In these cases, binary transformation was employed where LLOD values were replaced with 0. Values greater than LLOD were replaced with 1.

Hair sampling and determination of methylmercury (MeHg) exposure

MeHg exposure was quantified in hair samples, taken close to the scalp at the age of ~19 years of age, and analyzed by using Cold Vapor Atomic Absorption at the University of Rochester as described (Cernichiari *et al.,* 1995; van Wijngaarden *et al.,* 2017). Briefly, MeHg content was measured in the longest scalp of the hair segment available of approximately 1 cm length, by using cold vapor atomic absorption and applying Magos reagents, which are necessary for subsequent reduction of Hg ions to elemental Hg (Magos, 1971). All Hg results presented as MeHg are total Hg (THg) based on the assumption that ~80% of THg in hair is MeHg within the Seychellois population (Cernichiari *et al.,* 1995).

Long chain polyunsaturated fatty acids (LCPUFA)

Plasma phospholipid LCPUFA were measured as previously described (van Wijngaarden *et al.,* 2017). Briefly, total lipids were extracted from plasma samples, according to an adaptation of the method by Folch *et al.* (1957). A solid phase extraction using an NH-2 cartridge system conditioned with chloroform, followed by a series of solvent elutions was used to isolate phospholipids. Absolute amounts of LCPUFA were determined using gas chromatography mass spectrometry (GCMS) according to Bonham *et al.,* (2008) and included linoleic acid (LA, C18:2 n-6), α-linolenic acid (ALA, C18:3 n-3), arachidonic acid (AA, C20:4 n-6), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). Results were presented as mg/mL to indicate physiological quantities. Total n-6 LCPUFA (mg/mL) was calculated by the addition of LA and AA concentrations, and ALA, EPA and DHA were summed to calculate total n-3 LCPUFA (mg/mL). The n-6:n-3 ratio was calculated.

Statistical analysis

Descriptive statistics summarized the distributions of MeHg, serum autoimmune biomarkers (Th17 cytokines and alarmin IL-33), total n-3 and n-6 LCPUFA, n-6:n-3 LCPUFA ratio and covariates, which previously have been shown to be associated with MeHg exposure, including age, body mass index (BMI), sex, and maternal socioeconomic status (McSorley *et al.,* 2020). Based on the preliminary analysis, all values were natural log transformed for the linear regression models. All statistical analyses were performed using R, Version 3.5.1. Statistical significance in all analyses was determined using a two-sided approach $\alpha = 0.05$. Regression model assumptions were checked using standard methods (Weisberg, 2005). If violated, transforming the outcome, or fitting nonlinear additive models were considered (Hastie and Tibshirani, 1990).

Associations between serum cytokines and MeHg exposure were examined using three statistical models, which were determined a priori. Models were evaluated for extreme outliers and unduly influential points. All outcome models were evaluated using linear regression and were adjusted for set of covariates, which in every case included maternal socioeconomic status as well as BMI and sex of child reported at age of 19 years. Models were examined against the complete dataset and with outlier subjects with extreme cytokine status removed (n=2). Model 1 investigated the relationship between hair Hg and cytokines of Th17 axis and alarmin IL-33; following adjustment for total n-3 and n-6 LCPUFA concentrations in Model 2 and n-6:n-3 LCPUFA ratio in Model 3.

Results

The diagram (see figure 1) provides a summarized overview of the participants enrollment and assessment, as well as processes involved in data collection and analysis for the purpose of the current study.

Hg exposure and population characteristics

Participant demographic characteristics are presented in table 1. Cytokines associated with a Th17 and IL-33 response were analyzed for a total of 440 participants, consisting of 250 females and 190 males. Average hair Hg concentrations were 10.21 (5.98) ppm. In this cohort, males had significantly higher mean (SD) hair Hg than females (12.39 (6.85) ppm and 8.56 (4.59) ppm respectively; $p < 0.001$). Total n-6 LCPUFA and total n-3 LCPUFA were 0.15 (0.04) mg/mL and 0.05 (0.01) mg/mL, respectively. Mean BMI (SD) was significantly lower in males compared to females (21.28 (3.48) in males vs 22.8 (5.35) in females; $p < 0.010$).

Hg exposure and cytokines of Th17 axis and alarmin IL-33

Regression analyses for covariate-adjusted associations between MeHg exposure and autoimmune biomarkers are presented in table 2. Hair Hg was not significantly associated with any of the cytokines of Th17 axis or IL-33, either in unadjusted models or after adjustment for n-3 LCPUFA and n-6 LCPUFA concentrations, or after controlling for n-6:n-3 LCPUFA ratio.

Covariates

Concentrations of IL-27 were associated with maternal socioeconomic status ($\beta = -0.005$; 95% CI: -0.010, 0.000; $p = 0.044$) in the model adjusted for n-6:n-3 LCPUFA ratio. IL-27 concentrations were negatively associated with smoking status in adjusted for total n-6 and n-3 LCPUFA ($\beta = -0.157$; 95% CI: -0.284, -0.029; $p = 0.016$) and for n-6:n-3 LPCUFA ratio ($\beta = -0.158$; 95% CI: -0.285, -0.031; $p = 0.015$).

Similarly, cytokine IL-17F was negatively associated with female status in models controlled for total n-6 and n-3 LCPUFA ($\beta = -0.292$; 95% CI: -0.532, -0.052; $p = 0.017$) and n-6:n-3 LCPUFA ratio ($\beta = -0.290$; 95% CI: -0.529, -0.051; $p = 0.018$) (see table 3).

Discussion

In this high fish-consuming population of 19-year-old adults from the Republic of Seychelles, we found no statistically significant association between hair Hg concentrations and cytokines associated with the Th17 axis, in both unadjusted and adjusted analyses for n-3 and n-6 LCPUFA status. Based on the outcome of this study, Hg exposure, predominantly derived from fish, would appear to have no impact on autoimmunity in that it does not modulate the Th17 immune response.

The current study aimed to follow up on the study by McSorley *et al*. (2020) which demonstrated that the hair Hg content of the same cohort used in this study was associated with increased odds of having a higher combined score of ANA following adjustment for LCPUFA status (McSorley *et al*., 2020). The reported increased risk of presenting with ANA with higher Hg must be interpreted with caution as ANA it is not diagnostic of autoimmunity and many healthy individuals are known to have ANA in absence of clinical symptoms. The current study therefore aimed to investigate the impact of Hg on cytokines of the Th17 axis which have been reported as important mediators in the pathogenesis of autoimmunity. The lack of any significant association between Hg exposure and Th17 cytokines in the current study would suggest that fish consumption does not impact autoimmune risk through the Th17 axis. These findings align with a study by Monastero *et al*. (2017) which reported no relationship between Hg exposure (median, 4.58 μg/L (0.00458 ppm)) and the presence of IL-17 in a US cohort of avid fish and seafood consumers (n=287) (Monastero *et al*., 2017). These results, however, are in contrast to the study by Nyland et al. (2011a) which showed that hair Hg (14.1 ug/g) (as well as blood and urine Hg) was positively correlated with IL-17 in a population of Amazonian fish consumers (Nyland *et al*., 2011a). Disappearances in the results might be explained by the differential source of Hg exposure between the studies. Similarly, to the current study, US population (Monastero *et al*. 2017) account the main source of Hg exposure was fish intake, which correlated with higher Hg concentration in the blood, as well as mean concentrations of n-3 LCPUFA (Monastero *et al*., 2017). In contrast, in Brazilian Amazon, native residents were not only exposed to Hg through fish (MeHg), but also, they were exposed to inorganic Hg (iHg) through artisanal gold mining. Nevertheless, it must be noted that fish intake in US was lower than in the Seychelles (on average 2.4 cups of fish (~3 fish meals) a week

vs ~7 fishmeals a week), what has been reflected by the Hg exposure (median Hg in blood 4.58 μg/L (0.00458 ppm) in US vs median Hg in hair, 9.02 ppm). Nevertheless, analysis of women of childbearing age from Brazilian Amazon, similarly to both studies found no significant associations between total Hg (geometric mean of Hg in blood was 6.90 mg/L (6.9 ppm) and IL-17, due to low detection rates (Nyland *et al*., 2011b). Owing to the evidence to indicate that iHg, in contrast to MeHg, can produce more severe proinflammatory response, including a release of IL-17 (Gardner *et al*., 2010b; Crowe *et al*., 2017), it is plausible that co-exposure to iHg reported in the study of Nyland *et al*., (2011a), rather than differences in the levels of Hg exposure might be a reason of explain observed associations between Hg exposure and IL-17 in gold miners (Nyland *et al*., 2011a), but not in fish consuming populations (Nyland *et al*., 2011b; Monastero *et al*., 2017).

The evidence to support the immunotoxic effects of Hg is predominantly derived from *in vivo* animal studies, which indicate that Hg exposure in genetically susceptible animals results in an abnormal immune response with subsequent development of lupus-like syndrome, characterized by the presence of autoantibodies hypergammaglobulinemia along with the development of nephritis and arthritis (Hu *et al.,* 1997; Bagenstose *et al.,* 1999; Pollard *et al.,* 1999; Hansson *et al.,* 2003; Haggqvist *et al.,* 2005; Havarinasab and Hultman, 2005; Ramírez-Sandoval *et al.,* 2015). It is important to note, however, that these studies involved the administration of supranatural concentrations of Hg via intraperitoneal injection and thus are not reflective of the dose or route of exposure of Hg in humans. Although the evidence on the immunotoxic effects of Hg from human studies is limited, studies in adults occupationally exposed to Hg through gold-mining activities or through consumption of MeHg-contaminated fish, have reported positive associations between Hg status, increased concentration of ANA (Silva *et al.,* 2004; Nyland *et al.,* 2011a; Somers *et al.,* 2015; McSorley *et al.,* 2020) and concentrations of pro-inflammatory cytokines (IL-17, IL-1β, TNF-α, IL-1ra, IL-10, and IFN-γ) (Gardner *et al.,* 2010; Nyland *et al.,* 2011a). This evidence from human studies to date provides initial indications that Hg exposure has the potential to alter the immune response; however, the impact of such changes on autoimmune disease risk or subclinical autoimmunity remains elusive.

It is important to distinguish between Hg exposure from environmental sources (such as gold mining) and exposure through fish consumption, as the nutrients present in fish have been proposed to help mitigate the toxic effects of Hg particularly through the anti-inflammatory activities of the n-3 LCPUFA (EPA and DHA) which have previously been reported to favorably modulate immune function in autoimmune conditions (Duffy *et al.,* 2003; Wright *et al.,* 2008; Calder, 2015). An increased amount of EPA and DHA, from habitual dietary fish intake, has been shown to be associated with reduced biomarkers of inflammation (Calder, 2015), decreased concentrations of C-reactive protein (CRP), interferon (INF) γ, TNF-α, IL-6, IL-10 and with a higher TNF-α:IL-10 ratio (McSorley *et al.,* 2018). In the current study of healthy adults, concentrations of Th17 cytokines were low, and therefore the beneficial anti-inflammatory effects mediated by immunomodulatory n-3 LCPUFA is likely to be limited. Nevertheless, although the current study did not show any significant relationship between Hg exposure and Th17 cytokines, the adjustment for LCPUFA was shown to increase in the β-value (result not significant) for IL-17F, IL-23 and IL-31. Whilst changes were minimal, the direction of these changes may suggest that n-3 LCPUFA has a beneficial role in altering the association between hair Hg and Th17 cytokines (Costabile *et al.,* 2021).

Population of Republic of Seychelles is a described as high fish-eating population, eating on average 8 or more fish or fish-containing meals a week (Strain, 2014). Republic of Seychelles is also one of the countries with the highest per-capita-fish-intake in the world, with fish caput ~ 59 kg a year (FAO, 2022). In the current study, the data on the fish consumption in the population of 19 years old young adults from the main cohort was limited, as this information was provided by n=187 (42.5%) participants. In this population, the average fish intake was ~ 7 fishmeals/a week, which is lower when compared with fish intake of 12 fishmeals a week reported in the Main Cohort previously (Shamlaye et al., 1995), what might be a consequence of the transition into the Western type of diet between 1989 and 2011 (Cardoso *et al*., 2013).

A decreasing trend of fish intake reported in Seychelles (Shamlaye *et al*., 2004), might have a profound effect on the nutritional status of this population. A Western-style diet includes high intakes of

processed foods, white meat and eggs (Conway *et al*., 2018); while a traditional Seychellois diet is predominantly based on locally caught fatty fish (e.g., mackerel, tuna, and red snapper), and fish dishes (e.g., bouillon blan—a traditional fish soup) in conjunction with a high intake of fruit and vegetables (Bonham *et al*., 2009). In addition, fish eaten in the Seychelles is a main protein source for Seychellois (FAO, 2019), which additionally provide wide range of health promoting nutrients, including vitamin E, selenium and n-3 LCPUFA (Strain, 2014). The change in dietary pattern may also explain why previous research utilizing the SCDS cohort found no association between n-3 LCPUFA status (Bonham *et al*., 2008), whereas other studies have been shown that positive relationship between fish intake and n-3 LCPUFA status (Schiepers *et al*., 2010; Fuentes-Albero *et al*., 2019), while demonstrating that introducing 1-2 portions of fish a week may help to restore adequate n-3 LCPUFA status in the low fish consumers (Conway *et al*., 2020). In the current study, the mean (SD) of total n-3 LCPUFA, total n-6 LCPUFA and n-6:n-3 LCPUFA ratio was 0.05 (0.01) mg/ml, 0.15 (0.04) mg/ml and 3.77 (1.93), respectively. This is consistent with the previous analysis conducted in the same sample of young adolescents (n=491) (the mean (SD) concentrations of total n-3 LCPUFA was 0.04 (0.02) mg/ml and total n-6 LCPUFA was 0.15 (0.04) mg/ml; the n-6:n-3 LCPUFA ratio was 3.81 (1.97)) (McSorley *et al*., 2020), however differs from the evaluation of maternal samples (n=225) obtained from the same Main Cohort (the mean (SD) concentrations of total n-3 LCPUFA was 0.1 (0.03) mmol/L and of total n-6 LCPUFA was 4.3 (0.7) mmol/L; the n-6:n-3 LCPUFA ratio was 46.0 (13.5)) (Strain *et al*., 2012).

Consistently with other studies conducted in fish-eating populations, hair Hg was associated with fish consumption (McDowell *et al.,* 2004; Passos *et al.,* 2008; Faial *et al.,* 2015; Castaño *et al.,* 2015; McSorley *et al.,* 2020), age (McDowell *et al.,* 2004), smoking (Yokoo *et al.,* 2003; Gibb *et al.,* 2016) and maternal socioeconomic status (Reuben *et al.,* 2020). The SES of a family appears to be an important determinant of Hg exposure in developing countries, compared to Western populations (McDowell *et al.,* 2004). Higher concentrations of hair Hg (4.1 μg/g) has been observed in children from native Peruvian Amazon (Ruben *et al.,* 2020) compared to non-native children (mean hair Hg 1.6 μg/g) and suggests that lower SES is associated with higher Hg exposure as a result of local communities being more likely to be involved in artisan activities (Nyland *et al.,* 2011a). In the

Seychelles lower SES may be associated with higher consumption of fish although this is speculative, and more research is required.

In the current study, lower serum IL-27 concentrations were associated with smoking status and maternal socioeconomic status. The role of IL-27 in autoimmunity remains unexplored, however, there is some indications, which suggest that IL-27 can exert dual action on Th17/Treg responses, depending on the presence of absence of proinflammatory mediators (IL-17A) and release of danger signals, such adenosine triphosphate (ATP), which could be induced by cigarette smoke (Pouwels *et al.,* 2014). Nevertheless, these effects still need to be confirmed, owing to lack of reported difference in IL-27 concentrations between smoking and non-smoking individuals (Qiu *et al.,* 2016).

Furthermore, concentrations of IL-17F cytokine was (negatively) associated with female status, whilst the previous reports indicated that certain IL-17F polymorphisms can be associated with risk of recurrent pregnancy loss (Najafi *et al.,* 2014), as well as development and severity of autoimmune and inflammatory diseases, including rheumatoid arthritis (Marwa *et al.,* 2017), inflammatory arthritis (Shao *et al.,* 2020), osteoarthritis (Bafrani *et al.,* 2019; Zhao *et al.,* 2020), psoriasis (Choi *et al.,* 2019), multiple sclerosis (Wang *et al.,* 2014), asthma (Ramsey *et al.,* 2005), Crohn's disease and inflammatory bowel disease (Seiderer *et al.,* 2008). Moreover, when compared to the apparently healthy individuals/healthy controls, IL-17F concentrations were found to be higher in the patient cohort with systemic sclerosis, of which the majority were middle-aged women (84 females and 6 males) (Robak *et al.,* 2019), suggesting a potential role of IL-17F in determining autoimmune disease risk in females. Nevertheless, the role of cytokines associated with Th17 response in autoimmune pathology still requires further investigation, which would extend understanding of their contribution to the disease development.

A strength of this study is the sizeable cohort (n=440), who are high consumers of fish (approx. 7 fish meals per week) in a country where the environment is largely free from other pollutants. Our cohort

had a high MeHg exposure from an ocean fish rich diet with a wide range of MeHg concentrations (0.42-52.08 ppm). Although an available data on fish consumption was restricted to n=187 (42.5%) participants, utilizing measures of LPCUFA status provided an accurate determination of nutritional status of the population. Limitations of this study include its cross-sectional analysis which prevents determination of cause-effect relationship between Hg and autoimmune cytokines. Participants included in this analysis, similar to others (Nyland *et al.,* 2011a; Monastero *et al.,* 2017), were young, and this could have impacted our findings in that age is an important factor in the development of autoimmunity and it must be noted that autoimmune diseases tend to occur in the second half of adulthood, when immune competence has decreased (Goronzy and Weyand, 2012). Therefore, it would be interesting to follow this cohort over time to repeat these analyses or alternatively, recruit an older cohort, to see if progression through life and age-related changes in immune function might impact study outcomes; based on the fact that frequencies of Th17 cell subsets seem to be higher in older people (age ≥ 65), than in healthy middle-aged (age ≥ 45 and ≤ 64), and the young (age ≤ 44) (Li *et al.,* 2017).

In summary, MeHg exposure from the frequent consumption of ocean fish in young adults at 19 years of age was not associated with Th17 cytokines or IL-33 in both unadjusted and adjusted for LCPUFA status regression analyses. This finding is of particular public health interest given the important contribution of fish to nutrient intake and diet quality, as it provides 6.4% of total protein supply, including 19.8% of total animal protein consumed worldwide (Tacon and Metian, 2017). Whilst this study clearly shows no relationship between MeHg and Th17 cytokine status, there is a need to investigate these relationships in older, female populations, who are known to be at greater risk of autoimmune disease.

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Figure 1. A summarized overview of the participants enrollment, data collection and analysis for the purpose of the current study (n=440).

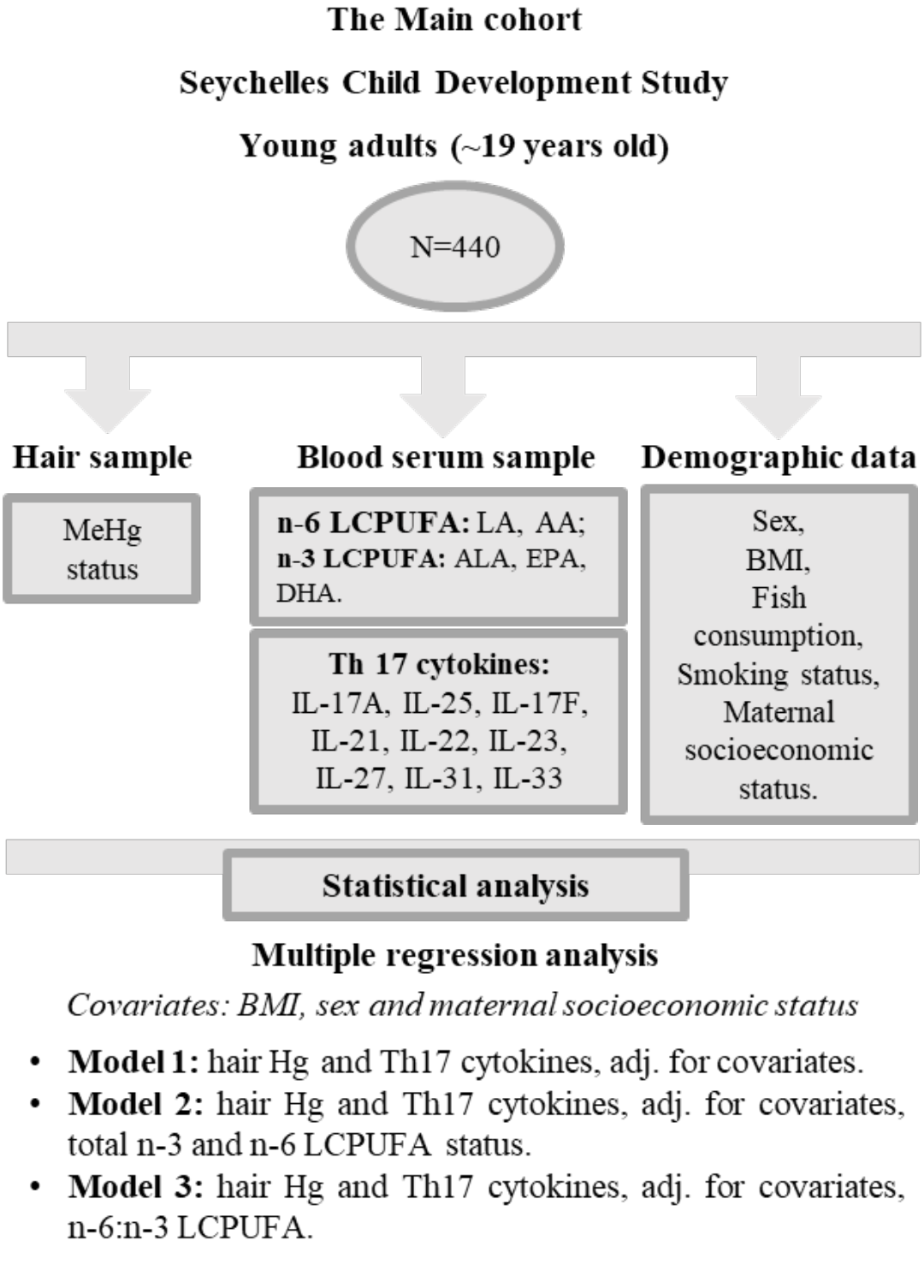

Table 1. Participant characteristics (n=440).

	N	> LLOD n, (%)	Mean	SD	Median	Range
Male:female	190:250					
- male, %	43.18%					
- female, %	56.82%					
Smoking status (male:female)	189:248					
- smoking male, %	43.25%					
- smoking female, %	56.75%					
Fish consumption (meals/week)	187		7.07	3.7		
BMI (kg/m2)	437		22.14	4.69	21.08	14.87-41.30
Y19 hair MeHg (ppm)	440		10.21	5.98	9.02	0.42-52.08
IL-17A (pg/mL)	440	39, (8.86)	1.46	1.25	1.22	1.22-15.37
IL-17E (pg/mL)	440	104, (23.64)	0.51	0.85	0.20	0.20-7.49
IL-17F (pg/mL)	440	159, (36.14)	391.93	683.02	79.90	79.90-5580.71
IL-21 (pg/mL)	440	409, (92.95)	33.27	50.93	17.33	0.53-718.66
IL-22 (pg/mL)	440	437, (99.32)	2.92	27.79	0.91	0.04-573.50
IL-23 (pg/mL)	440	374, (85.00)	7.84	45.03	3.60	0.70-937.37
IL-27 (pg/mL)	440	439, (99.77)	549.69	1300.62	378.09	5.80-2355.64

IL-31 (pg/mL)	440	229, (52.05)	23.59	28.39	9.68	3.89-143.96
IL-33 (pg/mL)	440	343, (77.95)	2.80	11.91	1.49	0.37-246.88
n-3 LCPUFA (mg/ml)	440		0.05	0.01	0.05	0.01-0.11
n-6 LCPUFA (mg/ml)	440		0.15	0.04	0.16	0.02-0.55
n-6:n-3 LCPUFA	440		3.77	1.93	3.16	0.96-17.73

MeHg, methylmercury; Y19, year 19; ppm, parts per million; IL, Interleukin; Lower Limit of Detection, LLOD; LCPUFA, long chain polyunsaturated fatty acids; BMI, Body Mass Index.

The lower limit of detection (LLOD) for each cytokine were 1.72 pg/ml for IL-17A, 0.28 pg/ml for IL-17E, 113 pg/ml for IL-17F, 0.75 pg/ml for IL-21, 0.06 pg/ml for IL-22, 0.99 pg/ml for IL-23, 8.2 pg/ml for IL-27, 5.5 pg/ml for IL-31 and 0.53 mg/ml for IL-33.

In cases where cytokine values were below or at the LLOD, values were replaced with LOD/√2 value. Cytokines IL17A, IL17E, IL17F, and IL31 had large number of LLOD replacements. In these cases, binary transformation was employed where LLOD values were replaced with 0. Values greater than LLOD were replaced with 1.

Table 2. Main effect models reporting covariate-adjusted associations between methylmercury (MeHg) exposure, long chain polyunsaturated fatty acids (LCPUFA) status and cytokines of T helper cells (Th) 17 axis with and without adjustment for LCPUFA.

		Y19Hg	n−3 LCPUFA	n−6 LCPUFA	n−6:n−3 LCPUFA
IL-17A	Unadj.	0.0 (-0.004, 0.004) p = 0.860			
	Adj. for n-3, n-6 LCPUFA	0.000 (-0.004, 0.004) p = 0.873	-0.145 (-1.710, 1.421) p = 0.856	0.073 (-0.460, 0.606) p = 0.787	
	Adj. for n-3:n-6 LCPUFA	0.000 (-0.004, 0.004) p = 0.873			0.001 (-0.010, 0.013) p = 0.807
IL-17E	Unadj.	-0.003 (-0.009, 0.002) p = 0.241			
	Adj. for n-3, n-6 LCPUFA	-0.003 (-0.009, 0.002) p = 0.256	0.428 (-1.798, 2.655) p = 0.706	-0.173 (-0.931, 0.584) p = 0.653	
	Adj. for n-3:n-6 LCPUFA	-0.003 (-0.009, 0.003) p = 0.270			-0.009 (-0.026, 0.008) p = 0.309
IL-17F	Unadj.	-0.014 (-0.032, 0.005) p = 0.156			
	Adj. for n-3, n-6 LCPUFA	-0.013 (-0.031, 0.006) p = 0.192	5.672 (-1.631, 12.975) p = 0.128	-1.246 (-3.732, 1.239) p = 0.325	

	Adj. for n-3:n-6 LCPUFA	-0.012 (-0.031, 0.006) p = 0.196			-0.056 (-0.111, -0.001) p = 0.047
IL-21	Unadj.	-0.007 (-0.027, 0.014) p = 0.521			
	Adj. for n-3, n-6 LCPUFA	-0.007 (-0.027, 0.014) p = 0.517	-1.131 (-9.096, 6.834) p = 0.780	-1.196 (-3.906, 1.514) p = 0.386	
	Adj. for n-3:n-6 LCPUFA	-0.007 (-0.027, 0.014) p = 0.533			-0.008 (-0.068, 0.053) p = 0.798
IL-22	Unadj.	-0.004 (-0.013, 0.005) p = 0.371			
	Adj. for n-3, n-6 LCPUFA	-0.004 (-0.013, 0.005) p = 0.397	1.016 (-2.516, 4.547) p = 0.572	-0.399 (-1.601, 0.803) p = 0.514	
	Adj. for n-3:n-6 LCPUFA	-0.004 (-0.013, 0.005) p = 0.405			-0.013 (-0.039, 0.014) p = 0.354
IL-23	Unadj.	-0.002 (-0.015, 0.012) p = 0.818			
	Adj. for n-3, n-6 LCPUFA	-0.002 (-0.015, 0.012) p = 0.822	-0.188 (-5.417, 5.042) p = 0.944	-0.740 (-2.519, 1.040) p = 0.414	

	Adj. for n-3:n-6 LCPUFA	-0.001 (-0.015, 0.012) p = 0.848			-0.012 (-0.051, 0.028) p = 0.565
IL-27	Unadj.	-0.005 (-0.016, 0.005) p = 0.328			
	Adj. for n-3, n-6 LCPUFA	-0.005 (-0.015, 0.006) p = 0.371	2.114 (-1.953, 6.182) p = 0.308	-0.927 (-2.312, 0.457) p = 0.189	
	Adj. for n-3:n-6 LCPUFA	-0.005 (-0.015, 0.006) p = 0.369			-0.019 (-0.050, 0.012) p = 0.226
IL-31	Unadj.	-0.008 (-0.026, 0.010) p = 0.401			
	Adj. for n-3, n-6 LCPUFA	-0.007 (-0.026, 0.011) p = 0.427	1.732 (-5.367, 8.831) p = 0.632	-1.312 (-3.728, 1.104) p = 0.286	
	Adj. for n-3:n-6 LCPUFA	-0.007 (-0.026, 0.011) p = 0.437			-0.026 (-0.080, 0.028) p = 0.345
IL-33	Unadj.	0.001 (-0.010, 0.011) p = 0.913			
	Adj. for n-3, n-6 LCPUFA	0.001 (-0.010, 0.011) p = 0.913	-0.381 (-4.399, 3.636) p = 0.852	-0.760 (-2.127, 0.607) p = 0.275	

Adj. for n-3:n-6	0.001 (-0.010, 0.011)	-0.010 (-0.040, 0.021)
LCPUFA	p = 0.881	p = 0.527

Data presented as β-value (95% confidence interval) p value.

All values were natural log transformed before applying linear regression models.

Unadj.: model adjusted for sex, BMI, smoking status, maternal SES status and Y19Hg.

Adj. for n-3, n-6 LCPUFA: model adjusted for sex, BMI, smoking status, maternal SES status, Y19Hg and total n-3 LCPUFA and total n-6 LCPUFA.

Adj. for n-6:n-3 LCPUFA: model adjusted for sex, BMI, smoking status, maternal SES status and Y19Hg and n-6:n-3 LCPUFA ratio.

IL; interleukin, BMI, Body Mass Index, SES, maternal socioeconomic status, LCPUFA, long chain polyunsaturated fatty acids.

Table 3. Associations between variables of participant characteristics of cytokines associated with T helper cells (Th)17 axis controlling for methylmercury (MeHg) exposure at age of 19 (Y19Hg).

		Female status	BMI	Smoking status	SES
IL-17A	Adj. for n-3, n-6 LCPUFA	0.005 (-0.047, 0.056) p = 0.859	-0.004 (-0.008, 0.001) p = 0.164	-0.011 (-0.060, 0.038) p = 0.659	-0.002 (-0.003, 0.000) p = 0.092
	Adj. for n-3:n-6 LCPUFA	0.005 (-0.047, 0.056) p = 0.862	-0.003 (-0.008, 0.001) p = 0.164	-0.011 (-0.060, 0.038) p = 0.662	-0.002 (-0.003, 0.000) p = 0.092
IL-17E	Adj. for n-3, n-6 LCPUFA	-0.033 (-0.106, 0.041) p = 0.382	0.004 (-0.003, 0.011) p = 0.292	-0.017 (-0.087, 0.052) p = 0.629	0.001 (-0.001, 0.004) p = 0.392
	Adj. for n-3:n-6 LCPUFA	-0.032 (-0.105, 0.041) p = 0.387	0.004 (-0.003, 0.011) p = 0.300	-0.018 (-0.088, 0.051) p = 0.610	0.001 (-0.001, 0.004) p=0.379
IL-17F	Adj. for n-3, n-6 LCPUFA	-0.292 (-0.532, -0.052) p = 0.017	-0.004 (-0.027, 0.019) p = 0.715	-0.191 (-0.419, 0.037) p = 0.101	0.005 (-0.004, 0.013) p = 0.294
	Adj. for n-3:n-6 LCPUFA	-0.290 (-0.529, -0.051) p = 0.018	-0.005 (-0.028, 0.018) p=0.680	-0.196 (-0.424, 0.032) p = 0.092	0.005 (-0.004, 0.013) p=0.263
IL-21	Adj. for n-3, n-6 LCPUFA	-0.144 (-0.406, 0.117) p = 0.278	-0.005 (-0.030, 0.020) p = 0.675	-0.062 (-0.310, 0.187) p = 0.627	0.003 (-0.007, 0.012) p = 0.568
	Adj. for n-3:n-6 LCPUFA	-0.142 (-0.403, 0.120) p = 0.287	-0.005 (-0.030, 0.020) p = 0.685	-0.062 (-0.311, 0.187) p = 0.624	0.002 (-0.007, 0.011) p = 0.651

IL-22	Adj. for n-3, n-6 LCPUFA	-0.095 (-0.211, 0.021) p = 0.110	0.006 (-0.005, 0.017) p = 0.289	-0.069 (-0.179, 0.042) p = 0.222	0.000 (-0.004, 0.004) p = 0.987
	Adj. for n-3:n-6 LCPUFA	-0.094 (-0.210, 0.022) p = 0.112	0.006 (-0.005, 0.017) p = 0.296	-0.070 (-0.180, 0.040) p = 0.214	0.000 (-0.004, 0.004) p = 0.994
IL-23	Adj. for n-3, n-6 LCPUFA	-0.002 (-0.015, 0.012) p = 0.822	-0.001 (-0.018, 0.015) p = 0.882	-0.018 (-0.181, 0.146) p = 0.831	0.002 (-0.004, 0.008) p = 0.448
	Adj. for n-3:n-6 LCPUFA	-0.001 (-0.015, 0.012) p = 0.848	-0.001 (-0.018, 0.015) p = 0.884	-0.019 (-0.182, 0.144) p = 0.820	0.002 (-0.004, 0.008) p = 0.497
IL-27	Adj. for n-3, n-6 LCPUFA	-0.041 (-0.174, 0.093) p = 0.550	0.004 (-0.009, 0.016) p = 0.582	-0.157 (-0.284, -0.029) p = 0.016	-0.005 (-0.009, 0.000) p = 0.053
	Adj. for n-3:n-6 LCPUFA	-0.039 (-0.173, 0.094) p = 0.564	0.003 (-0.009, 0.016) p = 0.595	-0.158 (-0.285, -0.031) p = 0.015	-0.005 (-0.010, 0.000) p = 0.044
IL-31	Adj. for n-3, n-6 LCPUFA	-0.101 (-0.334, 0.132) p = 0.396	-0.009 (-0.031, 0.013) p = 0.431	-0.154 (-0.376, 0.068) p = 0.173	0.002 (-0.006, 0.010) p = 0.603
	Adj. for n-3:n-6 LCPUFA	-0.098 (-0.331, 0.135) p = 0.407	-0.009 (-0.031, 0.013) p=0.426	-0.156 (-0.378, 0.066) p = 0.167	0.002 (-0.006, 0.010) p = 0.649
IL-33	Adj. for n-3, n-6 LCPUFA	-0.074 (-0.206, 0.058) p = 0.272	-0.001 (-0.014, 0.012) p = 0.870	-0.019 (-0.145, 0.106) p = 0.760	0.001 (-0.003, 0.006) p = 0.609

Adj. for n-3:n-6	-0.072 (-0.204, 0.060)	-0.001 (-0.014, 0.012)	-0.020 (-0.146, 0.105)	0.001 (-0.004, 0.006)
LCPUFA	p = 0.283	p = 0.877	p = 0.750	p = 0.699

Y19Hg, year 19 concurrent MeHg exposure.

All values were natural log transformed before applying linear regression models.

Statistically significant values with p value < 0.05 are presented in bold.

Unadj.: model adjusted for sex, BMI, smoking status, maternal SES status and Y19Hg.

Adj. for n-3, n-6 LCPUFA: model adjusted for sex, BMI, smoking status, maternal SES status, Y19Hg and total n-3 LCPUFA and total n-6 LCPUFA.

Adj. for n-6:n-3 LCPUFA: model adjusted for sex, BMI, smoking status, maternal SES status and Y19Hg and n-6:n-3 LCPUFA ratio.

IL; interleukin, BMI, Body Mass Index, SES, maternal socioeconomic status, LCPUFA, long chain polyunsaturated fatty acids.

CHAPTER 4:

An observational study investigating the impact of environmental mercury exposure on the relationship between cytokines of T helper (Th) 17 axis and disease outcomes in Systemic Lupus Erythematosus.

Abstract

Background: Exposure to mercury (Hg), including inorganic Hg (iHg) and methylmercury (MeHg), has been associated with markers of autoimmunity and with interleukin (IL)-17, a cytokine implicated in autoimmune pathogenesis including Systemic Lupus Erythematosus (SLE). That exposure is proposed to exacerbate SLE symptoms and may do this via the T helper (Th) 17 immune response.

Objectives: The aim of this study was to investigate the impact of Hg exposure, determined in urine (iHg), hair (MeHg) and number of dental amalgams (elemental Hg, Hg^0) on the relationship between cytokines associated with the Th17 axis and disease outcomes, including disease activity and disease-associated damage in SLE.

Methods: Hg exposure was measured in the hair (MeHg) and urine (iHg) of 88 SLE patients and their self-reported dental amalgam number (Hg^0). SLE disease activity (SELENA SLEDAI, SLAM, BILAG) and damage (SLICC/ACR+) were determined and serum Th17 cytokines were quantified. Regression analyses investigated the effect of Hg exposure on the relationship between cytokines associated with the Th17 axis and disease activity or disease-associated damage. Models were adjusted for age, BMI, and total Hg exposure, including iHg (urine), MeHg (hair) and Hg^0 (dental amalgams).

Results: Concentrations of MeHg in hair (median 0.59 ppm) and/or iHg concentrations in urine (median 1.99 ng/g creatinine) did not significantly influence the relationship between Th17 cytokines and SLE disease activity. IL-17E was positively associated with SLICC/ACR in unadjusted ($\beta = 0.465$, 95% CI: 0.432, 0.786, $p = 0.005$,) and adjusted ($\beta = 0.355$, 95% CI: 0.050, 0.660, $p = 0.023$) models. Higher serum IL-17A concentrations was associated with increased odds of having a higher disease damage score (OR = 0.35, 95% CI: 0.13, 0.96) in adjusted models.

Conclusions: Low level exposure to Hg, measured in the urine (iHg), hair (MeHg) and number of dental amalgams (Hg^0), was not associated with SLE pathology or autoimmune associated Th17 cytokines. More research is needed to investigate these relationships in a population of autoimmune patients with higher Hg exposure.

Keywords: Mercury (Hg), Systemic Lupus Erythematosus (SLE), Th17 cytokines, IL-33, Autoimmune disease, disease outcomes.

Introduction

The autoimmune disease Systemic Lupus Erythematosus (SLE) presents as a result of interplay between genetics and environmental stimuli (Kamen, 2015). Many factors have been implicated in SLE disease etiology and the exacerbation of SLE symptoms, including a role for the heavy metal mercury (Hg).

Human exposure to Hg can occur through inhalation of Hg vapor (elemental Hg^0) from dental amalgams, polluted industrial emissions and drinking contaminated water, all of which can result in exposure to inorganic Hg (iHg) (Boerleider *et al.,* 2017). The predominant route of exposure to Hg is through the consumption of fish, which accumulates methylmercury (MeHg) within the edible tissue (Rice *et al.,* 2014). Evidence from both *in vitro* and *in vivo* research demonstrates that exposure to both iHg or MeHg can induce oxidative stress (Shenker *et al.,* 2000) and adversely affect inflammation and could potentially contribute to autoimmunity (Häggqvist and Hultman, 2003, Gardner *et al.,* 2009, Crowe *et al.,* 2017). Exposure of genetically predisposed murine models to iHg or MeHg has consistently been associated with the spontaneous development of a lupus-like condition (Pollard *et al.,* 1999) with symptoms of nephritis (Hirsch *et al.,* 1982), arthritis, cerebritis, skin rash and vasculitis (Pollard *et al.,* 1999), increased autoantibodies (Martinsson *et al.,* 2008) and immune complex (IC) depositions (Hultman and Hansson-Georgiadis, 1992). Chronic low-level exposure to Hg, associated with industrial Hg^0/iHg pollution (Dahlgren *et al.,* 2007), artisanal gold mining activities (iHg) or through the dietary intake of fish (MeHg) (Silva *et al.,* 2004; McSorley *et al.,* 2020) has been associated with increased risk of having markers associated with autoimmunity. Nevertheless, a study in SLE patients reported no association between Hg concentrations in urine (iHg), hair (MeHg), as well as umber of dental amalgams (Hg^0) and disease activity (Crowe *et al.,* 2015).

T helper (Th) 17 cells are a subset of pro-inflammatory interleukin (IL)-17-producing T helper cells, which have been implicated in neutrophil recruitment, apoptosis and production of other inflammatory mediators, such as IL-6, IL-8, IL-10, IL-1α (Lee, 2018). The Th17 cells activity is tightly regulated by

immunosuppressive actions of regulatory T (Treg) cells, that are critical for maintaining an immune homeostasis. An increased release of pro-inflammatory mediators, resultant from exposure to environmental factors, such as heavy metals or infections, by promoting Th17 differentiation, can cause imbalance between Th17 and Treg (Lee, 2018) and thereby contributing to the development of autoimmune disease, including SLE (Robert and Miossec, 2020).

There is growing evidence to support role of dysregulated Th17 response and Th17 cytokine imbalance in the development of autoimmune inflammation (Muhammad Yusoff *et al.,* 2020). Notably, an increased IL-17A expression on T cells has been reported in both the skin and kidney tissue of SLE patients (Hedrich *et al.,* 2012). In addition, concentrations IL-17A has been shown to be associated with increased disease severity (Henriques *et al.,* 2010; Khan and Ahmed, 2015; Schmidt *et al.,* 2018), and thereby suggesting a potential involvement of IL-17 in autoantibody-mediated cell damage (Martin *et al.,* 2014). Furthermore, Th17-associated cytokines, including IL-17 (Koga *et al.,* 2019; Yin *et al.,* 2021), have been shown to correlate positively with active disease in SLE patients (Cheng *et al.,* 2009; Robak *et al.,* 2013) and the SELENA SLEDAI index of disease activity (Wong *et al.,* 2008; Cheng *et al.,* 2009; Mok *et al.,* 2010; Tanasescu *et al.*, 2010; Chen *et al.,* 2010; Robak *et al.,* 2013; El-Gazzar *et al.,* 2017; Li *et al.,* 2019). The evidence from experimental murine models of lupus disease proposed that IL-17 may have an important role in inducing SLE pathology. Mechanistic study utilizing murine lupus model, *Roquin*$^{san/san}$ mice, indicated that loss of IL-17 through decreasing production of IgG, IgG1, and IgG2a resultant from inhibiting B cell differentiation can significantly improve symptoms of nephritis (Li *et al.,* 2019). Similarly, to IL-17, observations in SLE cohorts, suggest that IL-23, a cytokine involved in Th17 differentiation may additionally promote Th17 response and production of their effector cytokines. In addition, IL-23 concentrations have been found to be significantly higher in SLE patients than in controls (Fischer *et al.,* 2017); as well as levels of this cytokine was elevated in active phase of disease when compared to inactive SLE and controls (Ghanima *et al.,* 2012).

To date, no study has investigated the impact of exposure to all forms of environmental Hg (iHg, MeHg and Hg^0) on the clinical outcomes associated with autoimmune dysfunction mediated through Th17 response. Immunotoxic properties of iHg and MeHg may have a considerable impact of autoimmune severity, owing to MeHg induced necrosis and subsequent release of pro-inflammatory mediators, including alarmin cytokines and autoantigens which promote Th17-mediated inflammation and exacerbate disease activity. Therefore, based on the evidence indicating for associations between IL-17 as well as other Th17 cytokines, and measures of disease activity in the SLE cohorts (Cheng *et al.,* 2009; Lin *et al.,* 2014; Yin *et al.,* 2021), this study aims to investigate if different forms of environmental Hg exposures, measured in hair (MeHg), urine (iHg), and dental amalgam status (Hg^0) can impact the relationship between cytokines of Th17 axis and disease activity and disease-associated damage in SLE patients.

Materials and methods

Study design

This observational study used a convenient sample of 101 individuals, who were previously recruited between 2010-2011 (n=52) (Breslin, 2013) and 2013-2015 (n=49) (Crowe *at al.,* 2017) and fulfilled the criteria for SLE diagnosis and classification (Tan *et al.,* 1982, Hochberg, 1997). A total of 88 SLE patient samples were available for cytokine analysis. All participants were recruited through rheumatology clinics in the Belfast Health and Social Care Trust (BHSCT) and Western Health and Social Care Trust (WHSCT) in Northern Ireland as described elsewhere (Crowe *et al.,* 2015; Crowe *at al.,* 2017). A summarized overview describing patient enrollment, data collection and analysis was presented in the figure 1.

Ethical approval was obtained from the Office for Research Ethics Committees Northern Ireland (Reference numbers: 10/NIR02/43; 15/NI/0062). The research adhered to the standards outlined in the Declaration of Helsinki 1975. All participants provided written informed consent.

At the rheumatology clinic, participants donated a single, non-fasted blood sample and processed serum was stored at -80°C for future Th17 cytokine quantification. Patient information including age, body mass index (BMI), clinical assessment of disease and samples for the assessment of Hg exposure were collected.

Clinical assessment

Patients were evaluated by a consultant rheumatologist for disease activity using the British Isles Lupus Assessment Group Index (BILAG), Systemic Lupus Activity Measure (SLAM), the revised Safety of Estrogen in Lupus Erythematosus National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), and disease associated damage was assessed using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR+) damage index, as described elsewhere (Crowe *et al.* 2015; Crowe *et al.,* 2017).

Biochemical analysis

The serum cytokine concentrations were determined by the electrochemiluminescence based Meso Scale Discovery (MSD) immunoassay platform using the U-PLEX Th-17 Combo 1 multiplex panel (K15075K-2) (MSD, Gaithersburg, MD, USA). MSD plates were analyzed on the MS2400 imager. IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-31 and IL-33 concentrations were calculated using the manufacturer's software MSD Discovery Workbench Version 4.0 and results are presented as mean (± SD) in pg/ml.

The lower limit of detection (LLOD) and percentage coefficient of variance (CV %) for each cytokine were identified as follows: IL-17A: 1.72-2.8 pg/ml (1.76%), IL-17E: 0.28-0.95 pg/ml (0.80%), IL-17F: 113-214 pg/ml (0.56%), IL-21: 0.75-3.0 pg/ml (3.41%), IL-22: 0.06-0.19 pg/ml (2.22%), IL-23: 0.99-2.0 pg/ml (0.90%), IL-27: 8.2-15 pg/ml (1.72%), IL-31: 5.5-15 pg/ml (3.78%), IL-33: 0.53-0.60 pg/ml (2.59%).

Assessment of total Hg exposure

Analysis of Hg content in collected hair and urine samples were conducted at the University of Rochester, Rochester, NY, USA. Hair Hg concentrations were used as the biomarker of exposure to organic Hg, as over 90% of total Hg present in hair is presumed to be MeHg (Marinho *et al.*, 2014). MeHg concentrations were determined in the hair sample, consisting of 50-100 hair strands of hair, which were donated by SLE patients during clinical appointment. The strands were cut close to the scalp of the occipital region of the patient's head. The MeHg concentration in hair were quantified using cold vapour atomic absorption spectroscopy (Cernichiari *et al.,* 1995). MeHg below the limit of detection (< 0.75 ppm) was imputed as 0.75/√2 ppm (Glass and Gray, 2001). iHg concentrations were determined in a 2 ml spot urine sample, which was processed and stored at –80°C prior the urinary iHg quantification. Urinary iHg (ng/mL) was analyzed using atomic fluorescence spectrometry (PSA 10.035 Millennium Merlin, PS Analytical Ltd., Kent, United Kingdom) according to methods used previously (Pichichero *et al.,* 2009). Urine iHg concentrations were adjusted by creatinine concentration (μmol/L) as measured by the ILab 650 (SpA, Milan, Italy). The intra-assay CV % for creatinine was < 1.7%. Dental amalgams were quantified based on self-reported information provided by SLE patients. All amalgams were given a score of 1 regardless of their size or location.

Statistical Analysis

All statistical analyses were performed using SAS Version 9.4. Statistical significance in all analyses was determined using a two-sided approach $\alpha = 0.05$. Regression model assumptions were checked using standard methods (Weisberg, 2005). Similar to others, the distribution of MeHg concentrations in hair and iHg concentrations in urine (Gardner *et al.,* 2010; Crowe *et al.,* 2017), disease activity, disease damage (Crowe *et al.,* 2017), dental amalgams and cytokine concentrations were skewed. Cytokine concentrations are presented on their natural scale. Descriptive results are presented as medians and interquartile ranges (IQR). The detection rate (%) above the LLOD was 94.3% for IL-17A; 10.2% for IL-17E; 9.1% for IL-17F; 68.2% for IL-21; 86.4% for IL-22; 55.7% for IL-23; 13.6%

for IL-31 and 50.0% for IL-33. IL-27 was detected in all patients. For concentrations of cytokines, which had undetectable values (< LLOD), a value of LLOD/√2 was imputed as a replacement value (McSorley *et al.,* 2020).

Associations between serum Th17-associated cytokines, Hg exposure in hair and urine combined with self-reported number of dental amalgams and disease outcome measures, including disease activity and disease-associated damage indexes were analyzed through linear and logistic regression models. In the logistic regression patients were categorized as having either low or high disease activity/damage. The cutoffs were defined as: SELENA SLEDAI (low < 4; high ≥ 4) (Gladman *et al.*, 2000; Petri *et al.,* 2005), SLAM (low < 7; high ≥ 7) (Liang *et al.*, 1989; Romero-Diaz *et al.,* 2011), BILAG (low < 9; high ≥ 9) (Isenberg *et al.,* 2005; Petri, 2007); while for disease-associated damage, value 1 (no damage < 1; damage present ≥ 1) was applied (Gladman *et al.,* 1997; Ghazali *et al.*, 2018). The cut-off values were determined separately for each disease outcome measure based on the available clinical evidence.

Covariates, known or suspected to be associated with (subclinical) autoimmunity or inflammation, such as age and BMI (McSorley *et al.,* 2020) were included in the analysis. Crude models (unadjusted series of models) estimated the direct relationship between the concentration of each single cytokine of the Th17 axis and measures of disease activity (SLENA SLEDAI, BILAG, SLAM) and disease-associated damage (SLICC/ACR) (model series 1). These models were further adjusted for age and BMI (model series 2), and Hg exposures, that include hair MeHg (model series 3), or urinary iHg (model series 4) or total Hg exposures in hair, urine and dental amalgams (model series 5).

Results

A summarized overview describing patient enrollment, data collection and analysis was presented in the figure 1.

Study Population

Patient characteristics are presented in table 1. The median (IQR) age of the 88 SLE patients was 49 (41.50, 58.50) years and the median BMI (IQR) was 25.80 (23.81, 30.66) kg/m^2. In this study, 86 (98%) patients were females and 2 (2%) were males.

The median (IQR) disease activity and disease-associated damage indexes were SLAM 4 (2-6), SELENA SLEDAI 3 (1-5), BILAG 4 (2-9) and SLICC/ACR+ 1 (0-2). These figures indicate low levels of disease activity in most patients, and relatively limited damage, with 55 (63%) patients scoring '0' on the accumulated damage index (SLICC/ACR+).

Median (IQR) hair (MeHg) and urine Hg (iHg) concentrations were 0.59 (0.37, 1.57) ppm and 1.99 (0.95, 4.11) ng/g creatinine, respectively. The median (IQR) number of self-reported dental amalgams was 5 with a range of 0-9 amalgams.

Cytokine concentrations

The detection percentage (n, %), median and IQR of each of cytokine measured for 88 SLE patients are presented in table 2. The detection rate above LLOD (n, %) of samples was 83 (94.3%) for IL-17A; 9 (10.2%) for IL-17E; 8 (9.1%) for IL-17F; 60 (68.2%) for IL-21; 76 (86.4%) for IL-22; 49 (55.7%) for IL-23; 12 (13.6%) for IL-31 and 44 (50.0%) for IL-33. IL-27 was detected in all patients (n=88).

Impact of Hg exposure on the associations between cytokines associated with Th17 response and disease outcomes

The impact of Hg exposures on the associations between cytokines associated with Th17 axis and disease outcomes in the cohort of SLE patients (n=88) is presented in table 3 (unadjusted model) and table 4 (adjusted model).

In all models, serum concentrations of IL-17A were positively associated with disease-associated damage as determined by SLICC/ACR+ but this did not reach statistical significance. IL-17E was

significantly associated with SLICC/ACR+ score in crude analysis (β = 0.465, 95% CI: 0.143, 0.786, p = 0.005) and in the models adjusted for age, BMI (β = 0.421, 95% CI: 0.103, 0.738, p = 0.010) and total Hg exposure measured in hair (MeHg), urine (iHg) and number of dental amalgams (Hg^0) (β = 0.355, 95% CI: 0.050, 0.660, p = 0.023) (see table 3).

Further adjustment of the model that included age and BMI (β = 0.421, 95% CI: 0.103, 0.738, p = 0.010), for Hg exposure measured in hair (MeHg), significantly increased the strength of association between disease-associated damage (SLICC/ACR+ score) and IL-17E (β = 0.429, 95% CI: 0.112, 0.746, p = 0.009); while adjustment for urinary Hg (iHg) significantly reduced the strength of this relationship (β = 0.347, 95% CI: 0.042; 0.652; p = 0.026) (see table 3).

Logistic regression analysis identified a statistically significant association between disease-associated damage and IL-17A. A higher IL-17A concentration were associated with a 65% lower risk of having a high SLICC/ACR+ score (SLICC/ACR+ > 0), compared with having a low SLICC/ACR+ score (SLICC/ACR+ = 0) while adjusting for age, BMI, and total Hg exposure (OR = 0.35, 95% CI: 0.13; 0.96) (see table 4.). When these analyses were adjusted for hair MeHg or for urinary iHg, there was a statistically significant change in the OR estimates of associations between disease-associated damage SLICC/ACR+ and IL-17A concentrations ($p < 0.05$); however, the magnitude of these findings was small and considered not to be clinically meaningful (see table 4.)

Discussion

In this cohort of 88 SLE patients, neither MeHg hair or urinary iHg concentrations altered the relationship between cytokines of the Th17 axis and disease activity, regardless of age, BMI and biomarkers of Hg exposure measured in hair (MeHg), urine (iHg) and number of dental amalgams (Hg^0). IL-17E was positively associated with disease-associated damage (SLICC/ACR+ score) and higher serum concentrations of IL-17A concentration was associated with higher odds of having disease

associated damage in the crude analysis and following adjustment for urinary iHg or hair MeHg; suggesting that Hg exposure has no significant impact on the clinical outcomes associated with Th17 axis in SLE pathogenesis.

Long-term environmental exposure to toxicants, such as Hg, has been proposed as a potential risk factor for inflammation and autoimmunity (Hemdan *et al.,* 2013; Pollard *et al.,* 2019), which may promote and exaggerate autoimmune pathology through increasing effector cytokines of the Th17 axis, including IL-17, and IL-22 (Veldhoen *et al.,* 2008; Gardner *et al*., 2010; Nyland *et al.,* 2011). This contention is supported by epidemiological research, which demonstrated that Hg exposure through gold-mining activities (iHg) and high consumption MeHg-containing of fish (MeHg) has been associated with increased level of autoantibodies (Silva *et al.,* 2004; Nyland *et al.,* 2011; Somers *et al.,* 2015; McSorley *et al.*, 2020) and higher concentrations of pro-inflammatory cytokines (e.g., IL-17, IL-1β, TNF-α, IL-1ra, IL-10, and IFN-γ), including IL-17 (Gardner *et al.*, 2010; Nyland *et al.,* 2011). Both iHg and MeHg, by establishing proinflammatory environment and inducing Th17 response may have a significant contribution to exaggeration of disease activity, given that previous studies conducted in SLE patients, demonstrated that cytokines associated Th17 response are positively associated with disease activity and increased SELENA SLEDAI scores (Cheng *et al.,* 2009; Chen *et al.,* 2010; El-Gazzar *et al.,* 2017; Selvaraja *et al.,* 2019).

The results presented in recent study were in contrast with some of the reports that have found associations between Th17 cytokines and measures of disease activity, as well as disease-associated damage. Although observations in multiple cohorts demonstrated that Th17 cytokines, including IL-17, can increase in SLE patients, whereas in healthy controls this cytokine remains low or undetectable (Wong *et al.,* 2008; Mok *et al.,* 2010; Vincent *et al.,* 2013), most of these studies found no associations between effector Th17 cytokines, including IL-17 (Zhao *et al.,* 2009; Mok *et al.,* 2010; Vincent *et al.,* 2013), IL-17A (Tanasescu *et al.,* 2010; Robak *et al.,* 2013; Raymond *et al.,* 2017), IL-17F (Tanasescu *et al.,* 2010; Robak *et al.,* 2013), IL-22 (Yang *et al.,* 2013)and disease activity determined by SELENA

SLEDAI measure. In addition, lack of association has been also observed in other studies, which demonstrated no relationship between IL-23 (Wong *et al.,* 2008; Cheng *et al.,* 2009; Tanasescu *et al.,* 2010; Robak *et al.,* 2013), IL-27 (Li *et al.,* 2010; Branco Pinto Duarte *et al.,* 2013) and IL-33 (Yang *et al.,* 2010).

The low cytokine levels reported in the recent SLE cohort may also explain lack of statistically significant associations between disease activity and Th17 cytokines, including IL-17A, as other studies, demonstrating relationship between SELENA SLEDAI and effectors Th17 cytokines, including IL-17, IL-17A, IL-17E and IL-22, were obtained from the patients with active/severe SLE disease (Cheng *et al.,* 2009; Chen *et al.,* 2010; Robak *et al.,* 2013, Yang *et al.,* 2013), lupus nephritis (Selvaraja *et al.,* 2019) or skin involvement (El-Gazzar *et al.,* 2017), where concentrations of these cytokines were higher than in the recent study. For example, Chen *et al.* (2010) reported a significant association in the SLE patients (n=65) with mean (SD) IL-17A concentrations of 2.14 (3.82) pg/ml, whereas in the recent study, IL-17A concentrations were lower (IL-17A mean (SD) was 0.95 (1.35) pg/ml). Low level of IL-17A in the recent cohort might be indicative of inactive disease phase or used of anti-inflammatory treatment, as IL-22 concentrations have been shown to be lower in relapsing SLE than in the healthy controls (Lin *et al.,* 2013).

Some observational studies conducted in SLE cohorts demonstrated that concentrations of IL-17, as well as IL-17A, IL-17E, IL-17F tend to be significantly higher in the patients with an active phase of SLE disease (Cheng *et al.,* 2009; Chen *et al.,* 2010; Robak *et al.,* 2013; Yang *et al.,* 2013), whereas others found no differences between inactive or active SLE diseases (Mok *et al.,* 2010; Pan *et al.,* 2013; Vincent *et al.,* 2013; Li *et al.,* 2019), instead showing that IL-17 can significantly increase in SLE patients with central nervous system disease (Vincent *et al.,* 2013), lupus skin disease and lupus nephritis (Yang *et al.,* 2013). In the current cohort, both IL-17E and IL-17F had low detection rate (%), being n=9 (10.2%) and n=8 (9.1%), respectively. Observations in SLE cohorts indicated that IL-17E is assoctaied with disease activity determined by SELENA SLEDAI measure (Li *et al.,* 2019; Selvaraja

et al., 2019), and concentrations of this cytokine tend to be higher in SLE with lupus nephritis when compared to SLE patients without nephritis and healthy controls (Selvaraja *et al.,* 2019); whilst IL-17F concentrations has been found to increase in SLE patients with active disease (Robak *et al.,* 2013). Consequently, a low detection rate in the current study might be explained by relatively low disease activity in this cohort; and the fact that samples with detectable IL-17E and IL-17F concentrations might be obtained from the patients, who had active disease phase. Nevertheless, this is speculative as the additional data on disease activity and organ involvement was limited for this cohort.

Although in the current study the effector cytokines associated with Th17 response, IL-17A, IL-17E and IL-22 were positively associated with the measure of disease activity (SELENA SLEDAI scores), they were not correlated with any measure of Hg exposure, including hair, urine and number of dental amalgams, which is in contrast to previous study of Nyland et al. (2011), that found a significant association between MeHg/iHg exposure (median (range) Hg measured in hair 14.1 (1.1-62.4) μg/g) and IL-17 concentrations in the cohort of gold miners who were high consumers of fish. Discrepancies in the reported results can be explained by a relatively low level of Hg exposure in the current study, as hair Hg concentration in this patient cohort was much lower (median (range) (0.59 (0.37-1.57) ppm) compared to hair MeHg concentrations in high fish consuming populations who were also occupationally exposed to MeHg/iHg through artisanal gold mining (14.1 (1.1-62.4) ppm (Nyland *et al.,* 2011). In addition, urinary iHg concentrations (reported in the current study ((median (range), 1.99 (0.95-4.11) ng/g creatinine) were also lower than a cohort of gold miners ((median (range), (3.67 (0.87–8.93) μg/L urine (results unadjusted for creatinine)) (Gardner *et al.,* 2010), but close to those in a cohort of dental professionals (median (range) 1.6 (0-13) nmol/mmol creatinine) (Langworth *et al.,* 1997), who were exposed to iHg through work with amalgams. While exposure to dental fillings does not appear to be linked with the development of autoimmune disease (Bangsi *et al.,* 1998; Crowe *et al.,* 2016), urinary iHg has been reported to be associated with disease severity in patients with the autoimmune disease scleroderma (Arnett *et al.,* 2001), but not in SLE patients (Crowe *et al.,* 2016).

Furthermore, patients in this study had generally well-controlled SLE, which was reflected by low levels of disease activity and disease-associated damage scores and investigating the impact of total Hg exposures or IL-17 on such a limited range of disease activity score makes it more challenging to identify relationships. Although, we reported statistically significant associations between IL-27 concentrations in serum of patients with active phase of disease (SLAM score ≥ 7), the differences in ORs between those with high (SLAM score ≥ 7) and those with low (SLAM score < 7) SLAM score may be too small to be considered as clinically relevant, given that SLAM of 7 or 8 still represents a fairly low-level of disease activity. Similarly, the clinical relevance of the observed associations between disease-associated damage index SLICC/AACR+ and IL-17A/E serum concentrations should be interpreted with caution, as SLICC/ACR+ is an indicative of accumulative disease damage and does not reflect recent injury caused by disease development. This study is the first study reporting an association between IL-17A/E concentrations and disease damage measure determined by SLICC/ACR+ score in SLE patients. Previous studies investigating SLE cohorts reported an upregulated expression of IL-17E in the lensional tissues of several inflammatory skin diseases including atopic dermatitis and psoriasis, while showing associations between IL-17E status and disease activity determined by SELENA SLEDAI score in the SLE patients with lupus nephritis (Selvaraja *et al.,* 2019) and those being in active phase of the disease (Li *et al.,* 2019).

To the best of our knowledge, this study demonstrated for the first time, associations between IL-17A/E concentrations and disease-associated damage (SLICC/ACR+) and future studies should investigate if these cytokines actually contribute to long-term disease damage observed in SLE. Analysis identified that adjustment for MeHg concentrations in hair Hg has strengthened the relationship between IL-17E and SLICC/ACR+ score, whilst adjustment for urinary iHg significantly decreased the strength of this relationship. This observation may suggest that iHg might act as potential contributor to the severity of disease-associated damage; however more research is required to verify this relationship and to determine a causal role for iHg in SLE damage over time. The role for iHg in autoimmunity is supported by experimental studies, which demonstrated that exposure to low iHg concentrations had

proinflammatory effects including increased IL-17 concentrations *in vitro*, while MeHg was immunosuppressive and was not able to induce IL-17 production (Gardner *et al.,* 2010; Nyland *et al.,* 2011). In addition, we have previously reported a negative association between hair MeHg and decreased disease-associated damage in SLE patients (Crowe *et al.,* 2016), attributed to concomitant exposure to anti-inflammatory n-3 LCPUFA with fish consumption known to be beneficial in management of autoimmune diseases (Li *et al.,* 2019).

A limitation of this study includes an observational study design and the sample size which was small and restricted to the local population within Northern Ireland, who had relatively low total Hg exposure. The patients within this study had low levels of disease activity and relatively little disease-associated damage, making it difficult to demonstrate differences between Th17 associated cytokines and disease outcomes. Based on a retrospective sample size calculation, a sample ranging between 164 and 213 SLE patients would be required to observe any significant relationships between cytokines of Th17 axis and clinical measures of disease activity.

Conclusions

This study provides evidence that low-level of Hg exposure determined in hair (MeHg) or urine (iHg) has no significant impact on disease activity in SLE induced by Th17 response; however, there is some indication that different sources of Hg exposure (hair (MeHg) vs urine (iHg)) may change the strength of relationship between IL-17E and disease-associated damage in individuals with autoimmune condition.

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Figure 1. A summarized overview of the patients enrollment, data collection and analysis for the purpose of the current study (n=88).

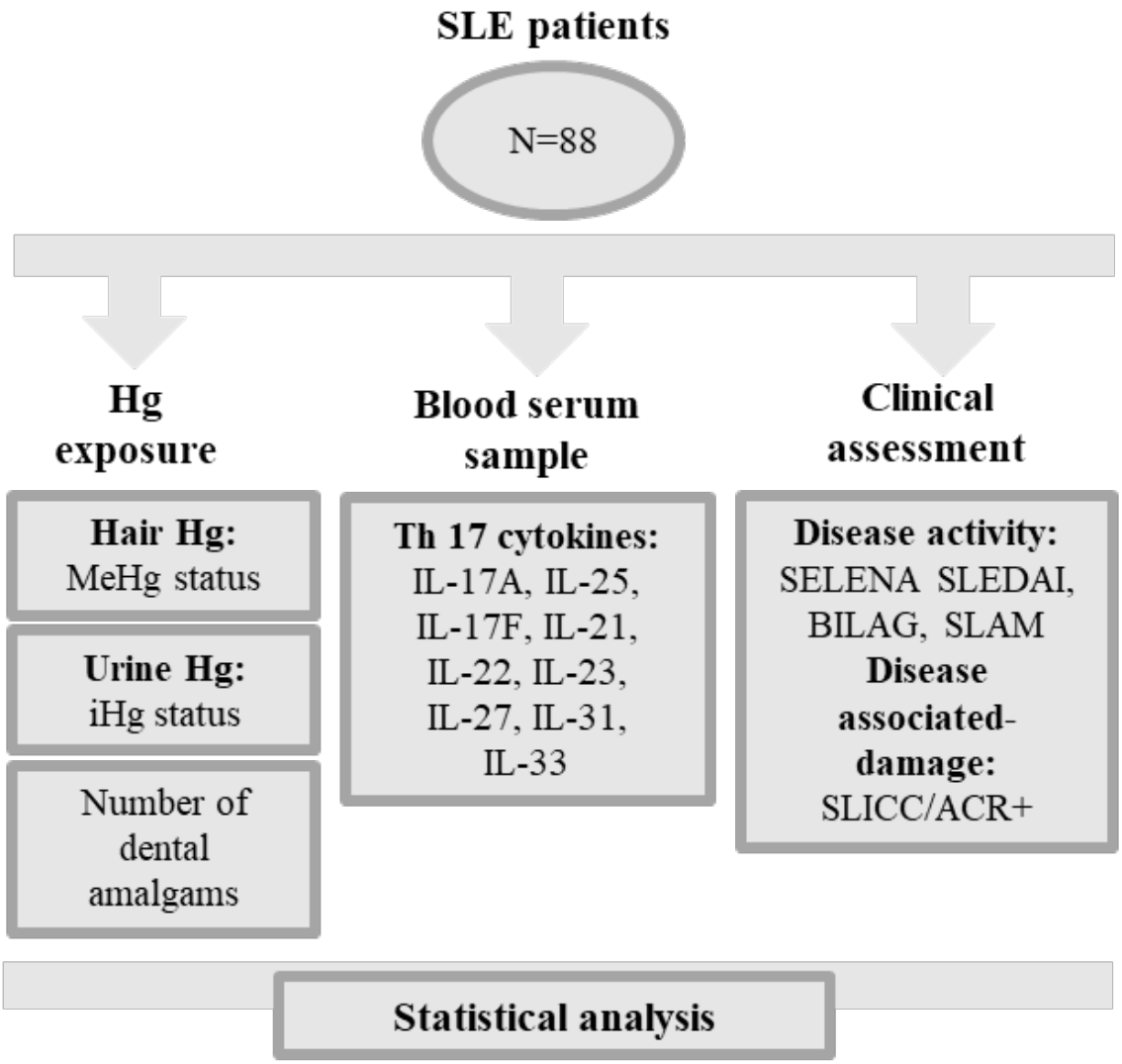

Table 1. Systemic lupus erythematosus patients' (n=88) disease activity and damage scores and biomarkers of Hg exposure.

	Mean (SD)	Median	Range
N		88	
Male (%)		2 (2)	
Female (%)		86 (98)	
Age	49.60 (13.04)	49	41.50-58.50
Body Mass Index (kg/m2)	27.12 (5.01)	25.8	23.81-30.66
SLAM	4.33 (2.66)	4	2-6
SELENA SLEDAI	3.65 (3.29)	3	1-5
BILAG	5.63 (5.09)	4	2-9
SLICC/ACR	1.33 (1.55)	1	0-2
Urine Hg (ng/g creatinine) (iHg)	3.12 (3.60)	1.99	0.95-4.11
Hair Hg (ppm) (MeHg)	1.19 (1.41)	0.59	0.37-1.57
Dental amalgams	5.26 (4.63)	5	0-9

BILAG: British Isles Lupus Assessment Group index; BMI: Body mass index; Hg: mercury; SELENA SLEDAI: Safety of Estrogen in Lupus Erythematosus National Assessment Systemic Lupus Activity Index; SLAM: Systemic Lupus Activity Measure; SLE: Systemic Lupus Erythematosus; SLICC/ACR: Systemic Lupus International Collaborative Clinics/American College of Rheumatology.

Table 2. Distributions of all measured autoimmune cytokines (pg/mL) (N=88).

	Detection N (%)	Mean (Std Dev)	Median	Range
IL-17A	83 (94.3)	0.95 (1.35)	0.66	0.35-1.08
IL-17E	9 (10.2)	0.36 (0.99)	0.20	0.20-0.20
IL-17F	8 (9.1)	341.06 (2220.74)	79.9	79.90-79.90
IL-21	60 (68.2)	7.16 (18.83)	2.57	0.53-7.23
IL-22	76 (86.4)	0.74 (1.55)	0.34	0.01-0.70
IL-23	49 (55.7)	3.30 (13.51)	0.70	0.70-2.32
IL-27	88 (100)	571.49 (332.39)	468.3	352.91-697.87
IL-31	12 (13.6)	13.33 (41.38)	7.78	7.78-7.78
IL-33	44 (50.0)	1.09 (4.06)	0.37	0.37-0.75

IL: Interleukin.

Detection is defined as cytokine concentration above LLOD.

The LLOD for each cytokine were IL-17A: 1.72-2.8pg/ml, IL-17E: 0.28-0.95pg/ml, IL-17F: 113-214pg/ml, IL-21: 0.75-3.0pg/ml, IL-22: 0.06-0.19pg/ml, IL-23: 0.99-2.0pg/ml, IL-27: 8.2-15pg/ml, IL-31: 5.5-15pg/ml, IL-33: 0.53-0.60pg/ml.

Table 3. The relationship between cytokines of Th17 axis and outcome scores for Disease Activity and Disease-Associated Damage (N=88).

		SLAM			SELENA SLEDAI			BILAG			SLICC ACR		
		Beta	(95% CI)	p	Beta	(95% CI)	p	Beta	(95% CI)	p	Beta	(95% CI)	p
IL-17A	Model 1	0.159	-0.263,0.582	0.455	0.211	-0.310,0.731	0.424	-0.234	-1.040,0.573	0.566	0.217	-0.025,0.459	0.078
	Model 2	0.129	-0.302, 0.560	0.554	0.219	-0.313, 0.752	0.415	-0.253	-1.083, 0.577	0.546	0.217	-0.021, 0.455	0.074
	Model 3	0.123	-0.310, 0.557	0.573	0.227	-0.308, 0.763	0.401	-0.264	-1.089, 0.561	0.526	0.223	-0.015, 0.462	0.066
	Model 4	0.128	-0.306, 0.563	0.558	0.167	-0.340, 0.673	0.515	-0.264	-1.089, 0.561	0.526	0.192	-0.033, 0.418	0.093
	Model 5	0.111	-0.328, 0.550	0.616	0.120	-0.363, 0.604	0.622	-0.330	-1.154, 0.495	0.429	0.191	-0.035, 0.417	0.097
IL-17E	Model 1	0.337	-0.237,0.912	0.247	0.625	-0.077,1.327	0.080	0.159	-0.946,1.263	0.776	0.465	0.143,0.786	0.005*
	Model 2	0.332	-0.250, 0.915	0.260	-0.613	-0.102, 1.33	0.092	0.201	-0.928, 1.330	0.724	0.421	0.103, 0.738	0.010*
	Model 3	0.326	-0.260, 0.911	0.272	0.624	-0.094, 1.342	0.088	0.186	-0.937, 1.310	0.742	0.429	0.112, 0.746	0.009*
	Model 4	0.338	-0.255, 0.930	0.260	0.453	-0.237, 1.14	0.195	0.186	-0.937, 1.310	0.742	0.347	0.042, 0.652	0.026*
	Model 5	0.328	-0.269, 0.926	0.278	0.455	-0.201, 1.110	0.171	0.098	-1.035, 1.231	0.863	0.355	0.050, 0.660	0.023*
IL-17F	Model 1	0.000	-0.212,0.000	0.797	0.000	0.000,0.000	0.895	0.000	0.000,0.001	0.506	0.000	0.000,0.000	0.878
	Model 2	0.000	-0.000, 0.000	0.700	-0.000	-0.000, 0.000	0.890	0.000	-0.000, 0.001	0.432	-0.000	-0.000, 0.000	0.919
	Model 3	0.000	-0.000, 0.000	0.751	-0.000	-0.000, 0.000	0.949	0.000	-0.000, 0.001	0.480	-0.000	-0.000, 0.000	0.975
	Model 4	0.000	-0.000, 0.000	0.700	-0.000	-0.000, 0.000	0.700	0.000	-0.000, 0.001	0.480	-0.000	-0.000, 0.000	0.717
	Model 5	0.000	0.000, 0.000	0.702	0.000	0.000, 0.000	0.948	0.000	0.000, 0.001	0.402	0.000	0.000, 0.000	0.877

IL-21	Model 1	-0.010	-0.187,0.020	0.504	0.000	-0.038,0.037	0.992	-0.014	-0.072,0.044	0.625	0.001	-0.017,0.019	0.924
	Model 2	-0.008	-0.039, 0.023	0.599	-0.000	-0.039, 0.038	0.988	-0.015	-0.071, 0.044	0.611	0.001	-0.016, 0.019	0.866
	Model 3	-0,007	-0.038, 0.024	0.642	-0,002	-0.040, 0.037	0.937	-0.013	-0.072, 0.046	0.658	0.000	-0.017, 0.018	0.957
	Model 4	-0.008	-0.040, 0.023	0.594	-0.001	-0.045, 0.028	0.660	-0.013	-0.072, 0.046	0.658	-0.002	-0.019,0.014	0.789
	Model 5	-0.006	-0.038, 0.025	0.690	-0.004	-0.039, 0.031	0.839	-0.016	-0.076, 0.044	0.603	-0.002	-0.019, 0.014	0.787
IL-22	Model 1	0.031	-0.162,0.400	0.868	-0.178	-0.632,0.275	0.437	-0.192	-0.895,0.511	0.588	-0.029	-0.244,0.185	0.788
	Model 2	0.018	-0.360, 0.396	0.924	-0.165	-0.632, 0.302	0.484	-0.224	-0.951, 0.504	0.543	0.010	-0.203, 0.223	0.927
	Model 3	0,011	-0.370, 0.392	0.954	-0.157	-0.627, 0.314	0.510	-0.237	-0.960, 0.486	0.516	0.017	-0.196, 0.231	0.871
	Model 4	0.018	-0.362, 0.399	0.923	-0.148	-0.591, 0.295	0.508	-0.224	-0.951, 0.486	0.516	0.018	-0.182, 0.218	0.858
	Model 5	0.024	-0.361, 0.410	0.900	-0.087	-0.511, 0.337	0.683	-0.173	-0.897, 0.551	0.636	-0.087	-0.511, 0.337	0.683
IL-23	Model 1	-0.016	-0.137, 0.027	0.465	-0.016	-0.068,0.036	0.550	-0.028	-0.108,0.053	0.493	-0.007	-0.032,0.017	0.553
	Model 2	-0.013	-0.056, 0.030	0.547	-0.015	-0.068, 0.038	0.569	-0.032	-0.114, 0.051	0.447	-0.005	-0.029, 0.019	0.694
	Model 3	-0,012	-0.056, 0.031	0.568	-0.016	-0.070, 0.038	0.549	-0.028	-0.110, 0.054	0.500	-0.005	-0.029, 0.019	0.694
	Model 4	-0.013	-0.056, 0.030	0.546	-0.021	-0.072, 0.023	0.405	-0.029	-0.112, 0.053	0.481	-0.008	-0.030, 0.015	0.508
	Model 5	-0.011	-0.055, 0.033	0.627	-0.013	-0.062,0.035	0.587	-0.026	-0.109,0.056	0.530	-0.013	-0.062,0.035	0.587
IL-27	Model 1	0.000	-0.111,0.002	0.942	0.000	-0.002,0.002	0.770	0.000	-0.003,0.003	0.976	0.000	-0.001,0.001	0.936
	Model 2	0.000	-0.002, 0.002	0.818	0.000	-0.002, 0.002	0.779	0.000	-0.003, 0.003	0.956	-0.000	-0.001, 0.001	0.971
	Model 3	0.000	-0.002, 0.002	0.810	0.000	-0.002, 0.002	0.788	0.000	-0.003, 0.004	0.931	-0.000	-0.001, 0.001	0.955

	Model 4	0.000	-0.002,0.002	0.820	0.000	-0.002, 0.002	0.835	0.000	-0.003, 0.004	0.940	-0.000	-0.001, 0.001	0.894
	Model 5	0.000	-0.002, 0.002	0.969	-0.001	-0.003,0.001	0.553	-0.001	-0.004,0.003	0.743	-0.001	-0.003,0.001	0.553
IL-31	Model 1	-0.006	-0.086,0.008	0.429	-0.006	-0.023,0.011	0.483	-0.010	-0.036,0.017	0.471	-0.002	-0.010,0.006	0.556
	Model 2	-0.005	-0.019, 0.009	0.515	-0.006	-0.023, 0.011	0.501	-0.011	-0.038, 0.016	0.429	-0.001	-0.009, 0.006	0.709
	Model 3	-0,004	-0.018, 0.010	0.540	-0,006	-0.023, 0.011	0.478	-0.010	-0.036, 0.017	0.479	-0.002	-0.010, 0.006	0.658
	Model 4	-0.005	-0.019, 0.009	0.514	-0.008	-0.024, 0.009	0.352	-0.010	-0.037, 0.017	0.455	-0.002	-0.010, 0.005	0.530
	Model 5	-0.004	-0.018, 0.011	0.613	-0.005	-0.020,0.011	0.572	-0.009	-0.036,0.019	0.531	-0.005	-0.020,0.011	0.572
IL-33	Model 1	-0.054	-0.061,0.087	0.448	-0.059	-0.233,0.114	0.497	-0.102	-0.370,0.166	0.451	-0.024	-0.106,0.057	0.555
	Model 2	-0.046	-0.188, 6.617	0.178	-0.058	-0.235, 0.118	0.513	-0.113	-0.386, 0.159	0.410	-0.017	-0.097, 0.064	0.680
	Model 3	-0,044	-0.190, 0.100	0.547	-0,061	-0.239, 0.116	0.495	-0.102	-0.375, 0.171	0.460	-0.019	-0.099, 0.061	0.640
	Model 4	-0.046	-0.190, 0.098	0.526	-0.078	-0.245, 0.090	0.358	-0.106	-0.381, 0.168	0.443	-0.026	-0.102, 0.050	0.497
	Model 5	-0.037	-0.183, 0.109	0.614	-0.047	-0.208, 0.113	0.559	-0.093	-0.367, 0.182	0.504	-0.047	-0.208, 0.113	0.559

IL: interleukin; BILAG: British Isles Lupus Assessment Group index; BMI: body mass index; CI: confidence intervals; Hg: mercury; SELENA SLEDAI: Safety of Estrogen in Lupus Erythematosus National Assessment; Systemic Lupus Activity Index; SLAM: Systemic Lupus Activity Measure; SLE: Systemic Lupus Erythematosus; SLICC/ACR: Systemic Lupus International Collaborative Clinics/American College of Rheumatology.

Statistical analysis was performed using linear regression.

Data presented as β-value (95 % confidence interval), p-value.

1 Unadjusted series of models

2 Model series adjusted for age BMI

3 Model series adjusted for age, BMI and hair Hg (MeHg)

4 Model series adjusted for age, BMI and urine Hg (iHg)

5 Model series adjusted for age, BMI and total Hg exposure measured in hair (MeHg), urine (iHg) and dental amalgams

*denotes significance $p < 0.05$

Table 4. The crude and adjusted odds ratios estimating the associations between cytokines of Th17 axis and outcome scores for Disease Activity and Disease-Associated Damage (N=88).

		SLAM			SELENA SLEDAI			BILAG			SLICC/ACR+		
		Low (<7)	High (≥7)	95%CI	Low (<4)	High (≥4)	95%CI	Low (<9)	High (≥9)	95%CI	Low (0)	High (>0)	95% CI
n, (%) patients		54 (51%)	34 (39%)		72 (82%)	16 (18%)		65 (74%)	23 (26%)		55 (63%)	22 (37%)	
IL-17A	Model 1	1.00	1.14	0.81, 1.58	1.00	1.38	0.87, 2.19	1.00	0.64	0.29, 1.43	1.00	0.36*	0.14, 0.91
	Model 2	1.00	1.08	0.77, 1.52	1.00	1.36	0.86, 2.16	1.00	0.63	0.28, 1.42	1.00	0.30*	0.11, 0.82
	Model 3	1.00	1.08	0.77, 1.53	1.00	1.36	0.89, 2.09	1.00	0.64	0.29, 1.43	1.00	0.32*	0.11, 0.88
	Model 4	1.00	1.07	0.76, 1.51	1.00	1.30	0.84, 2.02	1.00	0.63	0.28, 1.42	1.00	0.33	0.12, 0.87
	Model 5	1.00	1.06	0.75, 1.51	1.00	1.38	0.84, 1.96	1.00	0.63	0.28, 1.42	1.00	0.35*	0.13, 0.96

IL-17E	Model 1	1.00	1.46	0.79, 2.69	1.00	1.39	0.66, 2.94	1.00	0.76	0.25, 2.29	1.00	0.37	0.04, 3.71
	Model 2	1.00	1.53	0.82, 2.86	1.00	1.40	0.66, 2.95	1.00	0.76	0.25, 2.30	1.00	0.36	0.03, 4.43
	Model 3	1.00	1.53	0.82, 2.87	1.00	1.41	0.70, 2.85	1.00	0.76	0.26, 2.23	1.00	0.34	0.02, 4.89
	Model 4	1.00	1.48	0.79, 2.77	1.00	1.23	0.61, 2.51	1.00	0.76	0.25, 2.30	1.00	0.36	0.03, 4.89
	Model 5	1.00	1.48	0.75, 1.51	1.00	1.26	0.59, 2.70	1.00	0.75	0.24, 2.32	1.00	0.30	0.02, 4.96
IL-17F	Model 1	1.00	1.00	0.97, 1.03	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
	Model 2	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
	Model 3	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
	Model 4	1.00	1.00	1.00, 1.00	1.00	1.00	0.99, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00

	Model 5	1.00	1.00	1.00, 1.00	1.00	0.99	0.99, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
IL-21	Model 1	1.00	1.00	0.97, 1.03	1.00	0.99	0.97, 1.02	1.00	0.99	0.95, 1.03	1.00	1.01	0.99, 1.03
	Model 2	1.00	1.00	0.97, 1.03	1.00	0.99	0.97, 1.02	1.00	0.99	0.95, 1.03	1.00	1.01	0.98, 1.04
	Model 3	1.00	1.00	0.97, 1.03	1.00	0.99	0.96, 1.02	1.00	0.99	0.95, 1.03	1.00	1.01	0.98, 1.04
	Model 4	1.00	1.00	0.97, 1.03	1.00	0.99	0.95, 1.02	1.00	0.99	0.95, 1.03	1.00	1.01	0.98, 1.05
	Model 5	1.00	1.00	0.97, 1.04	1.00	0.98	0.93, 1.03	1.00	0.99	0.94, 1.04	1.00	1.02	0.98, 1.06
IL-22	Model 1	1.00	1.20	0.89, 1.62	1.00	0.86	0.59, 1.27	1.00	0.79	0.44, 1.42	1.00	1.14	0.85, 1.55
	Model 2	1.00	1.15	0.82, 1.61	1.00	0.86	0.59, 1.25	1.00	0.79	0.44, 1.41	1.00	1.08	0.78, 1.50
	Model 3	1.00	1.15	0.82, 1.62	1.00	0.87	0.58, 1.28	1.00	0.79	0.45, 1.39	1.00	1.07	0.75, 1.51

	Model 4	1.00	1.16	0.83, 1.62	1.00	0.86	0.59, 1.28	1.00	0.79	0.44, 1.41	1.00	1.08	0.77, 1.52
	Model 5	1.00	1.17	0.84, 1.63	1.00	0.78	0.44, 1.40	1.00	0.75	0.39, 1.43	1.00	1.07	0.72, 1.59
IL-23	Model 1	1.00	0.99	0.93, 1.05	1.00	0.98	0.93, 1.04	1.00	0.98	0.87, 1.09	1.00	1.02	0.97, 1.08
	Model 2	1.00	0.99	0.93, 1.06	1.00	0.98	0.93, 1.05	1.00	0.98	0.87, 1.09	1.00	1.02	0.97, 1.08
	Model 3	1.00	0.99	0.93, 1.06	1.00	0.98	0.93, 1.04	1.00	0.98	0.87, 1.10	1.00	1.02	0.97, 1.08
	Model 4	1.00	0.99	0.93, 1.06	1.00	0.98	0.91, 1.05	1.00	0.98	0.87, 1.09	1.00	1.03	0.97, 1.09
	Model 5	1.00	0.99	0.93, 1.06	1.00	0.98	0.89, 1.07	1.00	0.97	0.80, 1.16	1.00	1.03	0.97, 1.09
IL-27	Model 1	1.00	1.00*	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
	Model 2	1.00	1.00	1.00, 1.00	1.00	1.00	0.99, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00

	Model 3	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
	Model 4	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
	Model 5	1.00	1.00*	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00	1.00	1.00	1.00, 1.00
IL-31	Model 1	1.00	1.00	0.98, 1.02	1.00	0.99	0.95, 1.03	1.00	0.98	0.88, 1.09	1.00	1.01	0.99, 1.02
	Model 2	1.00	1.00	0.98, 1.02	1.00	0.99	0.95, 1.03	1.00	0.98	0.88, 1.09	1.00	1.01	0.99, 1.03
	Model 3	1.00	1.00	0.98, 1.02	1.00	0.99	0.95, 1.03	1.00	0.98	0.88, 1.09	1.00	1.01	0.99, 1.03
	Model 4	1.00	1.00	0.98, 1.02	1.00	0.99	0.93, 1.05	1.00	0.98	0.88, 1.09	1.00	1.01	0.99, 1.03
	Model 5	1.00	1.00	0.98, 1.02	1.00	0.99	0.93, 1.05	1.00	0.98	0.89, 1.09	1.00	1.01	0.99, 1.03
IL-33	Model 1	1.00	0.90	0.51, 1.58	1.00	0.93	0.74, 1.17	1.00	0.81	0.40, 1.64	1.00	1.05	0.93, 1.20

Model 2	1.00	0.87	0.44, 1.75	1.00	0.93	0.73, 1.19	1.00	0.81	0.40, 1.64	1.00	1.05	0.92, 1.21
Model 3	1.00	0.87	0.44, 1.75	1.00	0.93	0.74, 1.18	1.00	0.81	0.40, 1.61	1.00	1.06	0.92, 1.21
Model 4	1.00	0.87	0.43, 1.75	1.00	0.91	0.69, 1.20	1.00	0.81	0.40, 1.64	1.00	1.06	0.92, 1.22
Model 5	1.00	0.86	0.41, 1.78	1.00	0.92	0.68, 1.26	1.00	0.78	0.38, 1.61	1.00	1.06	0.92, 1.22

IL: interleukin; BILAG: British Isles Lupus Assessment Group index; BMI: body mass index; CI: confidence intervals; Hg: mercury; SELENA SLEDAI: Safety of Estrogen in Lupus Erythematosus National Assessment; Systemic Lupus Activity Index; SLAM: Systemic Lupus Activity Measure; SLE: Systemic Lupus Erythematosus; SLICC/ACR: Systemic Lupus International Collaborative Clinics/American College of Rheumatology.

Statistical analysis was performed using logistic regression.

Data presented as odds ratio (OR) (95 % confidence interval), p-value.

1 Unadjusted series of models

2 Model series adjusted for age BMI

3 Model series adjusted for age, BMI and hair Hg (MeHg)

4 Model series adjusted for age, BMI and urine Hg (iHg)

5 Model series adjusted for age, BMI and total Hg exposure measured in hair (MeHg), urine (iHg) and dental amalgams

*denotes significance $p < 0.05$.

CHAPTER 5:

The influence of fish consumption on methylmercury status and plasma concentrations of T helper 17-associated cytokines in women of child-bearing age.

Abstract

Background: Observational studies in high fish eating populations have reported associations between hair Hg status and markers of autoimmunity, including interleukin (IL)-17, a signature of T helper (Th) 17 response. There is currently a lack of studies that have investigated the impact of consuming the recommended fish intake (2 portions a week) on Hg status and autoimmune markers, including IL17.

Objectives: To investigate the effect of consuming two portions of fish (high and low MeHg-containing) per week on hair MeHg and plasma concentrations of cytokines IL-17A and IL-22 in women of child-bearing age.

Methods: A total 33 females of child-bearing age were randomised to consume meal with no fish, or either one/two portions of tuna (high MeHg) (n=15) or sardines (low MeHg) (n=7) per week for a period of 8 weeks. Hair Hg and plasma Th17 cytokines were measured at baseline (week 0) and post-intervention (week 8). Analysis of covariance (ANCOVA) was used to compare post-intervention MeHg, IL-17A and IL-22 concentrations across the three treatment groups (tuna; sardines; control - no fish) while controlling for baseline concentrations, age and BMI.

Results: Intake of tuna significantly increased MeHg concentrations in hair when compared to those who consumed sardines (the post-intervention mean difference in MeHg concentration was 312.7, 95% CI 129.1, 496.2, $p < 0.01$) and the control group (the post-intervention mean difference in MeHg concentration was 328.8, 95% CI 170.1, 487.6, $p < 0.01$). There was no significant change in IL-17A or IL-22 concentrations in any of the intervention groups.

Conclusions: Intake of one or two portions of either tuna or sardines had no impact on concentrations of cytokines (IL-17A and IL-22) associated with Th17 response, despite a significant change in MeHg status. Sardine intervention had no impact on Hg or IL-17 cytokines. These results provide evidence

that consumption of the recommended two portions of fish a week does not impact on Th17-induced autoimmunity in the women of child-bearing age.

Keywords: Th17 axis; IL-17; IL-22; Autoimmunity; Methylmercury; Fish

Introduction

Fish is an important component of human diet as it provides a high-quality protein, and other health-promoting nutrients, such as n-3 long chain polyunsaturated fatty acids (LCPUFA) which have been shown to promote neurodevelopmental and cardiometabolic health (Gribble *et al.,* 2016) and reduce risk of metabolic disease (Howe *et al.,* 2014). Dietary guidelines promote the inclusion of at least two portions of fish per week (SACN, 2004; EPA, 2015), of which one should be an oily fish (e.g., salmon, mackerel, herring, sardines), owing to the multiple health benefits for brain and heart health (Gribble *et al*., 2016), as well as the reduced risk of chronic inflammatory conditions (e.g., diabetes, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE)) or allergies (Swanson *et al*., 2012; *Li et al*., 2019). Nevertheless, all fish can contain a certain amount of methylmercury (MeHg), with the highest MeHg concentrations being found in the large long-lived predatory species (e.g., sharks, swordfish, tuna, mackerel) (Mahaffey *et al.,* 2011), as a result of their bioaccumulation of methylated mercury (Hg) which occurs naturally in the aquatic sediments (Silbernagel *et al.,* 2011).

Consumption of any type of fish, including commercially available fish products, has been shown to increase MeHg exposure (Kuras *et al.,* 2017). This is of significant public health concern, especially among women of child-bearing age and young children, as early life exposure to MeHg has been associated with damaging effects on the developing brain and nervous system (Rice *et al.,* 2014). In addition, evidence obtained from epidemiological studies conducted among high fish-consuming populations suggested a link between Hg exposure from fish consumption and increased concentrations of autoimmune biomarkers, including antinuclear antibodies (ANA), anti-nucleolar antibodies (ANoA) (Silva *et al.,* 2004; Nyland *et al.,* 2011a; Somers *et al.,* 2015; McSorley *et al.,* 2020) and interleukin (IL)-17 (Nyland *et al.,* 2011a), what may suggest a potential role of Hg in autoimmunity.

Autoimmunity can develop as the result of dysregulated immune system responses which are directed against endogenous tissue. The failure to distinguish between self and non-self-antigens results in the induction of autoreactive T-helper (Th) cells and the production of autoantibodies, which can

subsequently lead to chronic inflammation, and the development of autoimmune disease (Martin *et al.,* 2014; Rosenblum *et al.,* 2015). The incidence of autoimmune disease in the general population is relatively low and affects approximately 5-8% of population with a sex bias, favouring women over men (Fairweather and Rose, 2004, Haller-Kikkatalo *et al.,* 2017).

Although the aetiology of autoimmune conditions remains unclear, the scholastic effects of both genetic susceptibility and exposure to environmental factors may have a significant role in the disease initiation and symptom exacerbation (Rosenblum *et al*., 2015). Hg is one of the proposed environmental triggers of autoimmunity, supported by the experimental studies demonstrating that exposure to iHg/MeHg can result in the in the immune dysregulation (Shenker *et al.,* 2000; Gardner *et al.,* 2010a) and spontaneous development of lupus-like condition, characterized by nephritis (Hirsch *et al.,* 1982), arthritis, cerebritis, skin rash and vasculitis (Pollard *et al.,* 1999), increased autoantibodies (Martinsson *et al.,* 2008) and immune complex (IC) depositions (Hultman and Hansson-Georgiadis, 1999) in the genetically predisposed murine models.

The balance between pro- and anti-inflammatory responses is thought to be critical for maintaining immune tolerance, which is tightly regulated by actions of regulatory T (Treg) and effector Th17 cell subsets. Exposure to environmental toxicants, such as Hg has been associated with chronic immune activation resultant from increasing release of potent pro-inflammatory mediators (e.g., IL-1β, IL-6 and IL-21) (Umare *et al.,* 2014; Dolff *et al.,* 2011) and production of reactive oxygen species (ROS) (Kim and Sharma, 2004), which have been shown to promote Th17 cell formation, while decreasing Treg generation (Lee, 2018). The imbalance between Treg and Th17 responses, can result in subsequent loss of immune tolerance which can lead to the development of autoimmune conditions (Rosenblum *et al.,* 2015). Evidence supporting the role of Th17 cells in the development and propagation of autoimmune pathogenesis is obtained from observational studies which have reported increased concentrations of cytokines associated with Th17 response, including IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31 and IL-33 in individuals with autoimmune conditions such as psoriasis, RA, SLE and multiple sclerosis (MS)

(Cheng *et al.,* 2009; Chen *et al.,* 2010; Robak *et al.,* 2013; Zhao and Chen, 2014; Meka *et al.,* 2015; El-Gazzar *et al.,* 2017; Eyerich *et al.,* 2017; Raymond *et al.,* 2017; Yago *et al.,* 2017; de J Guerrero-García *et al.,* 2018; Gharibi *et al.,* 2019; Selvaraja *et al.,* 2019; McGinley *et al.,* 2020). In addition, the status of the main effector cytokine of the Th17 axis, IL-17, has been associated with clinical measures of disease activity, as well as biomarkers of autoimmunity, including ANA, rheumatoid factor and other pro-inflammatory cytokines implicated in the Th17 axis, including IL-22 (Chen *et al.,* 2010; Miletić *et al.,* 2012; Hu *et al.,* 2013).

Fish, being a part of well-balanced diet, but also a main source of toxic MeHg for humans, may affect the health status of vulnerable to autoimmunity populations, in particular women of child-bearing age. To date, there was no study which investigated an impact of MeHg exposure through consumption of commercial fish product according to the recommended by recent dietary guidelines fish intake (2 portions of fish a week) (SACN, 2004; EPA, 2015) on the Th17-induced autoimmunity. Therefore, by using a convenient a sample of female participants, who completed an eight weeklong intervention with consuming either of one or two portions of (high and low MeHg) fish a week, the main aim of this study is to evaluate if moderate fish intake can significantly change MeHg status and IL-17A and IL-22 cytokine concentrations compared to eating a meal without fish in a group of apparently healthy women of child-bearing age.

Materials and Methods

The data used in the current study were obtained from participants who were recruited to an 8-week randomized clinical intervention trial – iFish (registered at www.clinicaltrials.gov as NCT03765580). A modification of the original iFish study design (Conway *et al.,* 2021) is outlined in figure 1. The original iFish study was designed to investigate the influence of fish consumption on LCPUFA status whilst accounting for the fatty acid desaturase (FADS) genotype in women of child-bearing age from Northern Ireland, UK (Conway *et al.,* 2021). This study was reviewed and approved by the Ulster

University Research Ethics Committee (REC/16/0077). All research was conducted in accordance with the 1964 Declaration of Helsinki and its amendments.

A total of 66 women of childbearing age (aged between 18 and 45 years) were recruited between October 2016 and January 2017. The exclusion criteria included fish/seafood allergies, being a regular consumer of fish (< 2 portions of fish per week), use of fish oil/protein supplements, pregnancy or post-menopausal. Eligible participants were invited for two sampling appointments (baseline, week 0 and post-intervention, week 8) at the Human Intervention Study Unit (HISU) at Ulster University between January and May 2017 and were randomly allocated to one of the intervention groups (no fish or either 1 portion or 2 portions per week). Each of the groups received a lunch meal which contained a 140-g portion of fish (sardines or tuna) once or twice a week, or a control meal with no fish once a week for the duration of study. At baseline and following the 8-week intervention, participants provided biological samples including blood, hair, information on habitual dietary intake, anthropometric measurements and general health and lifestyle information.

In the current study, samples and data for 33 of the 49 participants who completed the intervention. There was insufficient sample available for 16 of the 49 participants (n=10 had insufficient plasma sample for cytokine analysis; n=6 had insufficient hair specimen for Hg analysis) who completed the original iFish intervention trial and thus the final number of participants used for the current study was 33. The overall compliance was high at 98%, and was 97%, 99% and 99% for no fish, tuna, sardines and no fish groups, respectively. Participants were grouped based on the type of fish they consumed during the intervention period. The intervention groups use for analysis includes a group consuming, no fish; 1-2 portions of sardines (low Hg) per week; or 1-2 portions of tuna (high Hg) (see figure 1).

Anthropometric measurements

Body weight (to the nearest 0.1 kg) was measured using TANITA digital scales (TANITA Europe, The Netherlands). Height was measured to the nearest centimetre using a stadiometer and body mass index (BMI, kg/m^2) was then calculated.

Blood sample collection

Fasting blood samples collected at baseline and post-intervention were processed to obtain serum and plasma by centrifugation at 3500 rpm for 15 min at 4 °C. All aliquots were subsequently frozen and stored at – 80 °C until batch analysis.

Analysis of cytokines associated with Th17 response (cytokines IL-17A and IL-22)

The plasma concentrations of two effector cytokines of Th17-assocaited axis, IL-17A and IL-22, were analysed according to manufacturer's instructions, using two separate singleplex electrochemiluminescence-based Meso Scale Discovery (MSD) immunoassays, S-PLEX Human IL-17A (K151C3S-Series) and S-PLEX Human IL-22 (K151H3S-Series) (Meso Scale Discovery, Gaithersburg, MD, USA). These cytokines were selected based on previous studies, which indicated that they are involved in the Th17 response associated with autoimmunity (Gardner *et al.,* 2010a; Nyland *et al.,* 2011a; Somers *et al.,* 2015). MSD plates were analyzed on the MS2400 imager MSD by using Discovery Workbench 4.0 software.

Results are presented as median and interquartile range (25^{th} and 75^{th} percentiles) in fg/ml, owing to high sensitivity of kit used to determine cytokine concentrations, as IL-17A has been reported to be low in apparently healthy individuals (Fujino *et al.,* 2003). The inter assay cytokine CVs were IL-17A (17.9%) and IL-22 (16.8%). The lower limit of detection (LLOD) for each cytokine were 57.8 fg/mL for IL-17A and 2.1 fg/mL for IL-22.

Hair sampling and assessment of MeHg exposure

Hair specimens were used to analyze MeHg exposure in those who completed the study, including the fish intervention and control group. Some 90% of total Hg present in the hair is assumed to be MeHg (Marinho *et al.,* 2014), therefore hair sample concentrations were used as biomarker of MeHg intake from the diet. Participants provided a hair sample consisting of approximately 200 hair strands, cut as close to the scalp as possible from the back of the head at week 0 (baseline) and week 8 (post-

intervention). All specimens were stored in high quality paper envelopes and labelled appropriately and transported to the University of Rochester, Rochester, NY, USA, where Hg exposure was determined by using cold vapor atomic absorption spectroscopy (Magos, 1981).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp, Armonk, NY). Descriptive statistics are expressed as mean and SD, whilst cytokine concentrations are presented as median and interquartile range (IQR). The differences in baseline characteristics between the intervention groups were tested using one-way analysis of variance (ANOVA). Analysis of covariance (ANCOVA) was used to compare differences in post-intervention hair MeHg concentrations; plasma cytokines status (IL-17A, IL-22) between the three treatment groups while controlling for baseline values and other relevant covariates, age and BMI.

Statistical significance in all analyses was determined using a two-sided approach $\alpha = 0.05$ with a Bonferroni correction for multiple comparisons. ANCOVA assumptions were checked using standard residual plots (Weisberg, 2005). If violated, logarithmic transformation of the outcome variable was considered. Hg concentrations in hair as well as LCPUFA measures are presented on their natural scale, whilst IL-17A and IL-22 cytokine concentrations were logarithmically transformed because of heavily skewed distributions and to reduce the impact of some large values evident in the data. Variables are presented as post-intervention (PI) mean differences (95% CI), whereas those, which are required log-transformation (cytokines IL-17A and IL-22) are displayed as the ratio of geometric means (95% CI). The post-intervention mean difference was calculated for MeHg hair and cytokine (IL-17A and IL-22) concentrations by subtracting the post-intervention mean from the control mean.

Results

Of the 49 participants who completed a intervention with fish (Conway *et al.,* 2021), a total of 33 are included in the analysis presented here which is grouped by high MeHg fish (tuna) (n=15), low MeHg fish (sardines) (n=7) or no fish (n=11).

Study Population

Participant demographic characteristics measured at baseline and at post intervention (PI) are presented in table 1. The mean (SD) age and BMI for the whole cohort (n=33) at the baseline was 25.88 (6.7) years and 23.99 (5.18) kg/m^2, respectively. Baseline MeHg concentrations in hair samples were mean (SD) of 440.7 (297.2) ppb. The median (IQR) plasma cytokine concentration at baseline was 1616 (1411-1866) fg/ml for IL-17A and 426.8 (235.7-828.3) fg/ml for IL-22. There were no significant differences in the baseline characteristics between the intervention groups ($p > 0.05$) (see table 1). Total mean MeHg concentrations of the sardine product was 63.2 ng/g dry weight (d.w.) and the tuna product was 437.9 ng/g d.w.

The effect of dietary fish intervention on MeHg concentrations in hair and cytokine IL-17A and IL-22

The effect of intervention with fish on hair MeHg, and plasma cytokine IL-17A and IL-22 concentrations at week 8, with adjustment for baseline concentrations, with or without adjustment for baseline age and BMI are presented in table 2. A significant increase in hair MeHg concentrations was observed in the group consuming tuna (high MeHg fish) for 8 weeks compared to the group consuming sardines (low MeHg fish) for 8 weeks (difference in post-intervention means 312.7, 95% CI 129.1, 496.2, $p < 0.01$) and the control group consuming no fish (difference in post-intervention means 328.8, 95% CI 170.1, 487.6, $p < 0.01$) (see table 2).

Discussion

In this cohort of healthy women of child-bearing age, intervention with up to two portions of low MeHg (sardines) and high MeHg (tuna) containing fish had no significant effect on Th17 response. Of interest, the consumption of tuna for 8 weeks significantly increased hair MeHg concentrations compared to those who consumed sardines or those in the control group who consumed no fish. Intake of one or two portions of fish, including sardines or tuna had no significant effect on concentrations of the cytokines IL-17A and IL-22 determined in the plasma. The results of the current study suggest that despite increased MeHg concentrations associated with consuming tuna or sardines no significant increase was observed for plasma concentrations of the Th17 associated cytokines, IL-17A and IL-22. Nevertheless, more caution should be taken regarding to the interpretation of the following findings, owing to the study limitations, including sample size and restricting cohort to local population of women of childbearing age.

To the best of our knowledge, this is the first study (conducted among women of child-bearing age) which demonstrated that consumption of the recommended two portions of fish, either low in MeHg (sardines) or high in MeHg (tuna), had no significant impact on concentrations of Th17 cytokines associated with autoimmune response, IL-17A and IL-22, despite a significant increase in MeHg status with tuna consumption These results are in contrast with other observational studies conducted among high fish consumers, which reported associations between MeHg status and increased IL-17 concentrations in the serum (Gardner *et al.,* 2010b; Nyland *et al.,* 2011a). Participants in the current study compared to those studies (Gardner *et al.,* 2010b; Nyland *et al.,* 2011a). were younger and had low fish intake at the baseline (< 2 fish portions a week), compared to native residents of Brazilian Amazon, who were reported to consume approximately eight fish meals a week (Passos *et al.,* 2003; Passos *et al.,* 2008).

The relatively low MeHg exposure in the current cohort of child-bearing age women might to explain why no significant change in IL-17A and/or IL-22 concentration was observed in this study. The MeHg concentrations determined in the hair samples of females in this study (the mean baseline MeHg

concentrations in hair was 440.7 (297.2) ppb; equal to 0.441 ppm) is similar to the hair MeHg concentrations reported in the cohort of reproductive-age women (geometric mean (geometric SD) of MeHg concentration in hair was 0.22 (0.03) ppm) from US (Somers *et al.,* 2015). Nevertheless, the MeHg concentrations of the current cohort was lower compared to the studies conducted among high fish consuming populations in the US, Seychelles or a population of Brazilian Amazonian gold miners where mercury status was reported as 4.58 µg/L (median in blood), 10.23 ppm (mean in hair) and 14.1 ppm (median in hair), respectively (Monastero *et al.,* 2017; McSorley *et al.,* 2020; Nyland *et al.,* 2011a). The study by Nyland *et al.* in Brazilian Amazonian gold mining community reported a positive association between MeHg status and IL-17 concentrations, raising concerns in relation the potential impact of Hg on immune function and its possible impact on autoimmune disease risk (Nyland *et al.,* 2011a).

The observed change in the hair MeHg concentrations upon intervention with fish in the current study is consistent with a number of epidemiological studies, which demonstrated that consumption of any type of fish can result in exposure to MeHg (Myers *et al.,* 2009) and that fish intake correlates with hair MeHg concentrations, in those who eat fish at least once a week (Hoang *et al.,* 2017), especially if they consume fish which is high in MeHg, such as shark, tuna, swordfish, marlin, cod (Nielsen *et al.,* 2014). In the current study noted that consumption of one or two portions of tuna a week, significantly increased MeHg concentrations in hair, when compared to those who consumed sardines and those who didn't consume fish. This is consistent with other studies (Kuras *et al.,* 2017; Yan *et al.,* 2017; Murata *et al.,* 2019), which demonstrated that increasing fish intake can result in an increase in the hair MeHg concentrations (Kuras *et al.,* 2017). Furthermore, those who ate tuna meals on a weekly basis (mean hair MeHg was 0.466 µg/g ± 0.328 in tuna customers; and 0.110 µg/g ± 0.105 in those who do not eat fish) (Murata *et al.,* 2019) may be at risk of having a high MeHg status (odds risk ratio was 2.086; 95% CI 1.041, 4.180, p = 0.03) (Yan *et al.,* 2017). Fish used for intervention in the current study had a relatively low MeHg content (total mean of MeHg were 63.2 ng/g d.w. for sardines and 437.9 ng/g d.w.in tuna), which was within the acceptable limit of 1 µg MeHg/g in fish/fish-based products (EC,

2006). Although, canned seafood is a known source of MeHg exposure (Siedlikowski *et al.,* 2016), and the canning process may itself increase Hg in fish (Boadi *et al.,* 2011), the total mean content of MeHg of the tuna and sardines used in the current study is aligned with concentrations reported in canned fish products (Alcala-Orozco *et al.,* 2017). Nevertheless, relative differences in the dietary habits between fish species and occupied position in the aquatic food chain, may explain why tuna is a high MeHg fish, whereas sardines are thought to be low in MeHg content. Sardines, being at the bottom of marine food chain are small fish which feed predominantly on zooplankton; whereas tuna, is a large predator, that resides at the top of food chain, and consumes a variety of prey thereby resulting in a higher MeHg content (Beckers and Rinklebe, 2017).

The effect of low-level MeHg exposure through fish consumption on immune function and health remains elusive. Although evidence from experimental studies indicated that exposure to high Hg concentrations can lead to immune dysregulation and the development of lupus-like syndrome in genetically predisposed animal models (Bagenstose *et al.,* 1999; Hu *et al.,* 1999; Pollard *et al.,* 1999; Hansson *et al.,* 2003; Haggqvist *et al.,* 2005; Havarinasab and Hultman, 2005; Ramírez-Sandoval *et al.,* 2015), although the evidence from humans is conflicting. The observed discrepancies observed in these previous studies may be attributable the individual being exposed to different forms of Hg (iHg in gold miners, MeHg in fish consumers) which could potentially result in their development of autoimmune biomarkers, including ANA (Silva *et al.,* 2004; Nyland *et al.,* 2011a; Somers *et al.,* 2015; McSorley *et al.,* 2020) and IL-17 concentrations (Gardner *et al.,* 2010; Nyland *et al.,* 2011a).

The results of the current study reported no significant change in the concentrations of the Th17 associated cytokines, IL-17A and IL-22, which has been recently implicated in autoimmunity. This finding is in alignment with other studies which found no relationship between Hg exposure and IL-17 concentrations among fish consumers (Nyland *et al.,* 2011b; Monastero *et al.,* 2017), except for one study which reported that IL-17 status was positively correlated with Hg concentrations in hair, blood and urine in the native residents of Brazilian Amazon (Nyland *et al.,* 2011a).

The presence of anti-inflammatory nutrients in fish, such as n-3 LCPUFA may explain why increased MeHg exposure have no influence on Th17 response. Previous studies have been shown that intake of ≥ 2 portions of canned fish products can increase n-3 LCPUFA status in the low consumers of fish (Conway *et al.,* 2021), and optimal n-3 LCPUFA status have been associated with positive birth outcomes (Ramón *et al.,* 2009) and improved metabolic profile, demonstrated by reduced concentrations of proinflammatory cytokines (e.g., IL-1β, IL-6, TNF-α) (Stratakis *et al.,* 2020). Therefore, intake of fish-derived n-3 LCPUFA has the potential to mitigate adverse effects of Hg exposure, as they have been shown to modulate immunity through anti-inflammatory and antioxidant activities (Calder, 2008; Gutiérrez *et al.,* 2019).

The current study utilized high sensitivity and specificity of techniques to quantify IL-17A and IL-22 cytokines and these methodologies are considered the were gold standard for the analysis of these biomarkers. Nevertheless, this study has certain limitations that include a small sample size which was not originally powered to investigate the influence of fish consumption on MeHg status or autoimmune biomarkers. Additionally, the study was restricted to young healthy adult females, who had very low fish consumption. Consequently, future research efforts should be expanded to include a broader population with age range groups and family history of autoimmune condition in the past, given that autoimmune disease vary widely with the age of onset (Angum *et al.,* 2020) and tend to aggregate within families (Cárdenas-Roldán *et al.,* 2013).

Conclusions

This study provides initial evidence demonstrating that consumption of the recommended two portions of fish a week, despite changing MeHg status, had no significant effect on cytokine IL-17A and IL-22 concentrations and thus are unlikely to impact autoimmunity through Hg modulated changes of Th17 cytokines. Although results of the current study may suggest that consuming fish according to dietary

guidelines does not contribute to Th17-induced autoimmune response in the population of women of childbearing age, more research is needed in order to confirm these findings in the general populations.

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Figure 1. Modified version of CONSORT flow diagram for study design of the current study (outlined in the red box).

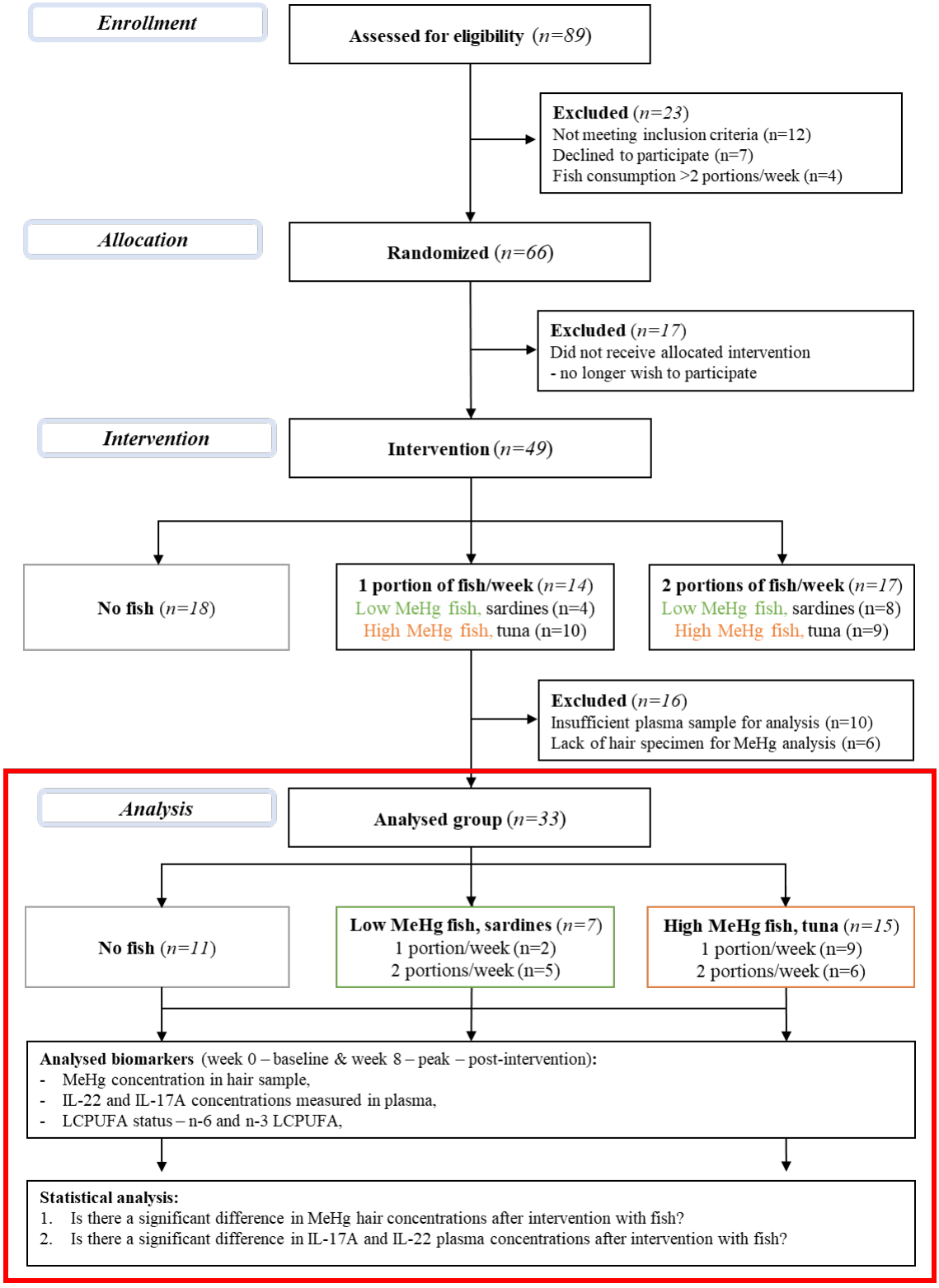

Table 1. Baseline and post-intervention (PI) characteristics of iFish study participants (n=33) for the whole cohort and according to the intervention group.

	Whole group (n=33)		No fish group (n=11)		Sardine group (n=7)		Tuna group (n=15)		p value*
	Baseline	PI	Baseline	PI	Baseline	PI	Baseline	PI	
Age (years)	25.88 (6.465)		27.18 (6.524)		27.00 (8.246)		24.40 (5.629)		0.501
BMI (kg/m2)	23.99 (5.176)		25.24 (5.925)		22.74 (3.404)		23.65 (5.390)		0.586
MeHg (ppb)	440.7 (297.2)	477.98 (323.7)	353.6 (275.6)	262.8 (208.1)	390.60 (208.3)	302.56 (123.1)	528.0 (337.2)	717.6 (297.9)	0.305
IL-17A (fg/mL)	1616 (1411-1866)	1453 (1155–1811)	1597 (1421-1901)	1265 (1010-1666)	1652 (1204-1853)	1665 (1435-2265)	1616 (1421-1880)	1529 (1178-1938)	0.971
IL-22 (fg/mL)	426.8 (235.7-828.3)	368.5 (237.7–510.2)	508.0 (297.5-915.9)	325.6 (227.7-393.9)	289.8 (227.5-480.1)	478.6 (227.0-949.4)	431.7 (219.6-884.2)	369.8 (274.2-527.9)	0.621
LA (mg/ml)	0.251 (0.047)	0.279 (0.045)	0.246 (0.060)	0.272 (0.040)	0.259 (0.045)	0.280 (0.063)	0.250 (0.038)	0.284 (0.043)	0.861
ALA (mg/ml)	0.013 (0.002)	0.012 (0.004)	0.012 (0.002)	0.012 (0.004)	0.012 (0.001)	0.011 (0.005)	0.013 (0.002)	0.013 (0.004)	0.469
AA (mg/ml)	0.065 (0.012)	0.069 (0.013)	0.067 (0.016)	0.070 (0.012)	0.063 (0.017)	0.062 (0.013)	0.064 (0.012)	0.072 (0.014)	0.847
EPA (mg/ml)	0.013 (0.002)	0.014 (0.006)	0.013 (0.004)	0.012 (0.004)	0.013 (0.002)	0.022 (0.006)	0.012 (0.002)	0.013 (0.004)	0.648

DHA (mg/ml)	0.021 (0.005)	0.024 (0.006)	0.023 (0.006)	0.022 (0.004)	0.021 (0.002)	0.030 (0.007)	0.020 (0.004)	0.024 (0.005)	0.306
Total n-6 LCPUFA (mg/ml)	0.316 (0.055)	0.348 (0.053)	0.313 (0.073)	0.342 (0.045)	0.322 (0.056)	0.343 (0.074)	0.315 (0.042)	0.355 (0.051)	0.948
Total n-3 LCPUFA (mg/ml)	0.046 (0.007)	0.051 (0.013)	0.048 (0.010)	0.045 (0.009)	0.045 (0.004)	0.063 (0.017)	0.046 (0.007)	0.050 (0.009)	0.569
n-6:n-3 LCPUFA ratio	6.829 (0.976)	7.115 (1.611)	6.503 (1.272)	7.793 (1.832)	7.072 (0.906)	5.658 (1.544)	6.955 (0.734)	7.297 (1.026)	0.397

Data are presented as mean (SD); cytokine IL-17A and IL-22 values are presented as median (IQR), where IQR is the 25th and 75th centiles.

Age and BMI are presented at baseline only.

p value for significant difference between intervention groups at baseline as determined using ANOVA.

$p < 0.05$ considered as significant.

Table 2. The effect of dietary intervention with high Hg (tuna), low in Hg (sardines) or control meal (no fish) on post-intervention (PI) mean concentrations of MeHg, IL-17A and IL-22.

			Intervention group		
			Tuna vs Control	Sardines vs Control	Tuna vs Sardines
MeHg	Adjusted for baseline MeHg	Differences in PI means (95% CI)	339.0 (186.7; 491.3)	15.15 (-164.1; 194.4)	323.9 (151.5; 496.2)
		p value	< 0.01	1.00	< 0.01
	Adjusted for baseline MeHg, BMI and age	Differences in PI means (95% CI)	328.8 (170.1; 487.6)	16.14 (-170.8; 203.1)	312.7 (129.1; 496.2)
		p value	< 0.01	1.00	< 0.01
IL-17A	Adjusted for baseline IL-17A	Ratio of PI geometric means (95% CI)	1.250 (0.889; 1.754)	1.466 (0.968; 2.218)	0.851 (0.575; 1.259)
		p value	0.32	0.08	0.92
	Adjusted for baseline IL-17A, BMI and age	Ratio of PI geometric means (95% CI)	1.256 (0.885; 1.778)	1.416 (0.925; 2.168)	-0.053 (-0.228; 0.123)
		p value	0.33	0.14	1.00
IL-22	Adjusted for baseline IL-22	Ratio of PI geometric means (95% CI)	1.556 (0.638; 3.784)	1.514 (0.508; 4.508)	1.026 (0.365; 2.884)

	p value	0.65	1.00	1.00
Adjusted for baseline IL-22, BMI and age	Ratio of PI geometric means (95% CI)	1.472 (0.578; 3.758)	1.455 (0.465; 4.571)	1.012 (0.340; 3.013)
	p value	0.90	1.00	1.00

PI: post-intervention; BMI: body mass index; MeHg: methylmercury; IL: interleukin.

IL-17A and IL-22 were log-transformed for statistical analysis; all values are presented on their natural scale.

Data are presented as differences in PI means (95% CI) or for IL-17A and IL-22 as ratio of PI geometric means (95% CI).

Analysis of covariance (ANCOVA) for comparison of PI.

p values are Bonferroni-corrected to allow for three pairwise group comparisons.

$p < 0.05$ considered as significant.

Table 3. The effect of dietary intervention with 1 (n=9) or 2 portions (n=6) of high Hg (tuna) compared to control no fish meal (n=11) on post-intervention (PI) mean concentrations of MeHg, IL-17A and IL-22.

Intervention group			1 portion Tuna *vs* Control	2 portions Tuna *vs* Control	2 *vs* 1 portions Tuna
MeHg	Adjusted for baseline MeHg	Differences in PI means (95% CI)	218.3 (83.10; 353.5)	516.8 (364.7; 669.0)	298.6 (144.7; 452.5)
		p value	< 0.01	< 0.01	0.01
	Adjusted for baseline MeHg, BMI and age	Differences in PI means (95% CI)	202.3 (68.28; 336.4)	521.3 (367.6; 675.1)	319.0 (163.9; 474.1)
		p value	< 0.01	< 0.01	< 0.01
IL-17A	Adjusted for baseline IL-17A	Ratio of PI geometric means (95% CI)	1.300 (0.845; 2.004)	1.172 (0.711; 1.932)	0.902 (0.533; 1.524)
		p value	0.11	1.00	1.00
	Adjusted for baseline IL-17A, BMI and age	Ratio of PI geometric means (95% CI)	1.279 (0.815; 2.009)	1.242 (0.743; 2.080)	0.971 (0.558; 1.687)
		p value	0.50	0.85	1.00
IL-22	Adjusted for baseline IL-22	Ratio of PI geometric means (95% CI)	2.099 (0.778; 5.649)	0.991 (0.323; 3.034)	0.472 (0.147; 1.510)
		p value	0.20	1.00	0.33

	Adjusted for baseline IL-22, BMI and age	Ratio of PI geometric means (95% CI)	2.028 (0.710; 5.808)	1.067 (0.330; 3.459)	0.505 (0.155; 1.791)
		p value	0.28	1.00	0.56

PI: post-intervention; BMI: body mass index; MeHg: methylmercury; IL: interleukin.

IL-17A and IL-22 were log-transformed for statistical analysis; all values are presented on their natural scale,

Data are presented as differences in PI means (95% CI) or for IL-17A and IL-22 as ratio of PI geometric means (95% CI).

Analysis of covariance (ANCOVA) for comparison of PI.

p values are Bonferroni-corrected to allow for three pairwise group comparisons.

$p < 0.05$ considered as significant.

CHAPTER 6:

General Discussion

This PhD thesis builds on the existing research and investigate the effect of exposure to mercury (Hg), specifically methylmercury (MeHg) and risk of autoimmunity in the high fish consumers from Republic of Seychelles (McSorley *et al.,* 2020), recruited through the Seychelles Child Development Study (SCDS) (Davidson *et al.,* 2006) and autoimmune patients with SLE disease (Crowe *et al.,* 2015).

There is compelling evidence to implicate role of Hg exposure in the development of autoimmunity, especially in the individuals with certain genetic predisposition (Pollard *et al.,* 2019; Bjørklund *et al.,* 2020). Although humans can be exposed to different forms of Hg, diet, in particularly consumption of the fish in the most common source of the neurotoxic MeHg (Silbergeld *et al.,* 2005). This has been also indicated in the findings of this literature review (**Chapter 2**), which additionally highlighted the importance of other foods, including plant and animal products, as potential sources of iHg/MeHg, especially if they are obtained from polluted with Hg sites. Although toxicological assessments provided evidence that increased Hg emission into the environment, particularly in Asian countries, may result in higher iHg/MeHg content detectable in the foods, the studies rarely investigated how consumption of contaminate foods could impact health outcomes. (Wang *et al.,* 2005; Rai *et al.,* 2019; Gebeyehu and Bayissa, 2020). Therefore, this review for the for the first time, comprehensively analyzed how intake of these foods may influence the Hg exposure in their consumers. In addition, this review considered the potential impact of other nutrients present in these products (e.g., n-3 polyunsaturated fatty acids (n-3 LCPUFA) and selenium (Se) in fish, phytochemicals in rice and vegetables), as well as the overall dietary pattern and applied food preparation methods, when assessing a total effect of Hg toxicity. Findings of the review were consistent with previous reports which demonstrated that fish is the most important source of MeHg in humans, while demonstrating that other foods such as rice, vegetables, and animal meat, may also be significant contributors to dietary Hg exposure in the residents of areas with high Hg-pollution.

Among all dietary sources of iHg/MeHg, fish consumption is considered as primary route of MeHg exposure MeHg, and regular consumption of any type of fish can increase customer MeHg status

(Myers *et al.,* 2009). Nevertheless, fish is also an excellent source of high-quality protein and other health-promoting nutrients, and its consumption has been associated with multiple health benefits, including those for brain and cardiometabolic function (Gribble *et al.,* 2016), as well as reduced risk of chronic inflammatory conditions (Swanson *et al.,* 2012; Li *et al.,* 2019). The majority of epidemiological investigations from fish consumers, including in those vulnerable to MeHg exposure, such as pregnant women and children (Valent *et al.,* 2013; Hsi *et al.,* 2014; Strain *et al.,* 2015; Golding *et al.,* 2017; Gustin *et al.,* 2017; van Wijngaarden *et al.,* 2017), have rarely reported any adverse health outcomes associated with regular fish consumption, however, few studies indicate that high Hg exposure from the consumption of MeHg containing fish and/or involvement in gold-mining actives (exposure to iHg) and increased risk of autoimmunity, as demonstrated by increased concentrations of antinuclear antibodies (ANA), anti-nucleolar antibodies (ANoA) and interleukin (IL)-17 (Silva *et al.,* 2004; Alves *et al.,* 2006; Gardner *et al.,* 2010; Nyland *et al.,* 2011a; Somers *et al.,* 2015; McSorley *et al.,* 2020). Following results of SCDS whereby hair MeHg concentrations were associated with increased odds of higher combined score ANA in the young adults with reported high fish intake (McSorley *et al.,* 2020), **Chapter 3** was focused on investigating impact of MeHg exposure on concentrations of cytokines associated with Th17 response in the same cohort of young adults. Cytokine concentrations were used as novel biomarkers of autoimmunity, to provide better understanding of MeHg contribution to the risk of autoimmune pathogenesis. In contrast to the previous report (McSorley *et al.,* 2020), findings from this study indicate that MeHg concentrations in hair was not associated with any of the cytokines associated with Th17 axis. Furthermore, the LCPUFA status of the individuals did not alter this finding. Consequently, this may indicate that MeHg exposure in high fish consumers does not impact on markers associated with autoimmunity in early adulthood. It is important to note, that use of ANA as determinant of autoimmune response in previous study (McSorley *et al.,* 2020), may have limited specificity, as ANA is not a diagnostic marker of autoimmunity and can be detected in healthy individuals without autoimmune condition. Therefore, in this body of research, to investigate if Hg exposure contributes to the risk of autoimmune disease, the cytokines associated with T helper (Th) 17 responses were quantified, as they have been identified as important mediators of immunopathology in

several inflammatory and autoimmune conditions, including SLE (Singh *et al.,* 2014; Rother and van der Vlag, 2015; Koga *et al.,* 2019). Use of these cytokines appears to be more relevant for investigations of the autoimmunity, as their presence is a direct indicative of Th17-mediated immune response. In addition, a main effector cytokine of Th17 cells, IL-17 is not detectable in healthy individuals. Therefore, use of cytokines associated with Th17 axis, as autoimmune biomarkers, is a novel aspect of this PhD research.

Furthermore, evidence obtained from the research conducted in autoimmune patients with autoimmune disease, including systemic lupus erythematosus (SLE) indicates that exposure to the pro-inflammatory Hg have potential to trigger of immune response, as well as induce and exaggerate autoimmune pathology (Crowe *et al.,* 2017), depending on the individual's exposure and susceptibility (Bjørklund *et al.,* 2020). Hg acting through cellular receptors, such as aryl hydrocarbon receptor (AhR) can induce Th17 differentiation followed by increasing their cytokines, including IL-17. Observational studies conducted in SLE cohort reported that concentrations of cytokines associated with Th17 axis have been increased in the patients, compared to controls; and also they have been found to be associated with clinical measures of disease severity (Henriques *et al.,* 2010; Khan and Ahmed, 2015; Schmidt *et al.,* 2018), including disease activity and disease-associated damage indexes (Wong *et al.,* 2008; Cheng *et al.,* 2009; Chen *et al.,* 2010; Mok *et al.,* 2010; Tanasescu *et al.,* 2010; Robak *et al.,* 2013; El-Gazzar *et al.,* 2017; Li *et al.,* 2019). Although a previous observational study conducted on the SLE cohort found no associations between disease activity and biomarkers of Hg exposure, including hair MeHg, urinary iHg and number of dental amalgams; the MeHg concentrations in hair were inversely correlated with disease-associated index. To explore further if Hg exposure may impact autoimmune pathology mediated through Th17 axis in individuals with SLE, this cohort was further investigated in **Chapter 4**. The results of this study demonstrated that Hg exposure, determined as in urinary iHg and hair MeHg, as well as number of dental amalgams (elemental Hg) have no significant impact on the relationship between cytokines associated with Th17 responses and disease severity, measured by disease activity and disease-associated damage scores. These findings are important for understanding the role of

environmental factors in autoimmunity risk, showing that low-level of Hg exposure determined in urine (iHg) and hair (MeHg) does not promote Th17-induced autoimmunity in SLE disease.

The MeHg exposure through fish consumption is associated with the individual characteristics, which include fish choice, as well as frequency and number of consumed fish meals (Castaño *et al.,* 2015; Faial *et al.,* 2015; Kusanagi *et al.,* 2018; Wiseman *et al.,* 2019). Individuals who consume fish more than five times per month have been shown to have higher MeHg exposure than those who eat fish occasionally (one to two fish meals per month) (Nielsen *et al.,* 2014), nevertheless the effect of this changes in MeHg status on autoimmunity risk has never been investigated. To address this aim, the follow up of randomized controlled trial (iFish Study) (Conway *et al.,* 2021) investigating effect of fish consumption on LCPUFA status was carried out, in order to determine if consumption of one to two servings of canned fish a week could significantly change concentrations of cytokines (IL-17A and IL-22) associated with Th17 response in this group of women of child-bearing age (**Chapter 5**). Although the analysis demonstrated that intake of fish high in MeHg (tuna) significantly increased MeHg concentrations in hair, plasma IL-17A and IL-22 cytokine concentrations remained unchanged. These results are consistent with other studies, which have shown that increasing fish intake can significantly increase/elevate MeHg status (Kuras *et al.,* 2017; Yan *et al.,* 2017; Murata *et al.,* 2019), even though the MeHg content in the consumed fish is low and remains within the within the legal limit of 1 μg MeHg/g MeHg for the fish/fish-based products (EC, 2006). The findings presented in **Chapter 5** are of particular importance for public health, as they add on to existing evidence supporting current dietary guidelines of safe fish consumption (SCAN, 2004; EPA 2015).

Lack of observed associations between MeHg exposure through fish consumption in young adults and autoimmune-predisposed individuals/SLE patients reported in the observational studies (**Chapter 3** and **Chapter 4**), together with findings of intervention trail (**Chapter 5**) presented in this PhD thesis, provide evidence that increased MeHg concentrations in hair resultant from fish intake have no impact on cytokines associated with autoimmunity, what may suggest that fish-derived nutrients may have a

role in mitigating against MeHg toxicity. It has been shown that marine nutrients, such as -3 LCPUFA, especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), as well as Se, acting as negative confounders, may influence the reported associations between health outcomes and MeHg exposure in fish consuming populations (Strain *et al.,* 2008; Choi *et al.,* 2014). The mitigating potential of fish-derived nutrients on the toxic effects of MeHg exposure is attributed to the anti-inflammatory properties of n-3 LCPUFA (Stratakis *et al.,* 2020), which through restoring a tolerogenic phenotype (Lee, 2018) may promote homeostasis of immune system (Calder, 2015). A regular fish intake (of at least two portions a week, including one portions of oily fish) (SCAN, 2004) helps to maintain an adequate n-3 LCPUFA status (Conway *et al.,* 2021), which appears to be critical for counteracting some of the proposed adverse effects of MeHg exposure. This can be through enhanced formation of EPA and DHA-derived metabolites (e.g. such as the eicosanoids, 3-series prostanoids (PGE3 TXA3), 5-series leukotrienes (LTB5), E-series resolvins (RvE), pro-resolving lipid mediators, D-series resolvins (RvD), protectins (PD) and maresins (MaR)) (Calder, 2015), which by decreasing concentrations of potent inflammatory mediators (e.g. C-reactive protein (CRP), interferon (INF)-γ, TNF-α, IL-6, IL-10 (McSorley *et al.,* 2018; Stratakis *et al.,* 2020) can modulate immune function in a favorable direction (Calder, 2008; Gutiérrez *et al.,* 2019). In addition, there is plausible evidence to suggest that achieving Se:Hg molar ratio ≥ 1 through increasing dietary intakes of Se may also help to protect against consequences of MeHg exposures (Ralston, 2008), and by enhancing antioxidant capability may prevent oxidative damage among high seafood consumers (Grotto *et al.,* 2011).

Although findings of this PhD research consistently indicated that exposure to Hg through fish consumption is not associated with Th17-induced autoimmunity, the following considerations should be emphasized. Firstly, Hg exposure measured differ between the studies, depending on population investigated (see table 1). In **Chapter 3**, the Hg exposure in young native residents of Republic of Seychelles, resultant from high consumption of the locally sourced ocean fish (on average ~7 meals a week), determined in hair samples (MeHg) was the highest (median 5.98 ppm and IQR was 0.42-52.08

ppm) among the studies; whereas Hg exposure in **Chapter 4** (median 5.98 ppm and IQR was 0.42-52.08 ppm) and **Chapter 5** was approximately 10 times lower. These discrepancies might be explained by the differences in frequency and type of fish consume, as populations described in **Chapter 5** at the baseline was known as low-fish consumers (median Hg concentrations measured in hair at baseline was 0.396 and IQR was 0.187-0.663). Although, the Hg exposure, determined either in hair (median Hg in hair was 0.59 ppm and IQR was 0.37-1.57 ppm) and urine (median Hg in urine was median 1.99 ng/g creatinine and IQR was 0.95-4.11 ng/g creatinine) samples in SLE cohort described in **Chapter 4** is similarly to population in **Chapter 5**, was low; the data on fish intake in these patients is lacking.

Secondly, concentrations of Th17 cytokines were consistently low between the studies, despite that they were measured in the samples obtained from three different populations vary in Hg exposure levels, including high fish consumers (**Chapter 3)**, SLE patients **(Chapter 4)** and women of child-bearing age **(Chapter 5).** There is limited number of studies assessing concentrations of Th17 cytokines in fish eating populations. Up to date, there are two studies, which measured only IL-17 concentration in the fish-eating populations. Study of Monastero *et al*. (2017) which reported no relationship between Hg exposure (median 4.58 μg/L (0.00458 ppm)) and the presence of IL-17 in a US cohort of avid fish and seafood consumers (n=287) did not report exact concentrations of IL-17 in this population; however it must be noted that in this study individuals who had IL-17 above the limit of detection of 1.38 pg/mL, had mean (SD) Hg concentrations in blood of 6.5 (5.6) μg/L (0.0065 ppm) (Monastero *et al*., 2017). Furthermore, study conducted on native population from Brazilian Amazon, despite showing a correlation between with hair Hg (14.1 ug/g; equal to 14.1 ppm) IL-17, the concentrations of IL-17 in these individuals have not been reported (Nyland *et al*., 2011a). Therefore, limited information on Th17 cytokine status in fish consumers does not allow comparisons with the results reported in **Chapters 3** and **5 of** this PhD research. Furthermore, available studies conducted in SLE cohorts assessing concentrations of cytokines associated with Th17 axis are vary in results. For example, serum concentrations of IL-17A, IL-17E, IL-21, IL-22, IL-23, IL-31 and IL-33 reported in the **Chapter 4** appears to be low, when compared with others (Wong *et al.,* 2008; Cheng *et al.,* 2009; Chen *et al.,*

2010; Mok *et al.,* 2010; Tanasescu *et al.,* 2010; Yang *et al.,* 2010; Pan *et al.,* 2013; Robak *et al.,* 2013; Lan *et al.,* 2014; Zhang *et al.,* 2014; Huang *et al.,* 2016; El-Gazzar *et al.,* 2017; Raymond *et al.*, 2017; Wang *et al.,* 2018; Li *et al.,* 2019); whereas concentrations of IL-17F and IL-27 in the current SLE cohort seems to be much higher than in other studies (Li *et al.,* 2010; Gaber *et al.,* 2012; Branco Pinto Duarte *et al.,* 2013; Robak *et al.,* 2013). The low Th17 cytokines concentrations reported in the chapters of this PhD, might be explained by the measurement of the sensitivity of kits used as well as the sample collection and storage conditions. Although, concentrations of cytokines measured in this PhD were determined by utilizing an enzyme-linked immunosorbent assay (ELISA) (see table 2), which is known as a gold standard method used to detect and quantify immune markers (Alhajj and Farhana, 2021), the samples examined in **Chapters 3, 4 and 5** were pre-collected and stored frozen for about 8-10 years before the analysis, what could possibly impact on the samples stability and amount of cytokines within them. Furthermore, cohort of SLE patients described in **Chapter 4** had a well-managed SLE disease, characterized by low disease activity and disease-associated damage measures, what could be another reason why concentrations of Th17 cytokines were low, with levels being comparable those measured in apparently healthy populations described in **Chapters 3 and 5.**

Furthermore, when interpreting outcomes of this PhD thesis it is important to take into consideration of certain limitations. Since second half of this PhD occurred in the lockdown of COVID-19 pandemic (years 2019/2020 and 2020/2021), the restrictions introduced significantly affected the possibility to obtain samples directly from the participants, therefore measurements of Th17 cytokines were done on the samples, which have been already collected and stored. In addition, the quantity of Th17 cytokines might be potentially affected by the storage conditions, leading to low readings; what may partially explain lack of observed associations. Furthermore, the analyses in this PhD were focused on the individuals being within similar age group (**Chapter 3**) or restricted by small sample size (**Chapters 4 and 5**) limited to particular region (**Chapter 3** – Republic of Seychelles; **Chapters 4 and 5** – Northern Ireland), what could also impact findings of this research, as age and ethnicity are known as important factors in the development of autoimmunity (Goronzy and Weyand, 2012; Lee *et al.,* 2018). Lastly, the

detailed data on the health status of participants as well as family health history was limited, what compromised ability to discuss the outcomes in the wider context. The populations investigated in the **Chapters 3 and 5** were assumed to be apparently healthy young individuals, however with no information on other conditions in which Th17 response may also play role.

Future research efforts should aim to repeat following investigations in the larger cohorts, which would represent the general population, with broader age groups and information on dietary (fish intake, consumption of LPCUFA foods) and lifestyle habits (exposure to Hg, physical activity, smoking, pregnancy, area of living), as well as health status (presence of inflammatory/autoimmune conditions, obesity, medications used). It would be interesting to design a prospective study and follow these individuals to assess if the concentrations associated with Th17 response may change over the life course. Secondly, more research is needed in order to identify and understand impact of other sources of Hg contamination, which may pose a potential health risk in certain vulnerable population groups, such as young children living in polluted by Hg emissions areas in Asian countries. Furthermore, setting up collaborative research initiatives, in particular focused on the countries where Hg contamination has been previously recognized as an environmental issue, would bring additional benefits, owing to possibility to target individuals who might be at high risk of occupational Hg exposure.

The summarized conclusion of this PhD thesis is that exposure to Hg measured in hair (MeHg) as well as in urine (iHg) does not alter concentrations of Th17 cytokines, associated with the development of autoimmunity. These findings are of great importance for public health, as they support current dietary guidelines promoting fish consumption as a part of well-balanced diet, that can promote long-lasting health and prevent chronic illness during life course. Nevertheless, this research has several the limitations, such as the relatively young age of participants and the sample size being insufficiently powered to confirm significance of the presented results which should be considered when interpreting the significance of the results. Therefore, in further studies addressing dietary exposure to MeHg, it would be beneficial to also emphasize the role of other foods, as potential contributor to the MeHg

exposure, as well as nutritional value of their components that make up for overall diet quality along the lifetime.

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Table 1. Summarized Hg status of participants, including measure of hair Hg, urinary Hg and number of dental amalgams (where relevant) across the studies.

Population investigated	**Study design**		**Hg status**		
			Hg in hair (MeHg)	**Hg in urine (iHg)**	**Number of dental amalgams**
Young adults from Republic of Seychelles	Observational	Whole group (n=440)	5.98 (0.42-52.08)	N/A	N/A
SLE patients from Northern Ireland	Observational	Whole group (n=88)	0.59 (0.37-1.57)	1.99 (0.95-4.11)	5 (0-9)
Women of childbearing age from Northern Ireland	Randomized controlled trial	Whole group (n=33) - baseline	0.396 (0.187-0.663)		
		Whole group (n=33) - post-intervention	0.426 (0.281-0.682)		
		No fish group (n=11) - baseline	0.405 (0.077-0.633)		
		No fish group (n=11) - post-intervention	0.270 (0.070-0.387)		
		Sardines group (n=7) - baseline	0.396 (0.184-0.567)		

	Sardines group (n=7) - post-intervention	0.338 (0.152-0.426)
	Tuna group (n=7) - baseline	0.377 (0.242-0.789)
	Tuna group (n=7) - post-intervention	0.667 (0.454-0.867)

Hg status is presented as median (IQR).

Hg in hair (MeHg) is presented in ppm units.

Hg in urine is presented as ng/g creatinine.

Table 2. Summarized concentrations of Th17 cytokines measured across the studies.

Population investigated	**Study design**		**Cytokines**								
			IL-17A	**IL-17E**	**IL-17F**	**IL-21**	**IL-22**	**IL-23**	**IL-27**	**IL-31**	**IL-33**
Young adults from Republic of Seychelles	Observational	Whole group (n=440)	1.22 (1.22-15.37)	0.20 (0.20-7.49)	79.90 (79.90-5580.71)	17.33 (0.53-718.66)	0.91 (0.04-573.50)	3.60 (0.70-937.37)	378.09 (5.80-2355.64)	9.68 (3.89-143.96)	1.49 (0.37-246.88)
SLE patients from Northern Ireland	Observational	Whole group (n=88)	0.66 (0.35-1.08)	0.20 (0.20-0.20)	79.90 (79.90-79.90)	2.57 (0.53-7.23)	0.34 (0.01-0.70)	0.70 (0.70-2.32)	468.3 (352.91-697.87)	7.78 (7.78-7.78)	0.37 (0.37-0.75)
Women of childbearing age from Northern Ireland	Randomized controlled trial	Whole group (n=33) - baseline	1.62 (1.41-1.87)				0.43 (0.24-0.83)				
		Whole group (n=33) - post-intervention	1.45 (1.16–1.81)				0.37 (0.24–0.51)				
		No fish group (n=11) - baseline	1.60 (1.42-1.90)				0.51 (0.30-0.92)				

	No fish group (n=11) - post-intervention	1.27 (1.01-1.67)	0.33 (0.23-0.39)
	Sardines group (n=7) - baseline	1.65 (1.20-1.85)	0.29 (0.23-0.48)
	Sardines group (n=7) - post-intervention	1.67 (1.43-2.27)	0.48 (0.23-0.95)
	Tuna group (n=7) - baseline	1.62 (1.42-1.88)	0.43 (0.22-0.88)
	Tuna group (n=7) - post-intervention	1.53 (1.18-1.94)	0.37 (0.27-0.53)

Cytokines are presented as median (IQR) in pg/ml

Summary of findings

The main research findings of this PhD thesis are:

1. Fish is the main source of mercury (Hg) exposure in the diet, however other products of plant and animal origin (rice, green leafy vegetables, processed meats), if obtained from Hg-polluted sites, are also notable contributors to human exposure. The nutritional value of iHg/MeHg-containing foods, as well as the technological processes used prior the consumption should be considered, to determine burden of Hg exposure in their consumers.
2. Dietary MeHg exposure from high fish consumption is not associated with concentrations of cytokines associated with Th17 response in young adults and n-3 LCPUFA, also found in fish, had no effect on this outcome. These findings contribute to the evidence supporting safe fish consumption among consumers.
3. Low level environmental exposure to Hg determined in urine (inorganic Hg), hair (MeHg) and number of dental amalgams (elemental Hg) had no impact on the relationship between Th17-associated cytokines and measures of disease severity in SLE. This finding demonstrates that exposure to Hg, including iHg, MeHg and elemental Hg, has no significant contribution to Th17 response autoimmune pathology.
4. Consumption of commercial fish products, either low in MeHg (sardines) or high in MeHg (tuna) appears to have no significant impact on concentrations of the cytokines IL-17A and IL-22 associated with Th17 response, even though intake of tuna/sardines can increase MeHg status. Although this finding may suggest that MeHg exposure through fish consumption according to the current dietary guidelines, does not induce Th17 response in the young women, it must be followed by further studies, that would involve general population.

Future directions

1. The study described in Chapter 3, has been conducted on the data and used pre-collected samples obtained from young adults at only one time point (19 years old), without consideration of potential changes in concentration of cytokines associated with Th17 axis throughout the life. Therefore, analysing one point cytokine data, without contrasting this with other established autoimmune biomarkers might be insufficient to determine potential autoimmune risks linked to Hg exposure. Therefore, would be important to measure Th17 cytokine concentrations at multiple times points, especially in the second half of adulthood, when immune competence is known to be reduced. Consequently, following this cohort over time and subsequently repeating these analyses would allow to see if age-related changes in immune functioning might impact study outcomes; based on the fact that frequencies of Th17 cell subsets seem to be higher in people above 65 years of age than in healthy adolescents.
2. As peripheral mononuclear blood cells (PBMCs), including lymphocytes may be involved in Th17 response, including activation and proliferation, possibility to obtain the blood samples and isolating PBMCs in future work may be important in order to conduct mechanistic *ex vivo* studies evaluating potential differences of PBMCs activation and Th17 response between autoimmune-predisposed individuals such as patients and apparently healthy controls upon MeHg exposure.
3. Fish is one of the best dietary sources of health-promoting nutrients, including n-3 LCPUFA, DHA and EPA. Owing to the immunomodulatory properties of n-3 LCPUFA, which have been shown to be beneficial for restoring immune homeostasis following inflammation, it may be important to explore how n-3 LCPUFA status may influence associations between MeHg, and cytokines associated Th17 response. In addition, would be also beneficial to include in these analyses other nutrients with anti-inflammatory properties, such as vitamin D and selenium., in order to assess any synergies, which might be protective against development of autoimmune response.

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Appendix 1. Study protocol.

An *ex vivo* investigation into the role of mercury chloride exposure on the cytokines release associated with Th-17 axis and cytokine IL-33 from human peripheral blood mononuclear cells (hPBMCs) of Systemic lupus erythematosus (SLE) patients.

Protocol

Ulster University

Scientific title of research

An *ex vivo* investigation into the role of mercury chloride exposure on release cytokines of Th-17 axis and IL-33 from human peripheral blood mononuclear cells (hPBMCs) of Systemic lupus erythematosus (SLE) patients.

Scientific abstract of research including key goals

Systemic lupus erythematosus (SLE) is a systemic inflammatory autoimmune disease with no known cure and an unclear aetiology. Many genetic and environmental factors have been implicated in the initiation and progression of disease. One of such environmental factor is mercury (Hg). Humans are predominantly exposed to methylmercury (MeHg) from fish consumption. Fish also contain beneficial nutrients, including the anti-inflammatory long chain polyunsaturated fatty acids (LCPUFAs).

In vivo evidence from animal studies supports a role for MeHg as an environmental contributor to the exacerbation of autoimmunity, nevertheless the evidence from human studies is limited or shows no effect.

There are several hypotheses of how Hg may contribute to SLE pathogenesis. For example, its immune-toxic properties have been suggested to increase cell death and release of self-antigens and the pro-inflammatory cytokines, such as IL-33.

This effect may have considerable impact on SLE patients, as defective apoptosis seems to contribute to disease pathogenesis. Therefore, Hg in healthy individuals may have lesser impact on the immune function, as their apoptosis and phagocytosis mechanisms prevent against inflammation. In SLE, defective apoptosis, causes cells to undergo necrosis, which is associated with release of alarmins, such as IL-33 and cellular debris. Impaired phagocytosis leads to self-antigen accumulation and persistent stimulation of Th-17 cells, which cytokines have been linked recently with many autoimmune pathologies.

Therefore, the main goal of this study is to compare an impact of *ex-vivo* Hg exposure on release Th-17 axis associated cytokines and IL-33 from hPBMCs of SLE patients and healthy controls.

Background

Systemic Lupus Erythematosus (SLE) is an autoimmune, systemic, inflammatory disease of unknown aetiology, in which tolerance to self-antigens has been lost (Crowe et al. 2017).

The SLE symptoms vary widely, however the most common are systemic manifestations, such as fever, malaise, arthralgias, myalgias, headache, and loss of appetite and weight (Cojocaru et al. 2011). Hence, dermatologic, musculoskeletal and renal involvement have been identified (Zoma, 2004; Cervera et al. 2003; Ben-Menachem, 2010). Therefore, an increasing overload of immune complexes deposition in the kidney may result in renal failure and sepsis, which are two main causes of the death (Cervera et al. 2003).

SLE is a multifactorial disease with wide range of causes implicated in its pathology, including genetic factors, in the setting of environmental triggers and stochastic events (Choi et al. 2012). Also, altered signalling might directly affect the cell ability to undergo apoptosis, which was implicated in the development of SLE. Defective or prolonged clearance of apoptotic cells allows them to progress to secondary necrosis (Mok and Lau, 2003; Shao and Cohen, 2011; Cojocaru et al. 2012). The induction of necrosis leads to loss of membrane integrity, release of intracellular components into the intracellular space. The release of cellular self-antigen results in the stimulation of a localized immune response to facilitate cellular debris clearance. The release of innate cytokines and alarmins such as IL-33 (Shao and Cohen, 2011; Li et al. 2014) from necrotic cell(s) help facilitate this clearance process.

Recently, the Th17-producing cells has been suggested in the autoimmunity development (Lourenço and La Cava, 2009) in SLE, rheumatoid arthritis, psoriasis and multiple sclerosis (Hedrich et al. 2012; Martin et al. 2014). Interestingly, frequencies of IL-17-producing T cells found in skin and kidney lesions of these patients, displayed increased production of IL-17 and contrastingly, decreased levels of IL-17F, which was suggested to aggravate the pro-inflammatory phenotype of the disease (Hedrich et al. 2012; Martin et al. 2014).
Also, IFN-λ1, IL-33 and Th-2 cytokines have been found to correlate with cytokines of Th-17 axis in autoimmune condition (Liang et al. 2018).

A range of environmental factors that impact SLE disease activity are diverse including Hg, which humans are primarily exposed to through the consumption of fish.

In vivo evidence from animal studies supports a role for MeHg as an environmental contributor to the exacerbation of autoimmunity, for instance in the genetically susceptible murine model, Hg induces autoimmunity and SLE-like condition, characterized by arthritis, cerebritis, skin rash and vasculitis (Pollard et al. 1999).
Nevertheless, the evidence from human studies is limited or shows no effect, because fish-based products are also rich in anti-inflammatory nutrients, such as n-3 long chain polyunsaturated fatty acids (LCPUFA) and their metabolites (e.g., eicosanoids, resolvins and protectins). The study of Crowe at al. (2015), failed to find a relationship between the SLE disease activity and urinary Hg level, in the same time suggesting, that Hg exposure thru high fish consumption might counteract the pro-inflammatory effect of the MeHg present in the fish.

Hg has been suggested to contribute to the autoimmunity though its cytotoxic properties, which leads to an increased rate of apoptosis. Also, MeHg has been shown to induce necrosis and stimulate the release of the alarmin cytokine IL-33 (Li et al. 2014).

This effect may have considerable impact on SLE patients, as defective apoptosis has been suggested to contribute to disease pathogenesis. Therefore, Hg in healthy individuals may have limited impact on the immune function, as their apoptotic function and phagocytosis are functional and thus limited immune activation. Whilst in SLE patients, defective apoptosis will lead to IL-33 release to stimulate inflammation and the attenuated clearance of cellular debris and the subsequent presentation of self-antigen to Th-17 cells (sensitized to self-antigen) will facilitate immune activation to exacerbate disease activity (Figure 1).

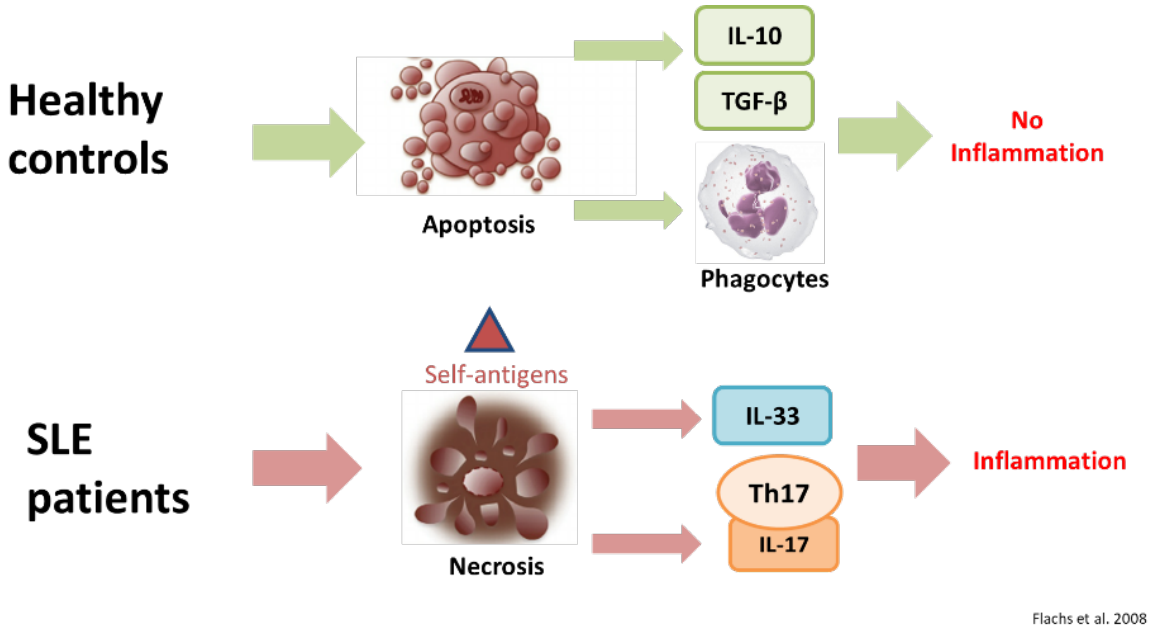


Figure 1. **Schematic diagram of PhD hypothesis**. The figure presents comparison between cell death in healthy individuals compared with the SLE patients. Briefly, in healthy controls cells are able to undergo programmed cell death (apoptosis), which does not result in inflammation, because their components are packed into apoptotic bodies (blebs) and they are removed during phagocytosis by macrophages. Furthermore, phagocytes release of TGF-b and

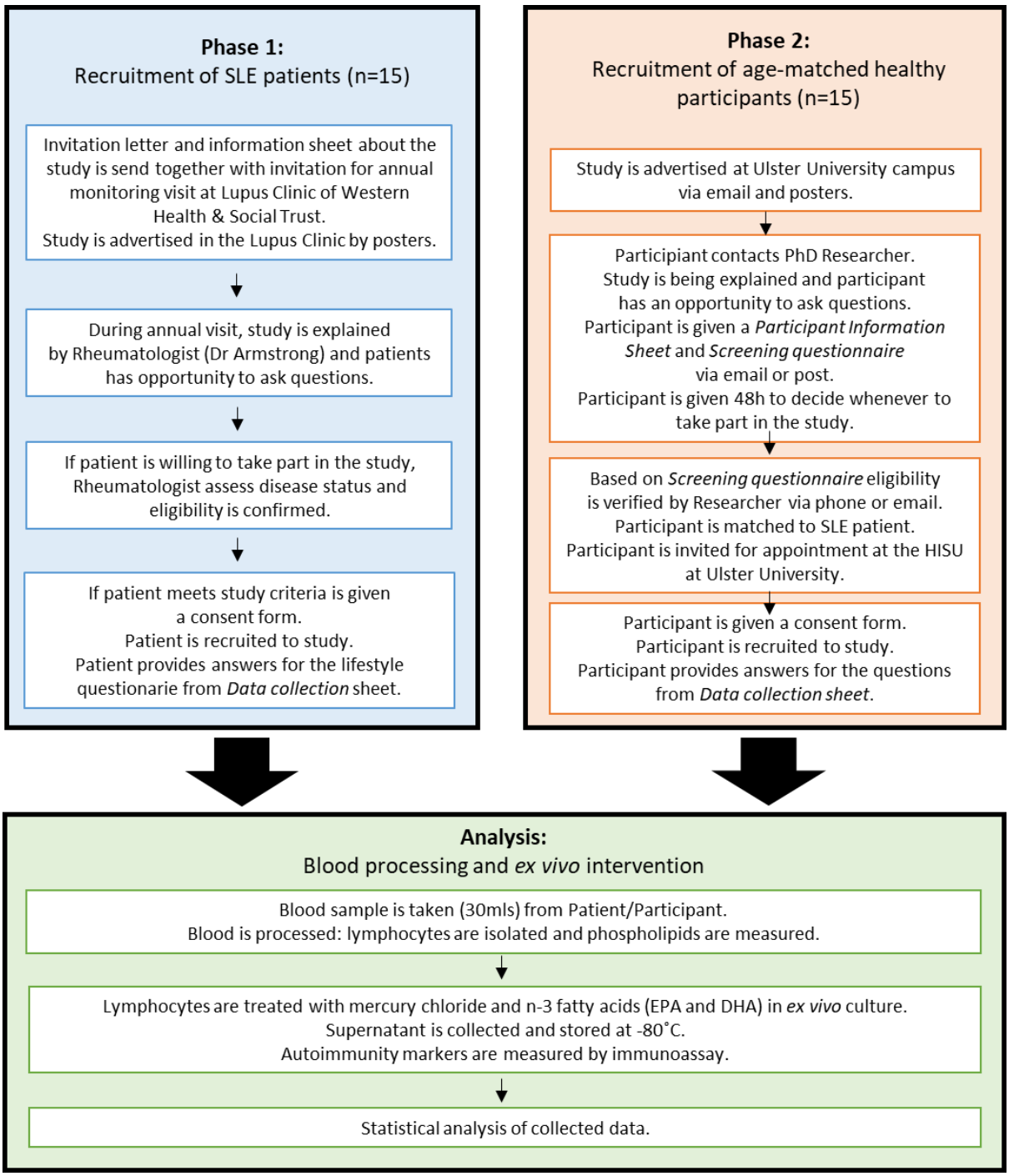
IL-10 to induce tolerance, in case of leakage of self-antigens. In SLE, defective apoptosis and prolonged clearance of cellular debris, allow the cells to progress to secondary necrosis. In the result, self-antigens accumulate. In SLE, tolerance to self-antigens is lost, therefore autoantibodies are generated, and pro-inflammatory is initiated.

To date no study has investigated the differences between Th-17 axis cytokines and IL-33 production in immune cells of SLE patients and healthy controls following *ex vivo* exposure to mercury. Also, no study has assessed the ability of n-3 fatty acids to negate the pro-inflammatory effect of mercury to potentially exaggerate inflammation in autoimmune-prone individuals.

Aim and purpose

The main aim of the research is to investigate an effect of Hg on hPBMCs ex vivo and its impact on release of IL-33 and cytokines associated with Th-17 axis in SLE patients and healthy controls. Furthermore, this study will explore if addition of anti-inflammatory n-3 LCPUFA, including Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) might impact the outcome of mercury chloride exposure.

Detailed plan of investigation and scientific procedures



Methodology

Brief outline of the study has been presented on the flow diagram (Appendix 10).

Recruitment process

The recruitment process has been outlined in Appendix 10.

The recruitment process of SLE participants will be carried out by rheumatologist consultant (Dr David Armstrong; Clinical Rheumatologist & Clinical Lead Fracture Liaison Service Altnagelvin Hospital) from the Western Health and Social Care Trust NI (WHSCT NI).

The recruitment process of healthy control participants will be carried out by PhD researcher.

The process will start from SLE patient recruitment following by healthy control participant recruitment, what will enable to match SLE participants with healthy control participants. Once a cohort of SLE participants will be recruited, healthy controls participants will be going to be recruit, in order to match with SLE group. The SLE participants will be matched with healthy control participants, based on gender, body mass index (BMI) and age (+/- 5 years of average age). A total of sample of 30 participants, including 15 SLE patients and 15 healthy controls, will be recruited.

Phase 1: Recruitment of SLE patients (n=15)

The study recruitment will be advertised in Lupus Clinics SLE within the WHSCT NI, by posters (Appendix 8).

Patient recruitment will be a part of annual appointment in the clinic. Consultant rheumatologist will send invitation for annual appointment together with the invitation letter (Appendix 4) and a patient information sheet (Appendix 2) with information about the opportunity to take part in the study. During the visit, study will be explained to the SLE patient and he/she will have a possibility to ask questions. If the patient will be willing to take part in the study, consultant rheumatologist will undertake the clinical assessment to confirm SLE diagnosis and provide disease activity measure, a British Isles Lupus Assessment Group (BILAG) index (Mikdashi and Nived, 2015).

Then, patient eligibility will be verified by filling screening questionnaire (Appendix 6).

If the SLE patient meet the eligibility criteria, PhD researcher will proceed with consent form (Appendix 5) and lifestyle questionnaire (Appendix 7), which includes anthropometric measurements, disease activity measure, and general information about lifestyle (smoking and alcohol consumption), used medications (n-3 fatty acids supplements, doses of steroids and anti-inflammatories) and dietary intake (n-3 PUFA sources). Finally, SLE participant will be asked to donate a one, 30mls (3x9ml sodium heparin and 3ml EDTA) blood sample taken by trained phlebotomist.

Phase 2: Recruitment of Age & Sex matched healthy control participants (n=15)

The study recruitment will be advertised at Ulster University, Coleraine Campus; by posters (Appendix 9) displayed at the main entrances, common areas; and invitation letters (Appendix 3) sending out by email. Interested in study participants, based on the contact details provided on the posters (Appendix 9) and invitation letters (Appendix 4), will be able to contact a PhD researcher by phone or email; and get more information about the study.

Healthy individuals, will consider taking part in the study, will be given a participant information sheet (Appendix 1) and Screening questionnaire (Appendix 6) by email or post.

They will be given 48h to decide whenever to take part in the study.

If they will be willing to participate, their eligibility will be verified by PhD researcher based on the screening questionnaire (Appendix 6) via phone call or email.

If the individual will meet the eligibility criteria, a PhD researcher will arrange one-off appointment with the volunteer at the Human Intervention Suite (HISU) at Ulster University (Coleraine).

During the appointment, PhD researcher will explain study in detail, answer participant's questions and provide consent form. Then, PhD researcher will ask questions in order to fill the questionnaire (Appendix 7), which includes anthropometric measurements, and general

information about lifestyle (smoking and alcohol consumption), used medications (n-3 fatty acids supplements, doses of steroids and anti-inflammatories) and dietary intake (n-3 PUFA sources). All the data will be recorded in data collection sheet (Appendix 7). Finally, healthy control participant will be asked to donate a one, 30 mls (3x9ml sodium heparin and 3ml EDTA) blood sample taken by trained phlebotomist.

Informed consent

Invited individuals, SLE and healthy controls participants, will receive both written and oral information regarding the study design and its requirements. Participants will be made fully aware that there is no obligation for them to take part in the study and it will be made clear that they are free to withdraw from the study at any time, without giving a reason of withdrawing. They will be given at least 48 hours to decide whether or not to participate. If they decide to take part in the study and meet the inclusion criteria informed written consent will be recorded (Appendix 5).

Inclusion/exclusion criteria

Inclusion and exclusion criteria will be confirmed based a screening questionnaire (Appendix 6), which will confirm patient participant /healthy control participant eligibility to take part in the study.

SLE patient is eligible to take part in the study, as the cohort of SLE participants, if:

• is aged between 18-65 years old;

• have a confirmed diagnosis of SLE based on criteria provided from American College of Rheumatology (ACR) (Aringer et al., 2019) and meet at least 4 out of 11 of those criteria;

• is free from other illnesses, that impact immune function;

• is not currently pregnant;

• is not currently (last 2 weeks) taking any medication, that includes high dose steroids (dose >10mg daily) and nonsteroidal antiinflammatory drugs (NSAIDs).
• is not currently (last 2 weeks) taking any n-3 PUFA supplements.

Healthy participant is eligible to take part in the study, as cohort of healthy controls participants, if:
• is aged between 18-65 years old;
• is free from other illnesses, that impact immune function;
• is not currently pregnant;
• is not currently (last 2 weeks) taking any medication, that includes high dose steroids (dose >10mg daily) and nonsteroidal antiinflammatory drugs (NSAIDs).
• is not currently (last 2 weeks) taking any n-3 PUFA supplements.

The reason why SLE patient participant recruitment and healthy control participant recruitment should be considered only for individuals younger than 65 years of age, is to limit risk that those individuals will suffer from other diseases or have complications related to their age.

Participant confidentiality

All information collected during the course of the research will be kept strictly confidential in adherence with the updated in 2018, Data Protection Act (1998).

All participants will be provided a clear information, what is personal data is used for and what is the reasoning behind using automated processing/decision on their data. They will be able to access their own data, once collected. If they want, they can request data revise, and if out of date – erase.

The research team is obligated to address these issues within month form the request.

All personal information will be collected and stored in a password protected file and each volunteer will be assigned a serial number that will be used on all information collected. Name, address or date of birth will not be released to any outside body or organisation. The unique ID will only be known to the research team, that including Dr Philip Allsopp, Dr Emeir McSorley, Ms Joanna Jurek and Dr David Armstrong; samples and information will not be identifiable to anyone without the unique code. All computers/laptops containing study information will be encrypted with a password.

All participant data will be kept on a password protected computer/laptop and in a password protected file. Other data will be stored in locked cabinets under the custodial care of the Chief Investigator.

The research team, that includes Dr Philip Allsopp, Dr Emeir McSorley, Ms Joanna Jurek and Dr David Armstrong; takes full responsibility to ensure the personal data is securely accessed, amended, and destroyed if necessary.

Data records

All personal information will be collected and stored in a password protected file and each participant will be assigned a serial number that will be used on all information collected. Name, address or date of birth will not be released to any outside body or organisation. The unique ID will only be known to the research team, samples and information will not be identifiable to anyone without the unique code. All computers/laptops containing study information will be encrypted with a password.

All participant data will be kept on a password protected computer/laptop and in a password protected file. Other data will be stored in locked cabinets under the custodial care of the Chief Investigator.

Sampling

Blood sampling: A blood sample of 30mls (3x9ml sodium heparin and 3ml EDTA) will be collected from each participant by a PhD researcher who is a trained Phlebotomist and has undertaken training in relation to the Human Tissue Act and research integrity.

Sample storage

All human tissue samples will be stored as per requirements of the Human Tissue Act (HTA). The researcher is aware of the 'UU Lone Working Guidelines', has received the appropriate vaccinations (e.g. Hepatitis B) and is familiar with handling of biological fluids. Custodial arrangements for blood samples will be made by the Chief Investigator.

Remaining blood samples will be stored in the restricted access laboratory situated in the Centre for Molecular Biosciences, Ulster University Coleraine, for up to six years following completion of the study. They will be stored in -80°C locked freezers with alarm. Samples will be separated into two freezers so that duplicates are available. Dr Philip Allsopp and the other named researchers will have access to the freezers and the samples.

All participants will be given a unique study identification code so participants will not be identifiable from the samples.

Phase 3: Analysis: Blood processing and ex vivo intervention (mercury challenge)

Experimental design of the study is presented in Appendix 11.

Blood processing

A 30ml blood (3 x 9ml sodium heparin and 3ml EDTA) sample will be taken. Lymphocytes will be isolated using the leucosep tube method as previously described (Nilsson et al., 2008) and stored at -80°C freezer.

The phospholipids levels will be assessed. The cytotoxic dose of mercury chloride will be determined using the MTT assay. Lymphocytes will be cultured at a concentration of 106 cells/ml in RPMI media supplemented with 10% heat inactivated foetal bovine serum, 1% streptomycin and 5% l-glutamine as per protocol (Gardner et al., 2010). Before the treatment with mercury chloride/n-3 PUFA baseline cytokine levels will be measured to allow later comparison between the treatment groups (all treatment groups have been presented in Appendix 11). Then, cells will be exposed to sub-toxic doses of mercury chloride in the presence of lipopolysaccharide (LPS) for a period of up to 48 hours with and without pre-treatment with n-3 PUFA, including EPA and DHA. Experimental concentrations used in the experiment have been predetermined by Crowe et al. (2018) (Crowe et al., 2018).

The release of autoimmune biomarkers, such as Th-17 axis cytokines (IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-31) and IL-33 will be measured by using the electrochemiluminescence-based Meso Scale Discovery (MSD) immunoassay, U-PLEX TH-17 Combo 1 (hu) SECTOR (K15075K-2) (Meso Scale Discovery, Gaithersburg, MD, USA). Autoimmune biomarkers released from lymphocytes exposed to mercury chloride with n-3 PUFA will be compared with that of exposed to mercury chloride alone. Results obtained from lymphocytes from SLE participants will be compared with results from lymphocytes from healthy controls.

Mercury (II) chloride handling

Potential risks of mercury handling identified in MSD of Mercury (II) chloride has been described in COSHH form for Mercury (II) chloride.

Briefly, to limit potential risk associated with Mercury (II) chloride handling, health and safety measures will be undertaken, including personal protective equipment (eye, skin and respiratory protection) and engineering adjustment to the workplace (adequate ventilation, fume hood). The material will be stored in well-ventilated locked cupboard according to manufacturer recommendations. Health and safety training will be provided.

Statistical analysis

Power calculation – sample size

Recent evidence has shown that exposure to inorganic mercury can increase release of the cytokines of Th-17 axis, IL-17 (Gardner et al., 2010), which has been suggested to play role in the autoimmune response (McGeachy et al., 2019).

IL-17A has been found to correlate with many serological measures in SLE patients, including immunoglobulin G (IgG), C-reactive protein (hs-CRP), proteinuria, and pre-albumin levels (Raymond et al., 2017).

To further explore an effect of mercury exposure on autoimmunity markers in the immune cells from SLE patients and healthy controls, we wish to investigate the effect of treatment with inorganic mercury (up to 200nM) with and without n-3 fatty acids on IL-17 cytokine release from human PBMCs, after 24h from LPS (50ng/ml) stimulation, with concentration mean of 63.6pg/ml (range: 48.9-75.1pg/ml) (Gardner et al., 2010).

In order to do that, we would need sample size of n=30, including 15 SLE patients and 15 healthy controls to ensure 80% power of calculations.

Statistical analysis

All data will be analysed using the statistical analysis package for the social sciences (SPSS version 21 SPSS Ina.,Chicago, IL, USA). Data will be examined for normality using the Kolmogorov Smirnov test and where necessary, data may be log transformed. The outcome will be tested using an independent T test to determine if the concentration of cytokine produced by lymphocytes from SLE participants, when exposed to mercury with and without n-3 fatty acids treatment, differs significantly from that observed in healthy controls under the same conditions. P values <0.05 will be considered as significant.

Timeframe for study tasks

Task	Date
Ethical application and approval	January 2020
Begin recruitment	June 2020
Sample collection (Blood)	June 2020 -December 2020
Data analysis & write up	January - March 2021

List of the attachments:

IRAS application form

Appendix 1: Participant information sheet

Appendix 2: Patient information sheet

Appendix 3: Invitation letter to participant

Appendix 4: Invitation letter to patient

Appendix 5: Consent form

Appendix 6: Screening questionnaire

Appendix 7: Data collection sheet

Appendix 8: Poster for Lupus Clinics

Appendix 9: Poster for Ulster University

Appendix 10: Flow diagram

Appendix 11: Experimental design

RG1C form – mercury chloride handling

COSHH form for Mercury (II) chloride

MSD of Mercury (II) chloride

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