Environment International 158 (2022) 106906

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

FLEXiGUT: Rationale for exposomics associations with chronic low-grade gut inflammation

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ARTICLE INFO

Handling Editor: Da Chen

Keywords:
Exposome
Gut inflammation
Environmental and food contaminants
Metabolomics
Microbiome
Chronic exposure

ABSTRACT

FLEXiGUT is the first large-scale exposomics study focused on chronic low-grade inflammation. It aims to characterize human life course environmental exposure to assess and validate its impact on gut inflammation and related biological processes and diseases. The cumulative influences of environmental and food contaminants throughout the lifespan on certain biological responses related to chronic gut inflammation will be investigated in two Flemish prospective cohorts, namely the "ENVIRONAGE birth cohort", which provides follow-up from gestation to early childhood, and the "Flemish Gut Flora Project longitudinal cohort", a cohort of adults. The exposome will be characterised through biomonitoring of legacy and emerging contaminants, mycotoxins and markers of air pollution, by analysing the available metadata on nutrition, location and activity, and by applying state-of-the-art -omics techniques, including metagenomics, metabolomics and DNA adductomics, as well as the assessment of telomere length and measurement of inflammatory markers, to encompass both exposure and effect. Associations between exposures and health outcomes will be uncovered using an integrated -omics data analysis framework comprising data exploration, pre-processing, dimensionality reduction and data mining, combined with machine learning-based pathway analysis approaches. This is expected to lead to a more profound insight in mechanisms underlying disease progression (e.g. metabolic disorders, food allergies, gastrointestinal cancers) and/or accelerated biological ageing.

1. Background

Exposure to environmental hazards, such as air pollution, legacy and emerging contaminants and toxins, is suspected to exert a negative impact on human health, leading to chronic diseases and accelerated biological ageing. The "exposome" concept, first described by Christopher Wild (Wild, 2005) and later refined by Gary Miller (Miller and Jones, 2014), refers to "the cumulative measure of environmental influences and associated biological responses throughout the lifespan including exposures from the environment, diet, behaviour and endogenous processes". The exposome is typically described by three overlapping and complementary domains: (i) a general external domain, including climate, urban/rural environment and social factors, which are mainly assessed at the community level by geographical mapping methods; (ii) a specific external domain, including environmental contaminants, diet, drugs and lifestyle factors, which are assessed at the individual level by questionnaires, environmental monitoring and biomonitoring; and (iii) an internal domain, including biological responses, such as metabolism, microbiota composition, inflammation, oxidative stress and ageing, which are all assessed by high-throughput molecular -omics methodologies (Wild, 2012).

Exposome research is challenging as it requires considering multiple life stages in the exposure assessment, repeated measurements of biomarkers in different time windows, the integration of data on biological

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https://doi.org/10.1016/j.envint.2021.106906
Received 30 June 2021; Received in revised form 3 September 2021; Accepted 23 September 2021

pathways to elucidate potential adverse outcome pathways, and the development of powerful statistical, bioinformatic and machine learning tools to analyse associations between exposures and health outcomes. The identification of environment-related risk factors can serve as a basis for instalment of effective preventive health policy measures (Lauriola et al., 2020).

FLEXiGUT is the first large-scale Flemish exposome project, which aims to characterize the life course influences of external exposures and internal biological responses in the context of the development and progression of chronic low-grade gut inflammation. It then further links this to related manifestations such as metabolic disorders (including diabetes, metabolic syndrome and obesity (Gholami et al., 2019)), food allergies (Wang and Sampson, 2011), gastrointestinal cancers (Cani and Jordan, 2018), and accelerated biological ageing (Jurk et al., 2014) (Fig. 1).

In the last years, several risk factors, including diet, exposure to environmental contaminants and (patho)physiological manifestations such as hyperglycaemia, dyslipidaemia and obesity, have been associated with gut barrier dysfunction and gut-specific as well as systemic low-grade inflammation (Franceschi et al., 2018; Leech et al., 2019; Phillips et al., 2019). In addition, it has been shown that the gut microbiota is crucial for the proper functioning of the mucosal immune system (Thursby and Juge, 2017) and to maintain mucosal barrier integrity (Natividad and Verdu, 2013), amongst many other benefits to the host, such as e.g. protection against pathogens (Bäumler and Sperandio, 2016) and metabolism of nutrients (Morrison and Preston, 2016). Association studies have linked microbiome alterations with common chronic human diseases, such as inflammatory bowel disease (Baumgart and Carding, 2007), obesity (Andoh et al., 2016), diabetes (Karlsson et al., 2013) and colorectal cancer (CRC) (Wirbel et al., 2019). The evidence for the involvement of the gut microbiome-inflammatory axis (MIA) in chronic diseases is supported by the recent discovery of the cross-disease Bacteroides2 enterotype and its link to faecal calprotectin as a gut inflammatory marker, as well as to serum C-reactive protein, a systemic inflammatory marker (Vieira-Silva et al., 2019). Specifically, two potential MIA-links are hypothesised: (i) oxidative stress and low-grade inflammation lead to gut microbiota dysbiosis, and subsequent chronic inflammation, which then lead to chronic diseases. At the same time, (ii) a healthy gut microbiota and their metabolites may contribute to the host immune balance and counteract inflammation.

The scientific questions to be addressed by the FLEXiGUT project are: What is the importance of the exposome in the development and progression of low-grade gut inflammation and related biological processes and diseases? What is the role played by the MIA? These two questions can be decomposed into several new elements: How do exposures affect gut microbial composition, the metabolome and MIA in early life and adulthood? What is the impact of the exposures and MIA regarding the development and progression of metabolic disorders and food allergies? Do these exposures induce DNA damage and can they be linked to e.g. gastrointestinal cancer? What is the impact of the exposures and MIA on (markers of) accelerated biological ageing?

The composition of the gut microbiota in adulthood is relatively stable (Arumugam et al., 2011) while it undergoes important shifts in early life, especially during the first 3 years (Rodríguez et al., 2015). In addition, there is strong evidence that the period of organ development during prenatal life and childhood is particularly vulnerable to impacts of environmental exposures, which can subsequently affect chronic disease risk later in life (Gluckman et al., 2008; Haug et al., 2018; Luyckx et al., 2017). Therefore, investigation of the impact of the exposome on the gut needs to be reflected both in early and later life stages.

Two prospective Flemish cohorts have been selected for the collection of detailed information on individuals in a wide age range: the "ENVIRONAGE birth cohort" (Janssen et al., 2017) and the "Flemish Gut Flora Project longitudinal cohort" (Falony et al., 2016) (Table 1). The ENVIRONAGE birth cohort is a prospective cohort from birth onwards, with children being followed up to the age of 10 years. The Flemish Gut Flora Project longitudinal cohort for microbiome studies comprises adult population.

Available biological samples include blood, urine, saliva, faeces and pregnancy-related samples (placenta and cord blood). The

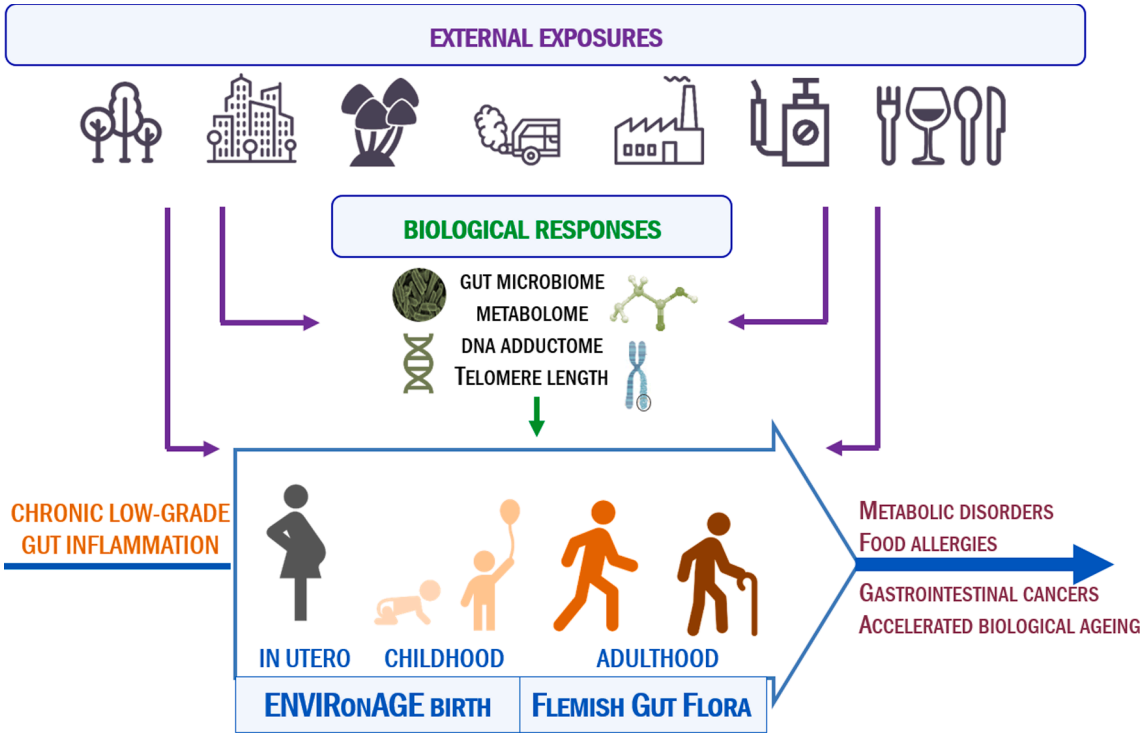

Fig. 1. FLEXiGUT conceptual framework: characterization of human life course exposure using a mother–child ("ENVIRONAGE birth") and an adult population ("Flemish Gut Flora Project longitudinal") cohort to assess the impact of the exposome on chronic low-grade gut inflammation, related diseases (e.g. metabolic disorders, food allergies and gastrointestinal cancers) and accelerated biological ageing.

Table 1
Characteristics of the two prospective Flemish cohorts used to assess the impact of the exposome on chronic low-grade gut inflammation, related diseases and accelerated biological ageing.

Type	Cohort	Characteristics
Mother-child cohort	ENVIRONAGE (ENVIROnmental influence ON AGEing in early life) birth cohort (Janssen et al., 2017)	Includes more than 1,600 mother–child pairs recruited in the South-East Limburg Hospital (Genk, Belgium) from 2010 onwards. In FLEXiGUT, 400 mother-children pairs will be selected. Biobanked placental tissue, cord blood, maternal blood, and faecal, urine and blood samples at childhood (age of 4–6 years) are available. Metadata include mothers' lifestyle and socio-economic status, questionnaires (food frequency, allergy, wellbeing and perceived stress) and clinical data (cognitive assessment, cardiovascular phenotyping and bone density at the age of 4 years).
Adult cohort	Flemish Gut Flora Project longitudinal cohort (Falony et al., 2016)	Initiated in 2017, this cohort includes more than 400 adults recruited across Flanders (Belgium). Biobanked blood, faeces and saliva samples are available at several time points during a monitoring period of 5 months. Metadata includes location tracking, lifestyle and socio-economic status, questionnaires (dietary recalls, food frequency, wellbeing and perceived stress) and clinical data (medical anamnesis, drug usage, blood test and measurement of inflammatory markers in faeces).

biomonitoring of contaminants and the gathering of -omics data for these biological samples, collected at different time points, complemented by the information available from questionnaires, lifestyle and clinical data, will allow capturing both the external and internal domains of the exposome from prenatal life onwards.

2. Exposure assessment

In FLEXiGUT, complex mixtures of already identified exposures are investigated in a targeted manner, while the complementary use of untargeted analysis will enable the discovery of relevant yet unidentified exposures. We will combine exposure science and high-throughput-omics technologies with epidemiological studies, focusing on biomonitoring of contaminants (Table 2**A**), analysis of biological responses (Table 2**B**) and metadata to assess the development and progression of chronic low-grade gut inflammation (Fig. 2).

2.1. Metadata

For all individuals, questionnaires, lifestyle and clinical data are available. Dietary exposure is traditionally assessed with self-reported methods, namely food-frequency questionnaires or dietary recalls. However, these methods are prone to recall bias and difficulties in assessing portion sizes (Scalbert et al., 2014). The resulting misclassification of subjects can influence observed associations between dietary exposures and disease outcomes (Marshall and Chen, 1999). Therefore, in trying to address these difficulties, in FLEXiGUT, the assessment of dietary intake using food-frequency questionnaires and diaries will be

Table 2
(A) Contaminants and **(B)** biological responses investigated in the FLEXiGUT project to assess the development and progression of chronic low-grade gut inflammation, related diseases and accelerated biological ageing.

	Sample matrix	Analysis approach	Analytical technique[a)]	In-house methods
(A) Contaminants				
Pesticides e.g. pyrethroids, neonicotinoids, organophosphate pesticides, etc.	Urine	Targeted	LC-MS/MS	(Gao et al., 2022)
Plasticizers e.g. bisphenol-A and substitutes, phthalates and alternatives, etc.			GC-MS/MS LC-MS/MS	(Gys et al., 2020) (Been et al., 2019)
Flame retardants e.g. organophosphorus flame retardants (PFRs)			LC-MS/MS	(Bastiaensen et al., 2018)
Persistent organic pollutants (POPs) e.g. polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs)	Blood		GC-ECNI/MS	(Dirtu et al., 2013)
Perfluorinated compounds e.g. per- and polyfluoroalkyl substances (PFAS)			LC-MS/MS	(Roosens et al., 2010)
New/emerging contaminants	Urine and blood	Untargeted	LC-HRMS	(Caballero-Casero et al., 2021)
Black carbon particles	Urine, blood and placenta	Targeted	Confocal microscopy	(Saenen et al., 2017; Bove et al., 2019)
Mycotoxins	Urine and blood	Targeted Untargeted	LC-MS/MS LC-HRMS	(De Ruyck et al., 2020)
(B) Biological responses				
Polar metabolome Including markers of food intake and inflammation	Urine, blood, saliva, faeces and placenta	Targeted and untargeted	LC-HRMS	(De Paepe et al., 2018)
Lipidome	Faeces and placenta			(Van Meulebroek et al., 2017; Rombouts et al., 2019)
DNA adductome	Placenta			(Hemeryck et al., 2015)
Telomere length	Blood and placenta	Targeted	qPCR	(Martens et al., 2017)
Microbiome profile	Faeces	Untargeted	Quantitative shotgun metagenomics	(Vieira-Silva et al., 2019; Vieira-Silva et al., 2020)
Inflammatory markers e.g. faecal calprotectin and serum C-reactive protein	Blood and faeces	Targeted	ELISA	

a) LC-MS/MS: Liquid chromatography-tandem mass spectrometry; GC-MS/MS: Gas chromatography-tandem mass spectrometry; GC-ECNI/MS: Gas chromatography-electron capture negative ion mass spectrometry; LC-HRMS: Liquid chromatography–high resolution mass spectrometry; qPCR: Quantitative polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay.

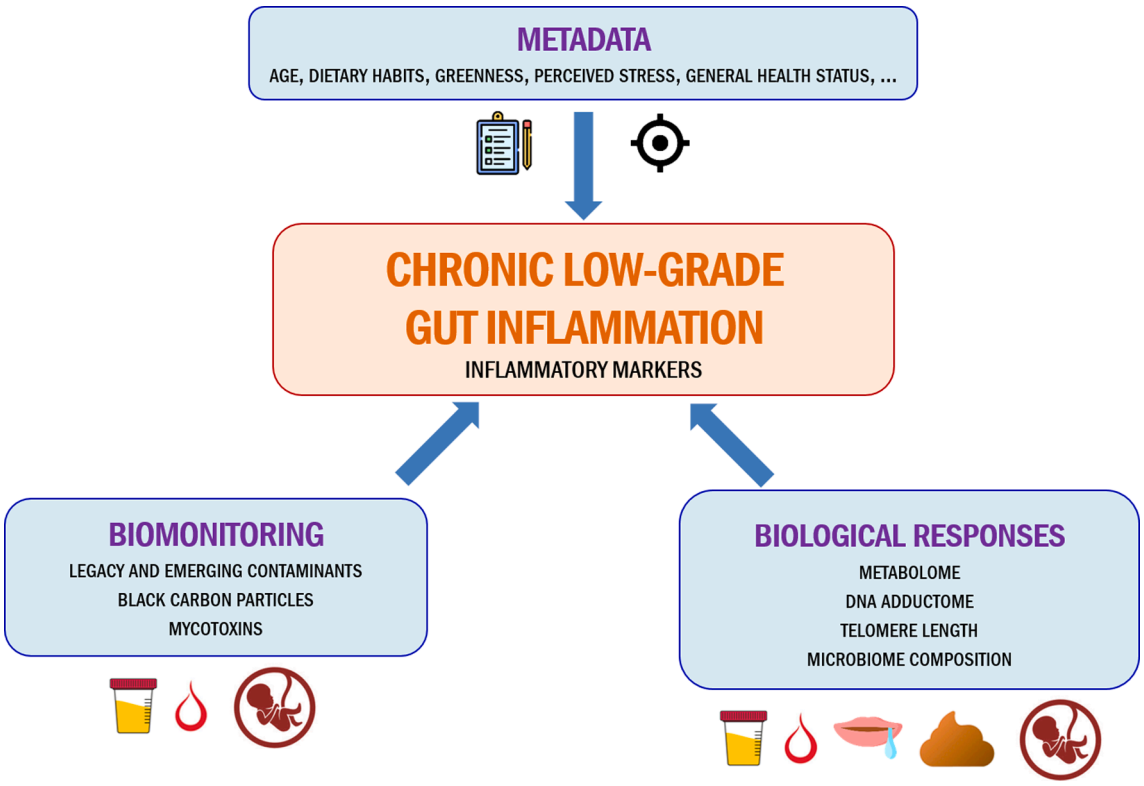

Fig. 2. Data produced in FLEX*i*GUT to assess the development and progression of chronic low-grade gut inflammation: analysis of metadata, biomonitoring of contaminants and analysis of biological responses using -omics techniques and telomere length measurement.

complemented by metabolomics analysis as a more objective measurement of certain dietary exposures.

Similarly, clinical data will be combined with gathered -omics data to investigate associations between exposures and health. In particular, the use of antibiotics will be linked to metagenomics data. Antibiotics have been reported to promote several adverse effects on the gut microbiota, including reduced species diversity (Lange et al., 2016), altered metabolic activity (Choo et al., 2017), and the selection of antibiotic-resistant organisms (Rolain, 2013). Exposure to antibiotics during infance has furthermore been associated with delayed gut microbiota development (Bokulich et al., 2016) and gastrointestinal and immunological conditions, e.g. Chron's disease (Ungaro et al., 2014).

2.2. *Biomonitoring of contaminants*

Targeted biomonitoring is the measurement of contaminants and/or their metabolites in body fluids and tissues to assess the exposure to particular substances, integrating all sources of external exposure (Dennis et al., 2017). FLEX*i*GUT will focus on biomonitoring exposures to legacy and emerging contaminants, markers of air pollution and mycotoxins as relevant environmental and dietary contaminants of interest that exert a wide range of toxicity and disturb gut homeostasis by inducing intestinal damage, inflammation and gut microbiota dysbiosis (Akbari et al., 2017; Deng et al., 2020; Vignal et al., 2021). For the analysis of biologically persistent contaminants, such as persistent organic pollutants (POPs) (Miniero et al., 2015), perfluorinated alkylated substances (PFAS) (Pérez et al., 2013) and black carbon particles (Xie et al., 2017), one time point biospecimens will be used to estimate the exposure. In contrast, non-persistent chemicals, such as plasticizers, pesticides, flame retardants and mycotoxins, are rapidly cleared from the body, with biological half-lifes ranging from a few hours to a couple of days (Huygh et al., 2015; Vidal et al., 2018). Accordingly, for these compounds, urine and blood samples of each individual will be collected at several time points to overcome exposure misclassification. Considering that the intraclass-correlation coefficients (ICC) values for most of this non-persistent contaminants range between 0.5 and 0.6, as has been reported in several mother–child cohorts (Casas et al., 2018), we have calculated that 4 samples per subject will allow achieving a target ICC of 0.80 using the Spearman-Brown equation (de Vet et al., 2017).

To evaluate the level of exposure to the contaminants, the population will be distributed into tertiles, classifying the subjects into high-, medium- and low-exposure groups. Two approaches will be explored for exposure assessment: (i) the level of exposure to each family of contaminants, to investigate interactions between these families and their associated biological effects (e.g. alterations of the gut microbiota, markers of effect, development of diseases and accelerated biological ageing); (ii) the Exposure Load metric, which integrates the total exposure to the contaminants, to identify vulnerable subpopulations disproportionately exposed to numerous chemicals at high concentrations (Willey et al., 2021).

2.2.1. *Legacy and emerging contaminants*

Humans are exposed daily to a wide range of man-made chemicals, including pesticides and plastic-related contaminants, some of them known as endocrine disruptors (EDCs), through food consumption and inhalation of dust. Recently, Deng et al. reported that microplastics can act as carriers and transport contaminants such as phthalate esters into the organism, showing evidence of these compounds affecting bowel health in mice, including alterations in gut microbiota composition, increased intestinal permeability and intestinal inflammation (Deng et al., 2020). Moreover, exposure to dibutyl phthalate (DBP) has been reported to disturb the homeostasis of the gut microbiota and cause gut inflammation in mice (Xiong et al., 2020). Exposure to phenolic compounds and parabens and to organophosphate flame retardants (PFRs), such as triphenyl phosphate (TPhP), has been associated with chronic bowel leakage symptoms and ulcerative colitis (de Silva et al., 2017) and to gut wall thickening, gut lumen shrinking, and cell swelling (Cui et al.,

2020), respectively.

In view of the above evidence linking emerging contaminants with microbiota dysbiosis and gut inflammation, the occurrence and levels of pesticides, plasticizers, flame retardants, and various legacy contaminants, such as POPs and PFAS, will be assessed within FLEXiGUT. Biologically persistent contaminants will be analysed in blood, while the presence of short-lived compounds in the body will be assessed in urine.

2.2.2. Air pollution

Ambient air pollution is recognised as one of the leading causes of the global burden of disease. Recently, particulate matter pollution has been associated with modifications of the gut microbiota (Liu et al., 2019), oxidative stress and low-grade gut inflammation (Vignal et al., 2017). Furthermore, it has been demonstrated that black carbon particles can pass through the placenta, suggesting direct foetal exposure to those particles during the most susceptible period of life (Bove et al., 2019). In view of the evidence above, the concentration of black carbon particles will be determined in placenta, blood and urine, to reflect both prenatal and postnatal exposure. Furthermore, it will be explored whether biomonitoring of black carbon particles can overcome the current limitations of environmental monitoring based on air pollution models, which only includes residential address but ignores often other exposures during commuting, work or leisure time.

2.2.3. Mycotoxins

Mycotoxins are secondary metabolites produced by fungi when growing on crops used for human and animal consumption (Arce-López et al., 2020). In contrast to the impact of acute mycotoxin poisoning, little is known on the risk of chronic multiple mycotoxin exposure (De Ruyck et al., 2020). The gut microbiota is capable of detoxifying mycotoxins, however mycotoxins can disturb gut microbial equilibrium and metabolism (Liew and Mohd-Redzwan, 2018). The mycotoxin deoxynivalenol, ubiquitously present in the human Western diet, can affect the intestinal barrier integrity (Akbari et al., 2017) and has been associated with significant CRC risk in the European EPIC cohort (Huybrechts et al.). Furthermore, deoxynivalenol revealed to exacerbate DNA damage induced by *E. coli*-producing colibactin both *in vitro* and in mice (Payros et al., 2017; Wilson et al., 2019). The analysis of validated multi-mycotoxin biomarkers of exposure in human biofluids can be a powerful tool to investigate their link with alterations of the gut microbiota. The analysis of small metabolites such as colibactin, which is hypothesised to regulate the causal relationship between mycotoxins, the gut microbiota and chronic gut inflammation (Du et al., 2017), will also be investigated. In addition, investigation of the metabolome and pathway analysis may potentially lead to new evidence for interpreting mycotoxin-induced metabolic dysfunctions.

2.3. Biological responses: multi-omics approach

The addition of -omics to a molecular term implies a comprehensive assessment of a set of molecules, e.g. small molecules (metabolomics), alterations and additions to biomolecules (adductomics) and the genetic material of the microbiota (metagenomics). By enabling the simultaneous analysis of large numbers of compounds without requiring prior hypotheses, these high-throughput -omics techniques provide information on the biological markers of external exposures and give insight into which pathways or processes are affected. However, analysis of a single type of -omics data is mostly limited to correlations (Hasin et al., 2017). The analysis of multiple types of -omics datasets in an integrative way, i.e. multi-omics, will return a more complete picture of the whole system than separately analysing them, including opportunities for the discovery of biomarkers of exposure and disease risk or early molecular events in the pathways leading to disease (Vineis et al., 2020).

2.3.1. Metabolomics

Metabolomic fingerprinting is the most revealing real-time quantifiable readout of the human phenotype (Suhre et al., 2016) with enormous potential for biomarker discovery and nutritional studies (Ulaszewska et al., 2019). Both targeted and untargeted metabolomics aids in accurately describing one's health state, with discrimination of the healthy vs. non-healthy metabolome (Rombouts et al., 2019; Rombouts et al., 2017; Vanden Bussche et al., 2015). To better understand the effect of the external exposome in the MIA, the polar metabolome and lipidome will be assessed using both targeted and untargeted approaches. This encompasses the assessment of both known and unknown small molecules that are linked to e.g. food intake, identification of the hence retrieved markers, and investigation of (patho)physiological processes as studied by means of pathway analysis. Identification of the detected metabolites will be achieved by means of comparison to authentic standards, spectral libraries and databases, as is detailed by Ulaszewska et al. (Ulaszewska et al., 2019); e.g. identification of food constituents, food additives and man-made compounds as well as compounds formed during food processing and/or digestion will be accomplished using the freely available FooDB database (Scalbert et al., 2014; Ulaszewska et al., 2019). Links with dietary intake will allow to assess the effects of the dietary exposome, whilst correlation with microbiome data will moreover provide a functional readout.

Indeed, for many of the above-mentioned (patho)physiological processes specific metabolites or metabolic pathway alterations have been evidenced in the context of the MIA. A specific example relates to allergic inflammation as observed in children with a history of anaphylaxis, where alterations in the pro-inflammatory 12-hydroxy eicosatetraenoic acid (12-HETE), which is a platelet-derived 12-lipoxygenase metabolite of arachidonic acid, were noted in plasma (Crestani et al., 2020). Similarly, linoleic acid and its oxidised derivates, 9- and 13-oxo-ODE, were found increased in the plasma of children with obesity (Tricò et al., 2019), reflecting advanced oxidative state in parallel to lipotoxicity in the development of insulin resistance (Zhao et al., 2016). These oxidised metabolites have been associated with lower linoleic acid-conjugating gut bacteria, which was referred to as a detoxification mechanism to reduce systemic low-grade inflammation (Tricò et al., 2019).

2.3.2. DNA adductomics

Exposure to exogenous or endogenous chemicals that attack, alter and/or covalently bind to DNA nucleobases can result in the formation of DNA adducts. These adducts can lead to mutations and chemically-induced carcinogenesis (Hemeryck and Vanhaecke, 2016; Krais et al., 2019), whilst the formation of DNA adducts has also been linked to the occurrence of developmental aberrations (Gorokhova et al., 2020). It is therefore of great importance to study the effects of the exposure to genotoxic chemicals early on as well as later in life.

It has clearly been established that the gut microbiome is implicated in the development of CRC (Wirbel et al., 2019). The differential abundance of microbiota species in CRC has been studied extensively, revealing several associations of gut microbes that may either contribute to or benefit from CRC tumorigenesis. Deciphering whether these associations are in fact cause or consequence is not straightforward, but there are several known examples of microbiota species with tumorigenic potential, including active involvement in DNA adduct formation.

A specific (high-impact) example of microbiome-modulated DNA adduct formation that indeed contributes to CRC risk involves the bacterial production of the earlier mentioned genotoxin colibactin (in *2.1.3. Mycotoxins*). Significant enrichment of colibactin-producing strains of *E. coli* in CRC patients is linked to e.g. cell cycle arrest, tumour progression, etc. (Wirbel et al., 2019), and in 2019, Wilson et al. demonstrated that colibactin alkylates DNA *in vivo*, resulting in the formation of DNA adducts (Wilson et al., 2019). This finding explains how colibactin may be involved in the development of CRC, is furthermore supported by a report on a mutational signature caused by colibactin-producing *E. coli* in CRC (Pleguezuelos-Manzano et al., 2020), and sparks further interest in the potential interfering role of mycotoxin

exposure. Noteworthy, the link between colibactin exposure and CRC was established by means of untargeted DNA adductomics. To the best of our knowledge, FLEXiGUT will be the first large-scale exposomics study to implement this state-of-the-art platform; targeted analysis of DNA adducts of interest combined with untargeted mapping of the DNA adductome will offer a direct reflection of overall genotoxin exposure, associated effects and potential disease risk (Hemeryck et al., 2015; Hemeryck et al., 2016).

2.3.3. Telomere length and ageing phenotype

It has been recently demonstrated that newborns who had higher exposure to air pollution during pregnancy have shorter telomeres at birth (Martens et al., 2017). Telomere shortening is a marker for biological cell ageing that can lead to a lower buffer capacity to deal with inflammatory conditions and oxidative stress followed by later-life disease susceptibility and shorter lifespan. Systemic chronic inflammation can accelerate biological ageing via reactive oxygen species-mediated exacerbation of telomere dysfunction and cell senescence (Jurk et al., 2014). However, the mechanisms behind inflammation-induced cellular ageing are understudied. This warrants the gathering of new insights by investigating the link between telomere length and exposomic markers in the context of gut microbiome inflammation.

2.3.4. Metagenomics

The gut microbiota plays a role in processing nutrients and contaminants. At the same time, the composition of the microbiome can be linked to the development of intestinal-related disorders. Metagenomics provides readouts of both microbiota composition and functionality, allowing uncovering associations to pathologies. Recently, faecal quantitative microbiome profiling enabled the discovery of an inflammation-associated enterotype linked to both auto-immune conditions (Vieira-Silva et al., 2019), as well as well-being and depression (Valles-Colomer et al., 2019) and obesity (Vieira-Silva et al., 2020). Possible links between disturbances to gut microbiome and intestinal diseases will be investigated through microbiome profiles in faeces, enabling quantification of microbial, gene and pathway abundances, and the assessment of enterotypes.

2.3.5. Inflammatory markers

Inflammatory markers are biomarkers used to evaluate inflammatory processes. For the assessment of gut inflammation, faecal calprotectin and serum C-reactive protein (CRP) are believed to be superior to erythrocyte sedimentation rate or leucocyte count (Lehmann et al., 2015).

Calprotectin is a calcium binding protein with antimicrobial properties and a role within the innate immune response (Ayling and Kok, 2018). This protein accounts for 60% of the cytosolic protein content of neutrophils. Accumulation of neutrophils at the site of inflammation in the gastrointestinal tract results in the release of calprotectin into faeces where it is stable and resistant to bacterial degradation (RØseth et al., 1992). The concentration of calprotectin in faeces is directly proportional to the extent of gut inflammation. Faecal calprotectin can be measured by quantitative enzyme-linked immunosorbent assay (ELISA) and the cutoff threshold is considered to be 50 μg/g (Lehmann et al., 2015). A *meta*-analysis of 670 patients has shown that calprotectin had a 93% sensitivity and 96% specificity for the diagnosis of gut inflammation (van Rheenen et al., 2010).

CRP is an acute-phase inflammatory protein that exhibits elevated expression during inflammation (Sproston and Ashworth, 2018). Serum CRP is a marker for systemic inflammation (Thompson et al., 1999). Low levels of serum CRP can be measured accurately, enabling identification of individuals with low-grade inflammation, defined as serum CRP concentration above 3 mg/L, but below 10 mg/L (Dinh et al., 2019).

In FLEXiGUT, faecal calprotectin and serum CRP will be analysed to respectively assess gut and systemic inflammation to uncover associations with certain external exposures and internal biological processes on the one hand, and health and disease outcomes on the other.

3. Data analysis: Processing and integration

Processing, analysing and mining the large volumes of data generated on the numerous external exposures and the molecular -omics profiles investigated is very challenging. FLEXiGUT has created a dedicated data infrastructure to store, manage, analyse and interpret the various types of data that will be obtained. Pseudonymised data will include targeted and untargeted data measurements on environmental and dietary contaminants, -omics data, inflammation markers, clinical data and metadata.

Data management will follow the FAIR-principle (Findable, Accessible, Interoperable and Reusable). Upon publication, the data will be accessible and reusable through public repositories, e.g. MetaboLights for metabolomics (Haug et al., 2020) and European Genome-phenome Archive for metagenomics (Lappalainen et al., 2015). Data will be made findable and interoperable by including metadata with controlled identifiers and using shared data analysis workflows within the consortium.

We have defined query methods, workflows for quality control and data pre-processing steps to harmonize and normalize the data for integrated analysis. Dimension reduction methods followed by data mining will be used to investigate correlations between features as well as for developing classifier and predictive models in relation to the experimental variables, e.g. inflammatory markers. The proposed integrative data processing strategy will not only allow to explore the link between the exposures and chronic low-grade gut inflammation, related diseases and accelerated biological ageing, but will also provide insights into the mechanisms by which the exposure might be exerting its effects through pathway module analysis (Vieira-Silva et al., 2016). In addition, combining the data analysis framework with machine learning-based pathway analysis approaches will identify relevant associations and targets for future biological validation.

4. Strengths, limitations and conclusions

One of the main strengths of FLEXiGUT is the parallel inclusion of mother-children and adult cohorts to characterize environmental exposure and associated biological responses during the human life course, including critical life stages, in order to investigate the interplay of exposure and the MIA. Chronic low-grade gut inflammation is hypothesised to be related to biological processes that cause health deterioration and increase the risk of chronic diseases and/or accelerated biological ageing. The multi-omics concept of FLEXiGUT aligns with the technology-driven paradigm change from reactive towards predictive, preventive and personalised (3P-concept) medical services as the medicine of the future benefiting the patient and healthcare at large (Gerner et al., 2020). The expertise of the participating research groups in several disciplines enables to assess both the exposures and biological responses taking a holistic approach. Furthermore, all the analyses for a group of exposures are carried out in the same laboratory, allowing to obtain consistent data and avoiding interlaboratory variability. For all individuals, questionnaires, lifestyle and clinical data are also available to obtain rich datasets of the exposome.

The sample size has been optimised *a priori* based on power analysis, but this approach is limited by the uncertainty associated to the FLEXiGUT hypotheses. The selected sample subsets from the "ENVIRONAGE birth" and "Flemish Gut Flora Project longitudinal" cohorts (n = 400 mother-children pairs and 400 adults, respectively) should allow to achieve statistically significant models for -omics validation and to complete the analyses in a reasonable time and budget. Furthermore, the specific associations and biomarkers detected in the two regional cohorts will be further validated in international cohorts. Given the time frame of the FLEXiGUT project, current research will be limited to the discovery phase and validation. Therefore, resulting associations and

identified target/markers molecules will form a basis for future follow-up projects, including further in-depth mechanistic and biological studies for causality determination. The ultimate goal is to develop evidence-based health prevention and intervention strategies regarding chronic low-grade gut inflammation and related diseases, such as metabolic disorders, food allergies, accelerated biological ageing and gastrointestinal cancers, providing pointers for policy makers in safeguarding public health.

In conclusion, the uniqueness of FLEX*i*GUT lies in the use of prospective cohorts at multiple life stages, the focus on environmental and dietary exposures and -omics profiles, and the central role of the gut microbiome with respect to research on the development and progression of chronic low-grade gut inflammation and related biological processes and diseases.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Interuniversity Special Research Fund (iBOF) from Flanders [grant number BOFIBO2021001102]. MDB is supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 946192, HUMYCO). GP is supported by the Exposome Centre of Excellence of the University of Antwerp (BOF grant, Antigoon database n. 41222).

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