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PH.D. THESIS

TITLE

COMPARATIVE CYTOKINE DYNAMICS: UNRAVELING THE ROLE OF LIFESTYLE AND DIETARY FACTORS IN SHAPING LOW GRADE CHRONIC INFLAMMATION ACROSS HEALTHY AND NEOPLASTIC PROFILES.

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SUMMARY

Abstract

Low Grade Chronic Inflammation (LGCI) is a common feature of non-communicable diseases. Cytokines play a crucial role in LGCI. This study aimed to assess how LGCI risk factors [e.g., age, body mass index (BMI), smoke, physical activity, and diet] may impact on specific cytokine levels in both healthy and neoplastic populations.

In total, 150 healthy volunteers and 132 neoplastic patients were recruited and subjected to questionnaires about the last 7-days lifestyle, including smoking habit, physical activity, and food frequency. A panel of circulating cytokines, chemokines, growth factors, metabolic markers and adipokines was analyzed by multiplex ELISA.

In healthy patients, BMI showed the heaviest impact on the correlation between LGCI-related risk factors and cytokines and was significantly associated with CRP levels. BMI, along with aging, smoking, physical activity, and eating habits were associated with an inflammatory pattern, characterized by a cluster that included IL-1β, IL-6, IL-8 and TNF-α. These markers retained their strong clustering feature in the neoplastic population, with the addition of other inflammatory markers, such as MIP-1α, MIP-1β, IL-4, and GM-CSF.

In conclusion, BMI, age, smoke, physical activity, and dietary habits are associated with specific cytokines that may represent potential markers for LGCI, and a first proof of this biomarker validation is given by the reappearance of this cluster in a neoplastic population.

Introduction

Low Grade Chronic Inflammation - LGCI

Acute inflammation is a pivotal element within the framework of innate immunity. It constitutes a localized response to cellular damage, marked by a blood flow increase, capillary vasodilation, leukocyte infiltration and localized production of chemical mediators, that contribute to the toxic agents' elimination and tissue repair. Acute inflammation is an indispensable component of immunosurveillance and host's defense mechanisms. The resolution of this type of inflammation is an active process that involves cytokines and various other anti-inflammatory mediators, predominantly of lipidic nature (Antonelli and Kushner 2017).

During the last decade of the 20th century, a distinct form of inflammatory process emerged and was named as Low-Grade Chronic Inflammation (LGCI) or Systemic Chronic Inflammation (SCI). This phenomenon was described as a prolonged inflammatory nature, chronical pathology-related event. Diverging from classical acute inflammation, LGCI is distinguished by its low-grade manifestation, involving the time-prolonged production of molecular mediators at lower concentrations. Furthermore, it exhibits a systemic impact rather than a localized effect (Furman et al. 2019).

Moreover, whereas the acute inflammatory event concludes with the resolution of the disease, LGCI is strictly linked to the perpetuation of the pathological state. It is induced by a combination of environmental, behavioral, and nutritional factors, closely tied to lifestyle, including malnutrition, sedentary behaviors, an inadequate sleep-time and stress (Freese et al. 2018).

A transition from short-lived to prolonged inflammatory responses is linked to a myriad of chronic, non-communicable conditions, such as cardiovascular diseases (CVD), Cancer, Metabolic-related diseases such as Type-2 Diabetes (T2D), Non-Alcoholic Fatty Liver Disease (NAFLD), or Metabolic Syndrome (METS), Immunosenescence, Neurodegenerative diseases, and so forth. Overall, these

conditions are responsible for around 71% of global mortality (Minihane et al. 2015; Furman et al. 2019).

LGCI and Smoke

LGCI is modulated by behavioral habits, such as, in example, exposure to cigarette smoke.

Despite the considerable risks associated with tobacco exposure, smoking prevalence still remains substantial. Cigarette smoke represents a primary source of chemical toxic substances for humans, serving as the leading cause of preventable diseases and premature deaths. The repercussions of smoking across various pathologies are mediated by its impact on immune and inflammatory systems (Elisia et al. 2020).

The aerial and particulate components of cigarette smoke constitute the initial interface with the immune system. Cigarette combustion produces a substantial amount of Reactive Oxygen Species (ROS), released into the aerial component, interacting mainly with the upper respiratory tract. The particulate components can potentially generate an even higher number of free radicals (Huang, Lin, and Ma 2005).

ROS damage the cellular epithelium inducing lipidic peroxidation and other forms of cell membrane damage, leading to DNA damage. Cigarette smoke components can activate the signaling cascades within epithelial cells, triggering the activation of inflammatory genes, such as Interleukin (IL)-8 and Tumor Necrosis Factor (TNF)-α (Churg et al. 2002). The dysregulated expression and secretion of these inflammatory mediators fosters the chronic recruitment of immune and inflammatory cells.

The impact of cigarette smoke on the immune systems is not exclusively stimulatory; it also influences T-lymphocytes, prompting the proliferation and production of cytokines involved in important biological functions. The adaptive immune and inflammatory response of T-Cells can be phenotyped as T-helper (Th)-1, Th2 or Th17. Cell differentiation in Th1 rather than Th2 or Th17 phenotypes reflect distinct activation patterns and subsequent production of specific molecules. For

instance, a Th1 phenotype exhibits elevated levels of Interferon (IFN)-γ, while a Th2 phenotype is characterized by increased levels of IL-4 (Zhou, Chong, and Littman 2009).

Cigarette smoke exerts an impact on T-cells differentiation by diminishing Th1 cellular levels in favor of Th2 (Cozen et al. 2004). Additionally, research indicates that dendritic cells exposed to cigarette smoke extracts are subjected to reduced expression of IL-12 (Th1 polarizing) and an increase of IL-23 (Th17 polarizing) (Vassallo et al. 2005). Conversely, circulating T-cells collected from smoking subjects and cultured *in vitro* showed concentrations of IL-13 (Th2 polarizing) significantly higher than their healthy control counterpart (Torres de Heens, van der Velden, and Loos 2009).

The molecular mechanisms through which cigarette smoke induces the Th1-to-Th2 switch, and Th17-mediated inflammation are not fully understood; however, they may entail the inhibition of Th1 differentiation-inducing cytokines (Vassallo et al. 2005).

Indeed, cigarette smoke disrupts various transduction pathways involved in cellular activation. Its components activate several pathways, like Mitogen-activated protein kinase (MAPK) (Iles et al. 2005), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (X. Liu et al. 2008), Signal Transducer and Activator of Transcription (STAT) (Kroening et al. 2008), and Activator Protein (AP)-1 (Smelter et al. 2010)-mediated pathways. Notably, the smoking-induced activation of NF-κB and AP-1 pathways play a pivotal role in altering key features, such as the production of inflammatory chemokines, corticosteroid resistance, responsiveness to pathogenic insults, and apoptosis (Figure 1) (Lee, Taneja, and Vassallo 2012).

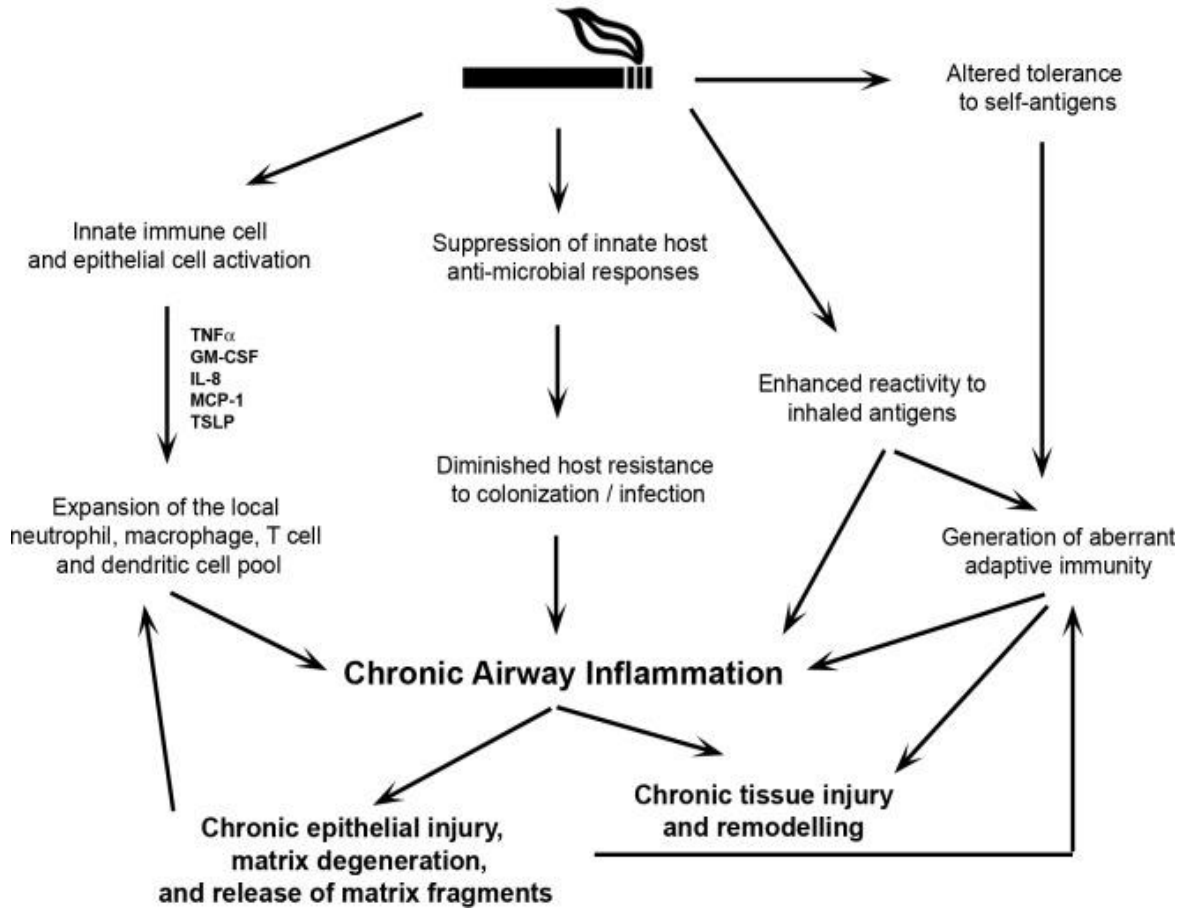

Figure 1 – Mechanisms underlying smoke induced LGCI in respiratory airways (Lee, Taneja, and Vassallo, 2011).

LGCI and Aging

LGCI is also a characteristic feature of physiological chronic conditions such as aging. One of the main alterations occurring during the aging process is the loss of regulation in the immune response, leading to a chronic inflammatory systemic state. Pro-inflammatory mediators, particularly cytokines and chemokines, represent the main factors responsible for the insurgence of chronic inflammation and immunosenescence. For instance, IL-6, TNF-α, along with their receptors, exhibit up-regulation with aging. Elevated levels of chemokines and C-Reactive protein (CRP) contribute to age-related pathogenesis (Gordon et al. 2011).

It is currently established that the molecular pathway associated with NF-κB represents the primary transduction process linked to inflammation. Numerous studies have documented that the NF-κB pathway up-regulates pro-inflammatory genes, including TNF-α, interleukins (IL-1β, IL-2, IL-6), chemokines (IL-8, CCL5) and adhesive molecules. Additionally, it influences insulin-related pathways and other transcription factors, such as insulin-like growth factor (IGF), mechanicistic target of rapamycin (mTOR), Sirtuin (SIRT) family, Forkhead box (FOXO)-1, and p53 (Haigis and Yankner 2010).

There are two hypotheses relative to age-related inflammation insurgence: molecular inflammation and Inflammaging. While these two models are synergistic in many aspects, they differ in their emphasis on different stages of the inflammatory process.

The molecular inflammation hypothesis relies on variations in inflammation-related transcription factors and the dysregulation of their target genes. According to this hypothesis, these changes constitute the molecular mechanisms underlying aging and age-related pathologies. The validity of this hypothesis is predicated on the heightened sensitivity of NF-κB to oxidative stress and alterations in cellular redox balance. Persistent oxidative stress and concurrent compromission of

antioxidative defense systems during aging lead to the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and lipidic peroxidation.

Despite younger subjects may rely on a well-functioning antioxidant system and a maintained redox balance, the functional decline associated with aging impairs the cellular redox homeostasis. This impairment triggers the activation of different pro-inflammatory signal pathways. These pathways, synergically with NF-κB ed AP-1, contribute to increased oxidative status- sensitive transcription factors during aging. Redox-mediated cell signaling involves kinase/phosphorylase proteins located near the cell membrane. These proteins activate other kinases, like MAPK and NF-κB activating kinase, further amplifying NF-κB activity. Conversely, thr gene expression levels of inflammatory cytokines, cyclooxygenases (COX), and lipoxygenases (LOX) are up-regulated through NF-κB hyperactivation during cellular aging (Chung et al. 2002).

It is then important to recognize how NF-κB acts as a master transcription factor for numerous pro-inflammatory genes, other mediators, and pathways (Jung et al. 2009).

The Inflammaging hypothesis describes an age-related phenomenon, characterized by a gradual increase of the pro-inflammatory status. The causes of such increase may be attributed to various nature factors, including accumulation of incompletely processed cellular and molecular debris, a progressive inability to interact and process gut microbiota and its products, evolving mitochondrial alterations culminating in the formation of multiprotein complex NLRP3 (inflammasome), cellular senescence, prolonged activation, or loss of regulation for complement proteins, age-related immune system alterations (Figure 2) (Franceschi and Campisi 2014; Sanada et al. 2018).

The convergence of these factors rise to the insurgence of Inflammaging, characterized by an increase of pro-inflammatory cytokines, normally observed during aging. Specifically, age-related alterations in innate immunity result in a loss

of inflammatory regulation, impacting the ability to initiate an effective adaptive immune response following antigens and environmental stimuli (Baylis et al. 2013).

Several studies on aging, conducted in both human and murine models, have generated data supporting the occurrence of Inflammaging. These studies reveal an increase of inflammatory cytokines, acute-phase proteins, coagulation factors, hormones, and oxidative stress. Consistent with the Inflammaging hypothesis, these immune system alterations contribute to the onset of organ-specific inflammatory diseases, such as atherosclerosis, Alzheimer's disease, and Type-2 Diabetes (Bauer and Fuente 2016). While the Inflammaging hypothesis has been useful for elucidating how age-related inflammation may modulate aging and the development of age-related diseases, the molecular mechanisms underlying Inflammaging are still not fully elucidated as of today.

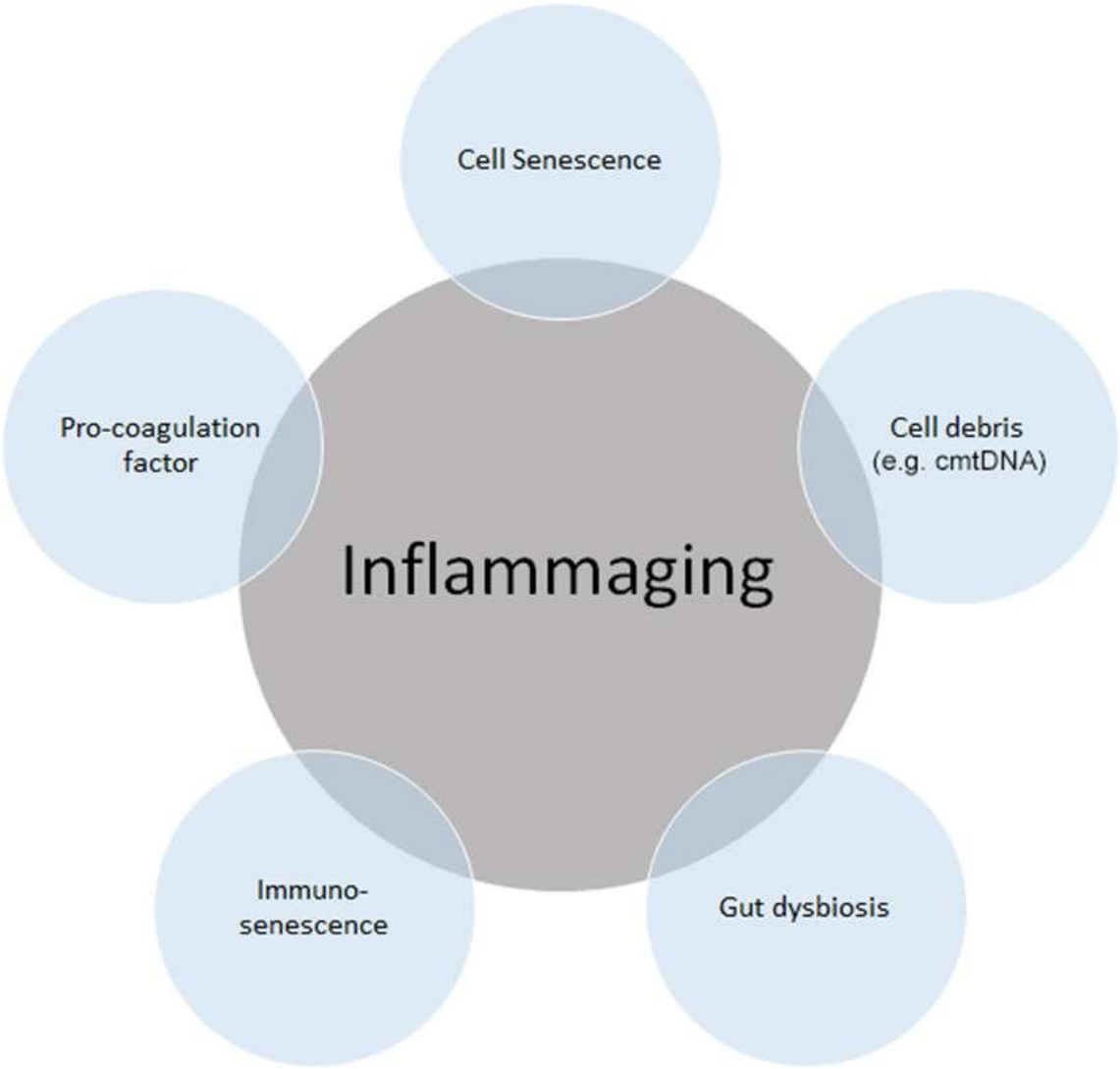

Figure 2 – Principal causes underlying the Inflammaging process. (Sanada et al, 2018)

LGCI and Obesity

When there is a sustained positive energy imbalance in the human body, with energy intake exceeding energy expenditure, a condition of obesity ensues. Obesity is marked by the excessive accumulation of adipose tissue in both visceral and subcutaneous areas. This accumulation can result from adipose cellular hypertrophy, meaning an increase in cell volume, and/or hyperplasia, which entails an increase in the number of adipose cells (Flier 2004).

With the onset of obesity, adipose tissue undergoes morphological and metabolic alterations. There is an increase of pro-inflammatory cytokines, including IL-6 and TNF-α, and a simultaneous reduction in the secretion of anti-inflammatory molecules, such as adiponectin. This imbalance in cytokine secretion contributes to a state of LGCI associated with obesity (Ellulu et al. 2017).

These alterations observed during obesity are not only confined to localized effects; they are indeed accompanied by an increase in serum levels of acute-phase proteins, leading to an inflammatory state that plays a critical pathophysiological role in the initiation and progression of obesity-related complications (Hotamisligil 2006). It is noteworthy that this inflammation shows improvement following weight loss, whether induced by a hypocaloric diet or bariatric surgery (Castanon, Lasselin, and Capuron 2014). This suggests the dynamic relationship between obesity, inflammation, and the potential for therapeutic interventions to modulate inflammatory responses.

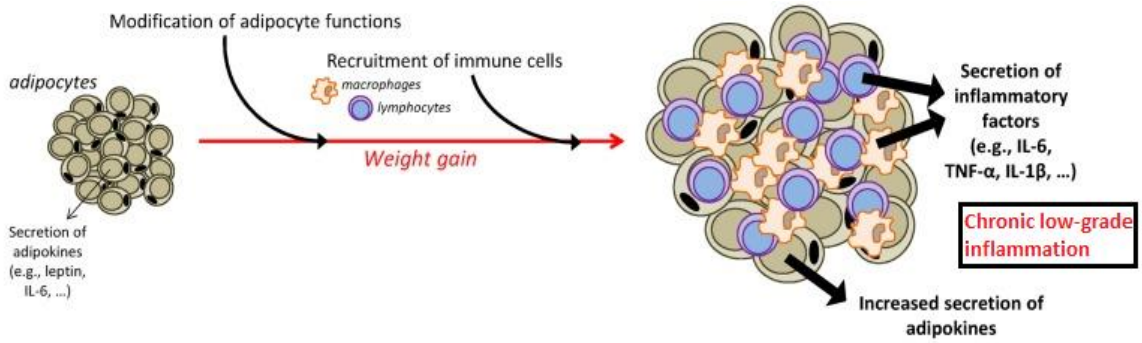

Figure 3 – Mechanism of Low-Grade Chronic Inflammation initiation following Adipokine secretion by Adipocytes (Castanon, Lasselin, and Capuron 2014).

Adipose tissue is comprised of approximately one-third by mature adipocytes, and the remaining two-thirds are constituted by the so-called Stromal-Vascular Fraction (SVF). SVF includes various cellular components, such as Adipose-derived Mesenchymal Stromal Cells (Ad-MSCs), regulatory T cells (Treg), endothelial progenitor cells (EPCs), fibroblasts, smooth muscular cells, pericytes, macrophages and pre-adipocytes at different stages of maturation (Zuk et al. 2002). Adipose tissue plays a pivotal role in regulating the entire body's activity by influencing physiological processes, such as the regulation of the basal metabolic rate, maintenance of metabolic homeostasis, immune response, as well as participating in pathogenic processes, like cardiovascular diseases (CVD), chronic inflammation, and cancer (Galic, Oakhill, and Steinberg 2010).

Several factors lie at the foundation of the inflammatory state, with the hypoxic condition emerging in adipose tissue being a prominent mechanism. An imbalanced food intake contributes to an uneven storage of fatty acids in adipose tissue, leading to hypertrophic alterations. As adipose storages enlarge, the oxygenation and vascularization of adipose cells and tissues become inadequate. Consequently, adipose cells undergo hypertrophic expansion, reaching a critical diameter of 140-180 μm, surpassing the critical threshold for oxygen diffusion, estimated to be around 100 μm. This establishes a state of hypoxia, inducing stress on the endoplasmic reticulum and resulting in altered production of adipokynes.

Significantly, it has been demonstrated that under low oxygen conditions, the production of Adiponectin and PPAR-γ mRNA is inhibited (Figure 3) (Hosogai et al. 2007; Chen et al. 2006).

Another crucial mechanism facilitating the insurgence of LGCI in obesity is associated with the infiltration of inflammatory cells into adipose tissue, predominantly macrophages and lymphocytes. These cells are attracted locally from the peripheral bloodstream through the adipocyte-mediated production and secretion of chemokines, such as the Monocyte Chemotactic Protein (MCP)-1 and the Vascular Cell Adhesion Protein (VCAM). The principal lymphocyte phenotypes inside the adipose tissue are the T helper 2 (Th2), regulatory T (Treg), and eosinophils, accompanied by M2 macrophages. Under normal physiological conditions, these cells contribute to maintain an anti-inflammatory, insulin-sensitive phenotype, by secreting anti-inflammatory cytokines and adipokines, such as IL-4 (Th2, eosinophils), IL-13 (eosinophils), and IL-10 (Treg, M2 Macrophages). Upon the initiation of inflammation associated with obesity, monocytes are lured to chemotactic signals and migrate into adipose tissue, undergoing a shift toward the pro-inflammatory M1 phenotype. Once recruited, M1 macrophages secrete more cytokines with paracrine activity, such as IL-1, IL-6, IL-12, MCP-1, IFN-γ. This secretion results in a reduction of eosinophils and Treg, along with an increase of $CD4^+$ Th1, $CD8^+$ effector T cells, and B lymphocytes. Collectively, these various cell types contribute to the increased local levels of pro-inflammatory cytokines (Figure 3) (Castanon, Lasselin, and Capuron 2014).

LGCI and Dietary Habits

Numerous evidence indicates that various foods and food components might modulate chronic inflammation. Several studies emphasize that LGCI is mitigated by adopting healthy behaviors, such as a healthy diet, physical activity, body weight control and smoking abstinence (Bonaccio et al. 2017).

In the cohort study MOLI-SANI, which involved over 7,000 healthy subjects (Centritto et al. 2009), those adhering to a Mediterranean-diet based regimen, rich in antioxidant nutrients such as plant-derived polyphenols, fibers, and 'good' fatty acid, exhibited significantly lower levels of glucose, lipids, CRP, blood pressure, and cardiovascular disease risk. Converesely, individuals with a higher intake of pasta and meats, adhering to a Western dietary model, showed elevated levels of glucose, lipids, and CRP. While there are some mixed reports in the literature, there is shared consensus on the positive correlation between glycemic index and LGCI (Minihane et al. 2015).

Furthermore, glycemic index has been found to be significantly associated with the progressive increase of oxidative stress markers in adults (Hu et al. 2006). Diets characterized by a low glycemic index and high fibers intake may exert a protective effect against systemic inflammation in individuals with diabetes (Qi and Hu 2007). Additionally, various epidemiological studies have highlighted an inverse correlation between CRP levels and fibers intake. Both the Dietary Approaches to Stop Hypertension (DASH) regimen and a normal diet supplemented with fibers effectively reduced CRP levels in normotensive subjects (King et al. 2007). Adherence to a high-carbohydrate, low-lipid diet with a substantial fiber and complex carbohydrates content, coupled with a suitable regimen of physical activity, resulted in a 50% reduction in the incidence of diabetes (Boer et al. 2013). In conclusion, it can be inferred that a low-postprandial glycemic diet is associated with lower inflammatory markers.

Fatty acids play a pivotal role in influencing inflammatory processes, exerting pro-inflammatory, anti-inflammatory, inflammation-resolving effects. Fatty acids derived from cell membranes may function as modulators of NF-κB and PPAR-α/γ transcription factors, and also serve as precursors for eicosanoids and docosanoids through epoxygenases, lipoxygenases and cyclooxygenases activity. Additionally, recent studies have demonstrated that IL-1β acts as a key sensor for metabolic stress induced by Saturated Fatty Acids (SFA) in the context of obesity and T2D (Minihane et al. 2015).

LGCI and Cancer

The association between chronic inflammation and development of cancer has been a subject of speculation since the 19th century, driven by observations of leukocytes in cancerous lesions. Numerous epidemiological studies support the notion that chronic inflammation serves as significant risk factor for cancer onset. In fact, up to 25% of human malignances are attributed to either chronic inflammation or microbiological infections (Perwez Hussain and Harris 2007). This underscores the substantial role that chronic inflammation plays in the pathogenesis of carcinogenesis.

Cancer-related chronic inflammation facilitates unlimited replicative potential, independence of growth factors, resistance to growth inhibition, escape of programmed cell death, enhanced angiogenesis, tumor extravasation, and metastasis. During the initial phase of tumor development, inflammatory mediators, including cytokines, ROS, and RNS derived from tumor-infiltrating immune cells, induce epigenetic alterations in pre-malignant lesions. This process results in the silencing of tumor suppressor genes (Grivennikov, Greten, and Karin 2010). More specifically, both tumor and immune cells secrete cytokines and chemokines, serving as survival and proliferation factors for malignant cells. The angiogenic lesion switch becomes crucial during this phase to ensure an adequate supply of tumor cells with oxygen, nutrition, growth, and survival factors (Zumsteg and Christofori 2009). The overall shift in cytokines and chemokines production leads to an increase in cell survival, motility, and invasiveness. A crucial process in tumor invasiveness is Epithelial-mesenchymal transition (EMT). It refers to the loss of carcinoma epithelial phenotype and the acquisition of mesenchymal features (Zeisberg and Neilson 2009).

The connection between tumorigenesis and inflammation is mediated through both intrinsic and extrinsic pathways. The intrinsic pathway is activated by genetic alterations that cause inflammation and neoplasia. These alterations include

mutation-driven proto-oncogene activation, chromosomal rearrangement and/or amplification, and the inactivation of tumor suppressor genes. Transformed cells secrete inflammatory mediators, thus generating an inflammatory microenvironment. The extrinsic pathway is driven by inflammation or infections, that elevate the risk for cancer development in organs at risk, such as the prostate, pancreas, colon, lung, breast, and skin. Both pathways interfere with tumor cells and induce the activation of several transcription factors, such as NF-κB, STAT-3, and Hypoxia-Inducible Factor (HIF)-1, leading to the formation of pro-inflammatory factors, including chemokines, cytokines, and PGHS-2. These molecules recruit and activate various leukocyte populations, such as macrophages, mast cells, eosinophils, and neutrophils, into the tumor microenvironment, involving stromal and endothelial cells as well as infiltrating cells. This concerted action of tumor and microenvironment results in a more pronounced generation of inflammatory mediators that drive the progression of a positive amplification loop which further triggers tumor growth and invasiveness. Tumor-associated inflammation requires the presence and activation of inflammatory cells such as macrophages and granulocytes in the tumor microenvironment, formation of inflammatory mediators by tumor and stromal cells, tumor remodeling, and angiogenesis (Kundu and Surh 2008; Colotta et al. 2009).

Accumulation of microbial pathogens and tissue necrosis activate transcription factors that are necessary for the expression of, in example, pro-angiogenic factors (IL-8, VEGF), growth factors (IL-6, GM-CSF), anti-apoptotic factors (Bcl-XL, c-FLIP), invasion-promoting factors (MMP-2, MMP-7, MMP-9, uPA), inflammatory enzymes (PGHS-2, LOX), prostaglandins, iNOS, chemokines (CCL2, CCL20, IL-8), and pro-inflammatory cytokines (IL-1, IL-6, IL-23, TNF, TGF-β, EGF) that support the malignant phenotype. All molecules mentioned above are regulated by the transcription factor NF-κB, a key orchestrator in innate immunity and inflammation that has emerged as a crucial tumor promoter (Karin 2006). Constitutively active NF-κB, was found to be associated with poor clinical outcome (Aggarwal and

Gehlot 2009). NF-κB activation in inflammatory cells in response to infectious pathogens, pro-inflammatory mediators as well as necrotic cell products results in the generation of secretable factors that support growth, survival, and vascularization of pre-malignant and malignant cells.

A crucial aspect of emerging cancer is the disruption of cellular energy levels. Nutrients are required for cell survival since they provide elements for energy catabolism and anabolism. The regulation of nutrient balance is intricately managed to uphold cell homeostasis and to control cellular proliferation. The onset of cancer disrupts nutrient homeostasis in affected organs and tissues, leading to metabolic stress, autophagy, and the induction of angiogenesis in the affected areas (Levine and Kroemer 2008). Consequently, proper nutrition and the bioavailability of nutrients emerge as additional lifestyle factors contributing to the initiation and aggressiveness of cancer (Hanahan and Weinberg 2011).

There is a growing recognition that lifestyle can influence cancer risk factors. The significant variations in cancer incidence across countries, notable shifts in these rates within populations, and rapid temporal changes within countries underscore the substantial impact of lifestyle, environmental, and/or behavioral factors, which may play a pivotal role, if not a predominant one, in the prevalence of common cancers in western societies. Addressing major modifiable risk factors holds the potential to reduce the risk of these cancers. Strategies such as improving dietary habits, reducing tobacco consumption, managing weight, limiting alcohol intake, and promoting smoking cessation are crucial elements in this endeavor. The modification of these factors presents a promising avenue in the therapeutic paradigm for cancer progression (Khan, Afaq, and Mukhtar 2010).

Aim

Inflammation constitutes a ubiquitous biological phenomenon, involving a high number and type of cells and molecular effectors. The qualitative and quantitative determination for these components depicts a crucial importance in diagnosis, prevention, and progressive evaluation of pathophysiological conditions (Rea et al. 2018).

Multiple cytokines preside over LGCI evolution. The cytokine network is a highly complex system that includes not only interleukins, chemokines, interferons, and tumor necrosis factors, but also cellular and soluble receptors and serum mediators. Cytokines are small, non-structural proteins with pleiotropic effects. They have pro-inflammatory and/or anti-inflammatory functions, and some of them are able to control their own production (Rea et al. 2018; Monastero and Pentyala 2017; C. Liu et al. 2021).

Obesity, aging, tobacco use, physical inactivity, and unhealthy diets are considered the main risk factors for LGCI. These physiologic, environmental, and/or behavioral stimuli modify the cellular homeostasis, leading to cell stress and to the production of cytokines. Thus, cytokine levels may vary upon a plethora of stimuli.

To date, critical levels of cytokines as biomarkers have not been defined yet, both in classical inflammatory diseases and in LGCI. Furthermore, only few studies have been performed to investigate cytokine levels in healthy subjects, and a limited number of LGCI-related risk factors have been explored when considering healthy subjects' cytokine profiles (Rea et al. 2018; Bonaccio et al. 2017).

Thus, aim of this PhD thesis is the research of potential soluble molecular biomarkers for the Low-Grade Chronic Inflammation (LGCI), through the cytokinome. A critical aspect for an effective inflammatory state evaluation is the fact that biomarkers must elicit certain features, such as their ability to reflect the

real condition of inflammation and their predictive power on the future state of health.

The first part of this study is based on the identification of circulating markers capable of identify the LGCI condition, based on the association with LGCI-related risk factors, such as age, Body Mass Index (BMI), smoking exposure, physical activity, and dietary habits. To this end, 150 healthy blood donors have been enrolled, a lifestyle questionnaire has been submitted to them, and a panel of 28 cytokines, chemokines, growth factors, and CRP has been screened in the serum.

The second part of this study is based on the phenotyping of a population of 132 patients with tumor, and the projection of the information gained in the first part, to both validate the observed results and to assess the impact of the neoplastic condition, and clinical comorbidities, on a panel of circulating cytokines, chemokines, and growth factors, metabolic markers, and adipokines.

Materials and Methods

Patients Enrollment and Serum Collection

A number of 150 blood donors were recruited at the Transfusion Medicine Unit of the Local Health Authority of Caserta, Italy. Exclusion criterion was ineligibility to donate blood, as indicated in D.M. 02/11/2015, i.e. documented infectious diseases or other proliferative, degenerative, and autoimmune diseases; altered blood count and blood pressure; and drug assumption).

Moreover, a number of 132 patients with neoplasies were recruited at the Local Health Unit of Naples-3, Italy.

All enrolled volunteers underwent detailed anagraphic, and bioimpedance phenotyping. including measurement of height, weight, waist, and hip. Body Mass Index (BMI) was calculated as ratio of body weight (Kg)/Height (m^2). Bioimpedance parameters calculated included Waist-to-Hip Ratio (WHR), Fat Mass (FM), Fat-Free Mass (FFM), Basal Metabolic Rate (BMR), Total Body (TBW) and Extra-Cellular Water (ECW), Visceral Adipose Tissue (VAT), Bone Weight, and Phase Angle.

In the onchologic population, clinical and pathological data were collected, including cancer type, presence of metastasis and eventual ongoing chemotherapy, as well as eventual comorbidities like Metabolic Syndrome (METS), Type-2 Diabetes (T2D), cardiovascular disease (CVD), Non-Alcoholic Fatty Liver Disease (NAFLD), Hyperlipaemia, and Anemia.

Moreover, a detailed questionnaire about anamnestic and anthropometric data, smoking habit, physical activity, and weekly food frequency, was administered to each subject (as described below).

A serum sample from all donors was obtained and stored at -20°C. Transfusion Medicine Unit performed cytometric blood counts and biochemical analyses (Aspartate transaminase, AST; Alanine transaminase, ALT, cholesterol, triglycerides, iron, total proteins, creatinine).

Investigations were carried out following the rules of the Declaration of Helsinki of 1975, revised in 2013. Informed consent was obtained from every volunteer before the procedure. The protocol was approved by the ethical committee of the University of Naples (protocol no. 349/18).

Lifestyle Questionnaire

Lifestyle questionnaire was face-to-face administered by an expert nutritionist and included information about the last 7 days of physical activity and food frequency. The physical activity of enrolled volunteers was recorded and analyzed with the short form of the International Physical Activity Questionnaire (IPAQ) (Craig et al. 2003). The questionnaire reports the activity of four intensity levels:

1) Vigorous-intensity activity, such as aerobics.
2) Moderate-intensity activity, such as leisure cycling.
3) Walking.
4) Sitting.

For each activity, the Metabolic EquivalenT (MET), a unit used to express energy spent and oxygen burned, was automatically calculated. MET value is given by the following equation (Jetté, Sidney, and Blümchen 1990):

$$MET = \frac{3.5\ ml\ (O_2)}{bodyweight\ [Kg] \times time\ [h]}$$

Based on the MET data analysis, as suggested by Craig et al. and by the Guidelines for Data Processing and Analysis of the IPAQ (www.ipaq.ki.se), and considering MET value distribution in the enrolled population, volunteers were classified into three groups, namely, volunteers with low or null physical activity (MET < 1,000), volunteers with an average physical activity (1,000 < MET ≤ 3.000), and volunteers with an intense physical activity (MET > 3,000).

Dietary information was collected with a weekly food-frequency questionnaire (FFQ) conceived on that used in the framework of the Italian EPIC study (Pisani et al. 1997; Simeon et al. 2015). Accordingly, a list of foods was developed in line with

the local availability, culturally specific and dietary habits (Mediterranean diet). Food frequency type with portion size was estimated by means of pictures. The participants were asked to report the frequency of consumption of food items. Pictures showing different portion sizes – arranged by increasing amount – followed the question on frequency and corresponded to a specific portion in grams. Additional questions addressed issues such as habitual cooking practices and types of cooking fats. Questionnaires were finally digitalized, and data relative to the weekly total consumption of each food item, expressed in grams, were gathered, and analyzed.

Determination of Cytokines, Chemokines, and Growth Factors

Serum samples were screened for the concentration of interleukin (IL)-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, basic Fibroblast Growth Factor (bFGF), Eotaxin, Granulocyte-Colony Stimulating Factor (G-CSF), Granulocyte and Macrophage-Colony Stimulating Factor (GM-CSF), Interferon-γ (IFN-γ), Interferon-γ inducible protein 10 (IP-10), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 (MIP-1α), MIP-1β, C-C motif chemokine ligand 5 (CCL5)/Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), Tumor Necrosis Factor-α (TNF-α), Platelet-Derived Growth Factor (PDGF-bb), and Vascular Endothelial Growth Factor (VEGF), using the Bio-Plex® Multiplex Human Cytokine, Chemokine, and Growth Factor Kit (cat. n. M500KCAF0Y, Bio-Rad, Hercules, Ca, USA) according to the manufacturer's protocol, as previously described (Cabaro et al. 2018; 2021). The magnetic bead-based assay was performed on a Bio-Plex® 200 System (Bio-Rad, Hercules, Ca, USA). All the values obtained were included within the detection limits indicated by the manufacturer (bio-rad.com/Bio-Plex/AnalyteGuide).

Additionally, a second array of metabolic markers, including C-Peptide, Ghrelin, Gastric Inhibitory Peptide (GIP), Glucagon-Like Peptide-1 (GLP-1), Glucagon, Insulin, Leptin, total Plasminogen Activator Inhibitor-1 (PAI-1), Resistin, and Visfatin, was screened using the Bio-Plex Pro® Human Diabetes 10-plex Assay (cat. n. 171A7001M, Bio-Rad, Hercules, Ca, USA) according to the manufacturer's protocol, as previously described (D'Esposito et al. 2021; Caso et al. 2019; Pisano et al. 2017).

High sensitivity C-reactive protein (CRP, cat. n. L2KCR2, Siemens, USA) chemiluminescence (CLIA) assay was performed using the IMMULITE® 2000 Analyzer (DPC, Los Angeles, Ca, USA), according to the manufacturer's protocol. High Molecular Weight Adiponectin (cat. number 296752, Fujirebio, Tokyo, Japan) CLIA assay was performed using the Lumipulse® G600II analyzer (Fujirebio, Tokyo, Japan).

Statistical Analysis

Statistical analyses were performed using the GraphPad® Prism 8.4.2 software (GraphPad Software Inc., La Jolla, Ca, USA) and the R version 4.1.2 programming language (R Foundation for Statistical Computing, Vienna, Austria) through the RStudio® Integrated Development Environment (Posit PBC, Boston, Ma, USA). Packages used for the analyses were: *base* (R Team 2014), *CCA* (Ignacio González and Déjean 2023), *CCP* (Menzel 2022), *corrplot* (Wei and Simko 2021), *cowplot* (Wilke 2023), *DescTools* (Signorell 2022), *dunn.test* (Dinno 2017), *factoextra* (Kassambara and Mundt 2020), *FactoMineR* (Lê, Josse, and Husson 2008), *FSA* (Ogle et al. 2023), *GGAlly* (Schloerke et al. 2021), *ggeasy* (Carroll, Schep, and Sidi 2023), *ggcorrplot* (Kassambara 2022), *ggrepel* (Slowikowski 2022), *janitor* (Firke 2023), *patchwork* (Pedersen 2022), *pheatmap* (Kolde 2019), *stats* (R Team 2014), *tidyverse* (Wickham et al. 2019), *tsne* (Donaldson 2022), and *umap* (Konopka 2023).

Categorical variables were described by the number of occurrences and relative frequency as percentage, and then compared using the Fisher's exact test.

Continuous variables were described as either mean ± standard deviation or median and Interquartile range (IQR, 25^{th} to 75^{th} percentile). The Shapiro-Wilk normality test was used to evaluate whether the continuous data were normally distributed. Correlations among two continuous variables were reported and analyzed with either Pearson's *r* correlation coefficient or Spearman's ϱ rank correlation and their respective 95% confidence intervals. For two-group comparisons, either a Welch's two-tailed t-test for independent samples (for parametric data) or a Mann-Whitney U-test (for non-parametric data) were used. Multiple comparisons among more than two groups were made using the ANOVA test with Tukey's correction (for parametric data) or the Kruskal Wallis Test with Dunn's post hoc correction (for non-parametric data). Moreover, the non-parametric Jonckheere-Terpstra test was used to analyze the trend within an ordinal independent variable. Correlations among two continuous variables were reported and analyzed with either Pearson's

r correlation coefficient or Spearman's ϱ rank correlation and their respective 95% confidence intervals.

Outliers have been detected and accordingly removed with the ROUT (Robust regression and Outlier removal) method of regression. Briefly, a robust nonlinear regression is used to fit a curve that is not influenced by outliers; then the residuals of the robust fit are analyzed to identify any outliers. This step used an outlier test adapted from the False Discovery Rate approach of testing for multiple comparisons; finally, outliers are removed and a least-squares regression on the remaining data is performed (Motulsky and Brown 2006).

High dimensional data visualization algorithms, such as t-distributed stochastic neighbor embedding (t-SNE) (Maaten and Hinton 2008) and Uniform Manifold Approximation and Projection (UMAP) (McInnes, Healy, and Melville 2020), were used for visualizing the high dimensionality data on two-dimensional projections. Moreover, Multiple Factor Analysis (MFA) (Escofier and Pagès 2016) was used to both analyze a dimensionality reduction with a proper variable categorization, single-variable impact on the cumulative overall variance, and relative tumor clustering.

To investigate the collinearity effects among risk factors and food groups, data were analyzed with a multivariable linear regression analysis. Generated models were analyzed with an ANOVA test to evaluate the goodness of fit. Regression coefficients were reported as an estimate and its 95% confidence interval.

All p-values <0.05 were considered statistically significant.

Results

Healthy Population: Anthropometric and Clinical Features

The enrolled population was represented by 150 blood donors, with 63 female participants and 87 male participants. Overall, the mean age was of 40.9 years, while the BMI median was of 25.92 Kg/m^2; forty-three individuals (28.66%) were smokers (Table 1). Female and male subgroups did not display statistically significant differences for age, smokers' percentage, physical activity, cytometric blood counts, and biochemical analyses. However, the male subgroup showed significantly higher weight, height, and BMI values compared with the female subgroup (Table 1).

Table 1: Clinical phenotyping of the enrolled population. Age is expressed as mean ± standard deviation. Smokers are expressed are count and percentage. All other data are reported as median and range [min; max]. p-values in bold are considered statistically significant ($p < 0.05$).

Parameters [Unit]	Total Population (N: 150)	Females (N: 63, 42%)	Males (N: 87, 58%)	p-value
Age [Years]	40.9 ± 11.2	40.33 ± 11.84	39.62 ± 10.88	0.707
Weight [Kg]	75 [50; 120]	65 [50; 94]	82 [64; 120]	**<0.001**
Height [m]	170 [145; 186]	165 [145; 177]	175 [160; 186]	**<0.001**
BMI [Kg/m^2]	25.92 [19.16; 37.87]	23.44 [19.16; 36.51]	26.42 [20.98; 37.87]	**<0.001**
Smokers	43 (28.66%)	22 (34.92%)	21 (24.14%)	0.2
Phis. Act [MET]	1.026 [0; 12.000]	1.026 [0; 11.874]	1.026 [0; 12.000]	0.828
RBC [10^6/μl]	5.05 [3.91; 7.25]	4.51 [3.91; 5.67]	5.23 [4.39; 7.25]	0.581
Hb [gr/dl]	14.7 [12; 20.3]	13.35 [12; 16.6]	15.2 [13.1; 20.3]	0.248
HCT [%]	43.8 [35.6; 60.1]	40.3 [35.6; 49.5]	44.4 [39.5; 60.1]	0.368
MCV [fl]	86.9 [69.4; 95.2]	88.85 [79.9; 95.2]	85.4 [69.4; 94.2]	0.908
MCH [pg]	29.5 [21.5; 31.8]	29.7 [25.7; 31.1]	29.2 [21.5; 31.8]	0.378
MCHC [g/dl]	33.6 [31; 35.8]	33.1 [31.8; 35.7]	33.8 [31; 35.8]	0.233
PLT [10^3/μl]	232 [135; 364]	227.5 [135; 364]	234 [135; 313]	0.175
WBC [10^3/μl]	6.2 [3.33; 12.5]	6.435 [3.97; 11.4]	6.15 [3.33; 12.5]	0.695
Neutrophils [%]	57.1 [30.5; 73.9]	58.2 [3.05; 73.9]	55.8 [32.7; 68.8]	0.902
Lymphocytes [%]	31.9 [26.6; 52.6]	32.55 [2.66; 49.6]	31.9 [22.7; 52.6]	0.263
Monocytes [%]	7.4 [0.48; 11.9]	6.75 [0.48; 8.8]	7.8 [4.7; 11.9]	0.174
Eosinophils [%]	2.4 [0.1; 12]	1.6 [0.1; 6.1]	2.6 [0.6; 12]	0.122
Basophils [%]	0.6 [0.03; 1.4]	0.55 [0.03; 1.4]	0.6 [0.2; 1.2]	0.294
NLR	1.79 [0.62; 3.62]	1.77 [0.75; 3.62]	1.79 [0.62; 3.03]	0.885
AST [UI/l]	23 [15; 41]	21 [16;29]	23 [15; 41]	0.873
ALT [UI/l]	19 [8; 52]	15 [8; 30]	20 [10; 52]	0.102
Cholesterol [mg/dl]	177 [115; 268]	190 [132; 268]	174 [115; 253]	0.887
Triglycerides [mg/dl]	75 [42; 234]	61 [42; 188]	79 [43; 234]	0.21
Serum Iron [μg/dl]	95 [43; 271]	97 [46; 271]	93.5 [43; 228]	0.671
Total Proteins [g/dl]	7.1 [6.4; 7.9]	7.2 [6.8; 7.7]	7.1 [6.4; 7.9]	0.67
Creatinine [mg/dl]	0.88 [0.52; 1.12]	0.64 [0.52; 0.96]	0.905 [0.58; 1.12]	0.382

MET: Metabolic EquivalenT, Hb: Hemoglobin, HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; PLT: Platelets; WBC: White Blood Cells; NLR: Neutrophils to Lymphocytes ratio; AST: Aspartate transaminase; ALT: Alanine transaminase.

Serum samples of blood donors were then screened for the concentration of a panel of cytokines, chemokines, growth factors, and C-Reactive Protein (CRP). Overall, all factors were detectable in serum specimens, with a defined cytokine/chemokine profile for every individual (Figure 4, Table 2). 22% of total individuals had CRP concentrations higher than 3 mg/l; however, these subjects did not display any significant difference in cytokine concentrations, compared to those with CRP < 3 mg/l. No statistically significant differences were observed between female and male participants for any of the circulating factors (Figure 4, Table 2).

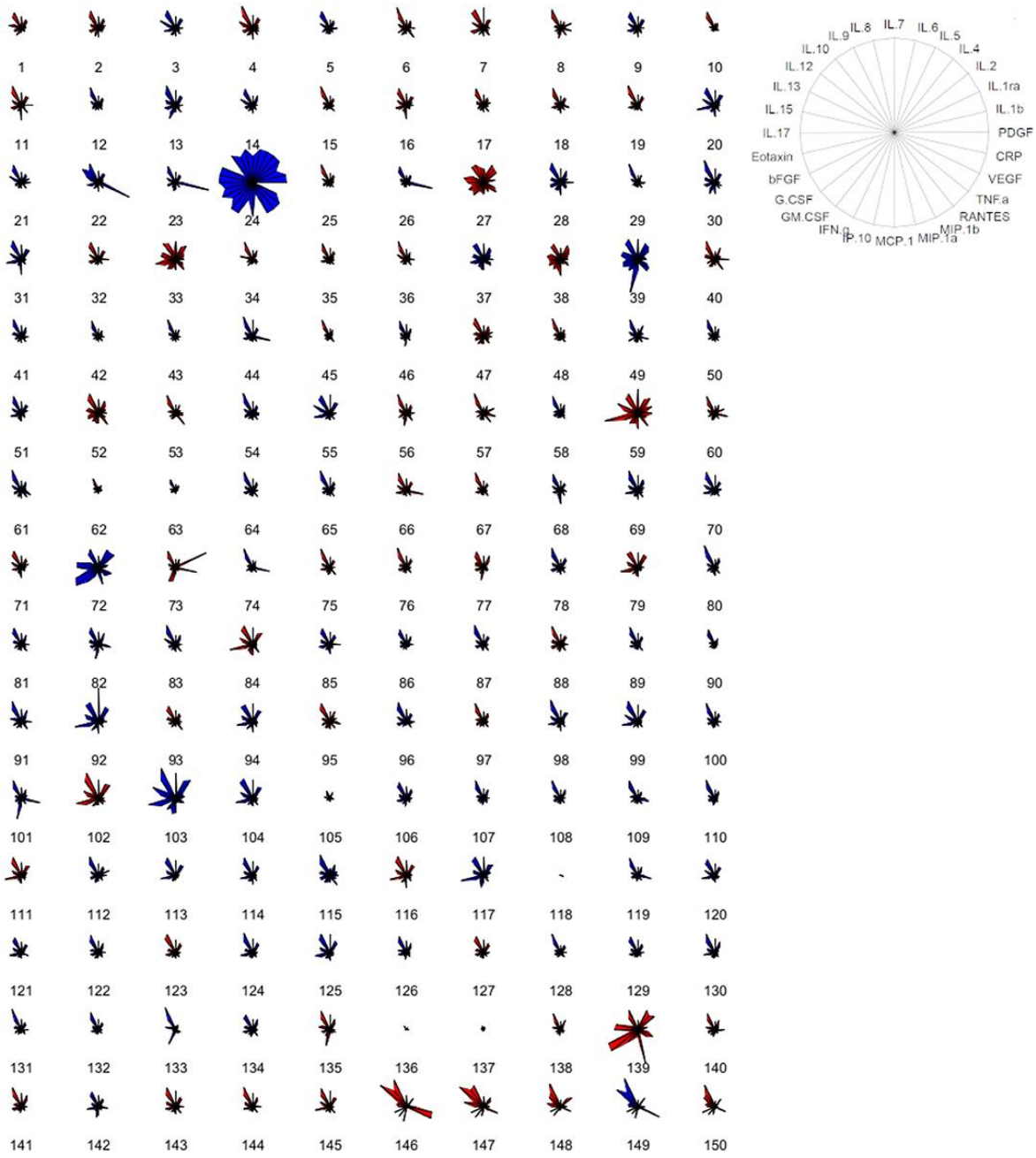

Figure 4: Cytokine-based pattern of volunteers. Star plot obtained by multivariate data analysis of the whole cytokinome of every subject, consisting of a sequence of equi-angular spokes [radii), with each spoke representing one cytokine, as indicated in in the right legend. Data length of a spoke is proportional to the magnitude of the variable for the data point relative to the maximum variable across all data points. A line is drawn connecting all data values for each spoke. Blue stars represent male subjects, and red stars represent female subjects.

Table 2: Serum concentration of cytokines, chemokines and growth factors. hsCRP values are expressed as mg/l, while every other concentration is reported as pg/ml. All values are reported as median and IQR [25th – 75th percentile].

Marker	Concentration (total)	Concentration (Females)	Concentration (Males)	p-value
IL-1β	1.89 [1.81; 2.22]	1.89 [1.81; 2.22]	1.89 [1.81; 2.22]	0.74
IL-1ra	352.5 [288.1; 463.6]	381.8 [284.4; 546.7]	341.7 [289.3; 400]	0.16
IL-2	15.46 [14.84; 17.20]	16.07 [14.52; 17.58]	15.46 [14.84; 16.68]	0.97
IL-4	5.93 [5.21; 7.22]	6.1 [5.27; 7.4]	5.81 [5.21; 7.04]	0.39
IL-5	55.51 [50.69; 61.5]	55.51 [49.42; 61.5]	55.51 [50.69; 61.5]	0.17
IL-6	7.85 [7.18; 8.91]	7.85 [7.29; 9.17]	7.85 [7.01; 8.91]	0.09
IL-7	43.69 [40.38; 46.93]	43.69 [40.38; 46.93]	43.69 [40.79; 48.52]	0.88
IL-8	18.82 [16.34; 23.02]	19.86 [16.19; 25.51]	18.56 [16.34; 20.98]	0.17
IL-9	252.1 [233.6; 264.4]	252.4 [234.6; 263.7]	251.3 [232.6; 266.2]	0.87
IL-10	24.85 [22.65; 26.9]	24.85 [22.65; 27.56]	24.85 [22.65; 26.73]	0.48
IL-12	11.26 [10.84; 12.88]	12.08 [11.26; 14.05]	11.26 [10.41; 12.08]	0.27
IL-13	5.41 [4.72; 6.08]	5.41 [4.72; 6.08]	5.41 [4.81; 6.16]	0.78
IL-15	354.9 [338.4; 379.4]	360.1 [344.1; 390.6]	349.6 [338.4; 372.4]	0.34
IL-17	26.28 [23.78; 30.3]	26.28 [23.56; 31.67]	26.69 [23.78; 29.51]	0.92
Eotaxin	47.79 [33.82; 63.48]	47.62 [29.51; 59.58]	47.88 [36.96; 67.44]	0.4
bFGF	78.43 [73.34; 85.86]	78.43 [73.34; 87.07]	78.43 [73.34; 83.41]	0.25
G-CSF	293.3 [260.3; 353.7]	283.8 [249; 360.1]	293.3 [263.1; 352.4]	0.75
GM-CSF	12.89 [12.45; 13.47]	12.97 [12.45; 13.96]	12.75 [12.3; 13.18]	0.3
IFN-γ	12.44 [11.43; 13.47]	12.78 [11.56; 14.36]	12.24 [11.36; 13.84]	0.13
IP-10	250.5 [203.5; 306.5]	260.8 [202.8; 333.6]	242.5 [203.8; 281.1]	0.09
MCP-1	33.63 [26.58; 44.53]	33.74 [25.94; 43.64]	33.32 [27.65; 44.77]	0.72
MIP-1α	2.6 [2.39; 2.91]	2.66 [2.39; 3]	2.53 [2.39; 2.82]	0.36
MIP-1β	81.52 [75.51; 90.01]	81.72 [74.91; 87.94]	81.37 [76.52; 91.43]	0.5
PDGF	1538 [1180; 1994]	1497 [1180; 2114]	1553 [1151; 1956]	0.92
RANTES	6573 [4951; 7858]	6843 [4825; 8706]	6251 [5150; 7557]	0.32
TNF-α	48.87 [44.63; 55.39]	48.49 [44.63; 56.91]	49.26 [45.4; 55.39]	0.27
VEGF	416.9 [398.7; 446.9]	421.4 [402.2; 455]	412.5 [397.5; 436.5]	0.29
CRP	1.22 [0.57; 2.59]	1.1 [0.47; 2.61]	1.29 [0.76; 2.47]	0.99

Cytokines and LGCI Risk Factors in healthy population

Firstly, we investigated the correlation between LGCI risk factors (Age, BMI, Smoke, and physical activity) and the inflammatory cytokines IL-1β, IL-6, IL-8, IL-9, Eotaxin, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, and CRP. A Canonical Correlation Analysis (CCA) revealed that LGCI risk factors and inflammatory cytokines displayed a correlation coefficient of 0.521 (95% CI: 0.382 – 0.66), with a goodness of fit (R^2) equal to 0.501 (Figure 5A). The standardized weights for the single risk factors indicated a stronger relevance of BMI, which concurred for 78.27% of the correlation; smoke contributed to 13.28%, while age and physical activity contributed to 8.44% and <0.002%, respectively (Figure 5B). The standardized weights for the single inflammatory cytokines showed that IL-1β, CRP, IFN-γ, and IL-6 were the principal components of the correlation, concurring for 47.83, 23, 11.23, and 7.88% respectively (Figure 5C).

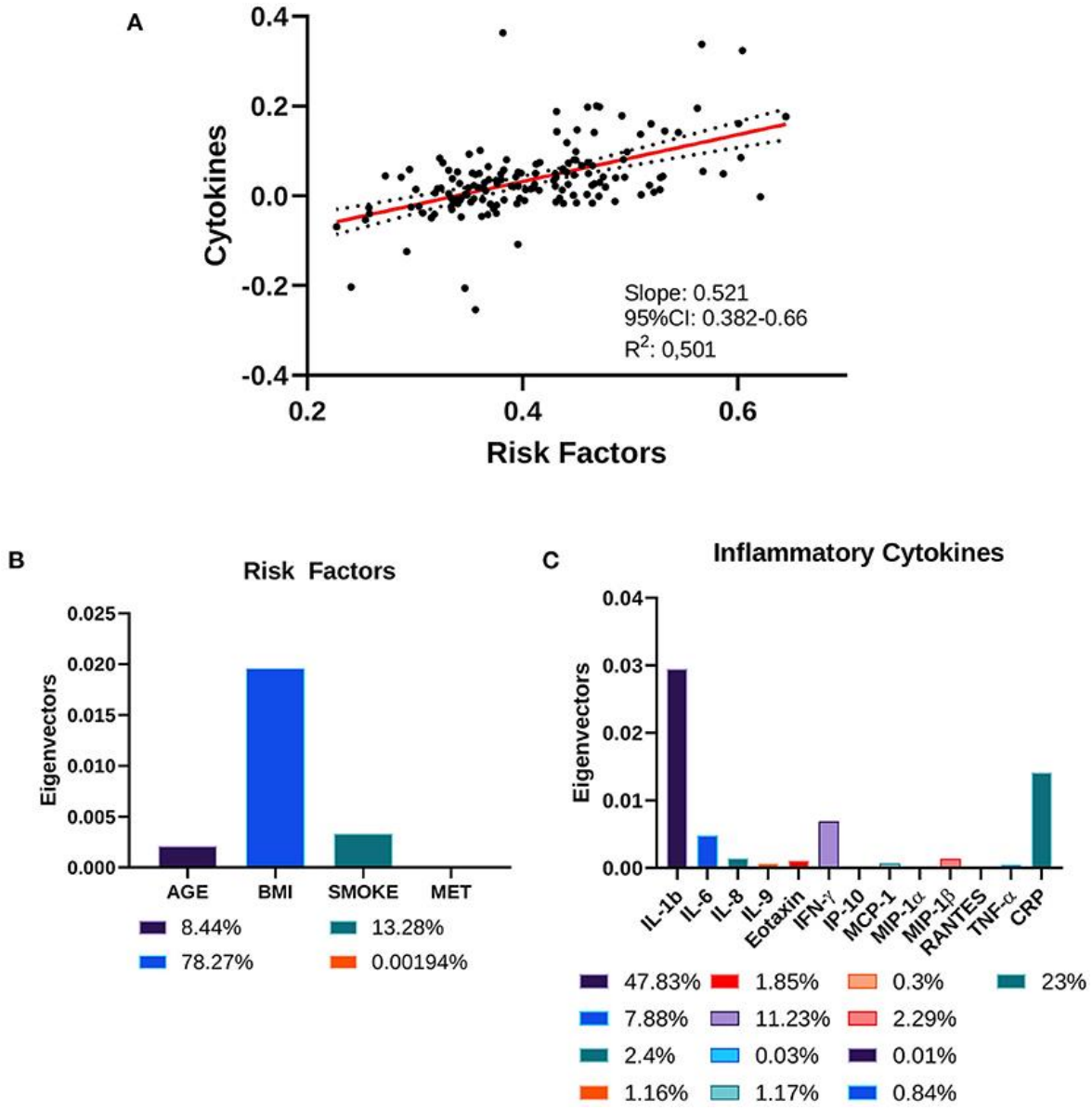

Figure 5 – Canonical Correlation Analysis of cytokines and Risk factors. (A) Two away CCA setting; each subject is described by two canonical variates per mode, represented on a scatter plot. The two variate groups are maximally correlated, and their linear regression slope corresponds to the canonical correlation coefficient. (B) Risk factors' and (C) cytokines' standardized weights are represented as absolute values and as percentage in the color legends.

Next, the association between specific cytokines and LGCI-risk factors was investigated. Interestingly, IL-1β, Eotaxin, MCP-1, and MIP-1α displayed a significant overall difference among four age-related groups (i.e, 20-29, 30-39, 40-49, 50-59 years). Eotaxin and MCP-1 showed also an increasing trend associated with age (20–29 ≤ 30–39 ≤ 40–49 ≤ 50–59 years; Figure 6). In smokers, a significant increase in IL-1β and RANTES was detected (Figure 6). Overweight individuals (BMI: 25-30 Kg/m^2) showed significantly higher levels of CRP, compared with normal weight

individuals (BMI: 20-24.9 Kg/m^2). Finally, physical activity was associated with higher levels of MIP-1α (Figure 6).

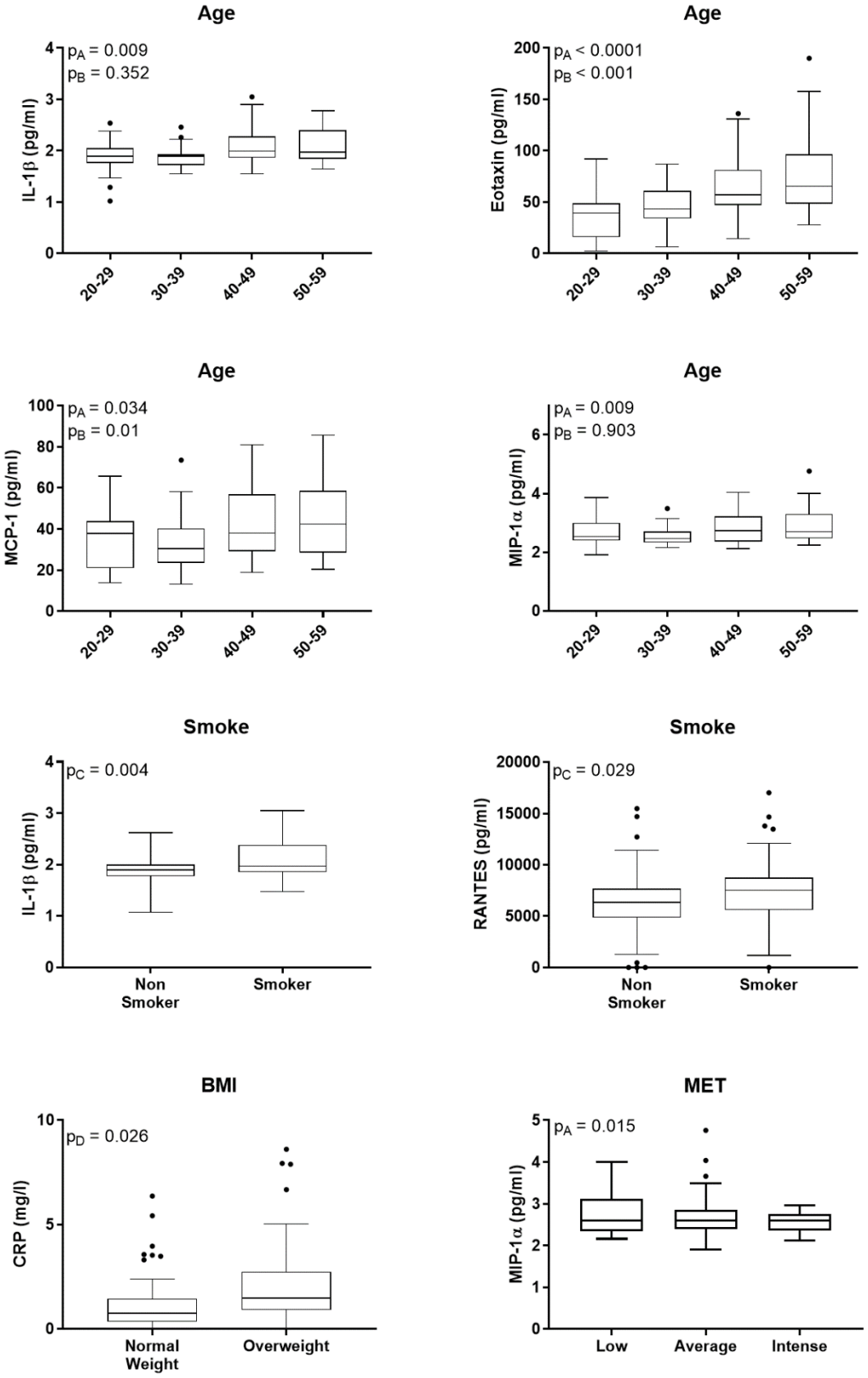

Figure 6 – Age, smoke, BMI and Physical-activity related cytokines. Boxplots indicate cytokine concentrations in subjects classified for age (20-29 years, n: 29; 30-39 years, n: 53; 40-49 years, n: 33; 50-59 years: n: 29), smoke habit (Non-smokers, n: 107; smokers, n: 43), BMI (Normal Weight, n: 65; Overweight: n: 60), and physical activity (Low physical activity, n: 74; average physical activity, n: 53; intense physical activity, n: 23). IL-1β, Eotaxin, MCP-1, MIP-1α, and RANTES concentrations are expressed pg/ml. CRP concentration is expressed as

mg/l. Figure only reports factors with statistically significant differences. p_A: overall ANOVA test; p_B: Jonckheere-Terpstra trend test; p_C: Welch's t-test; p_D: Mann-Whitney U test.

Cytokines and Dietary Habits

Dietary information was collected with a weekly food-frequency questionnaire. Data relative to the weekly total consumption of each food item (in grams) were collected, included in 11 food groups, and analyzed. The main components for the dietary intake of the volunteers were "Grains", "Greens", "Fresh Fruits", and "Milk and Dairy", which accounted for 29.79, 18.07, 17.17, and 11.01% of the total food consumption, respectively (Figure 7).

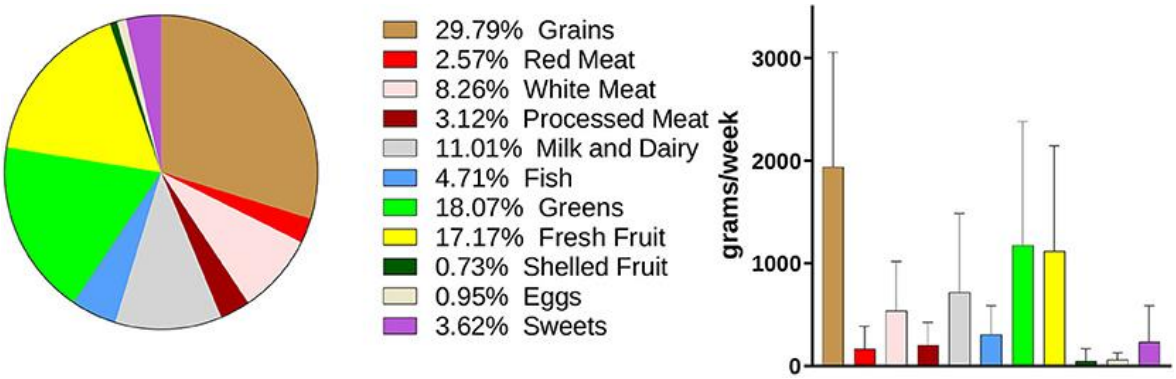

Figure 7 – Dietary habits of the enrolled volunteers. Weekly consumption of food is expressed in grams. Data are showed in a pie-chart as percentage, compared with the whole weekly food intake, and in a histogram, as a mean ± standard deviation for the single food groups.

Food groups and inflammatory cytokines displayed a correlation coefficient of 0.462 (95% CI: 0.319 – 0.607), with an R^2 equal to 0.482 (Figure 8A). The standardized weights for the single food items indicated a stronger relevance of Red Meats and Shelled Fruits, which concurred for the 28.7 and 28.15% of the correlation, respectively (Figure 8B) Among the inflammatory cytokines, IFN-γ, IL-1β, and IL-6 were the principal components of the correlations, contributing to 36.62, 29.5, and 20.5%, respectively (Figure 8C).

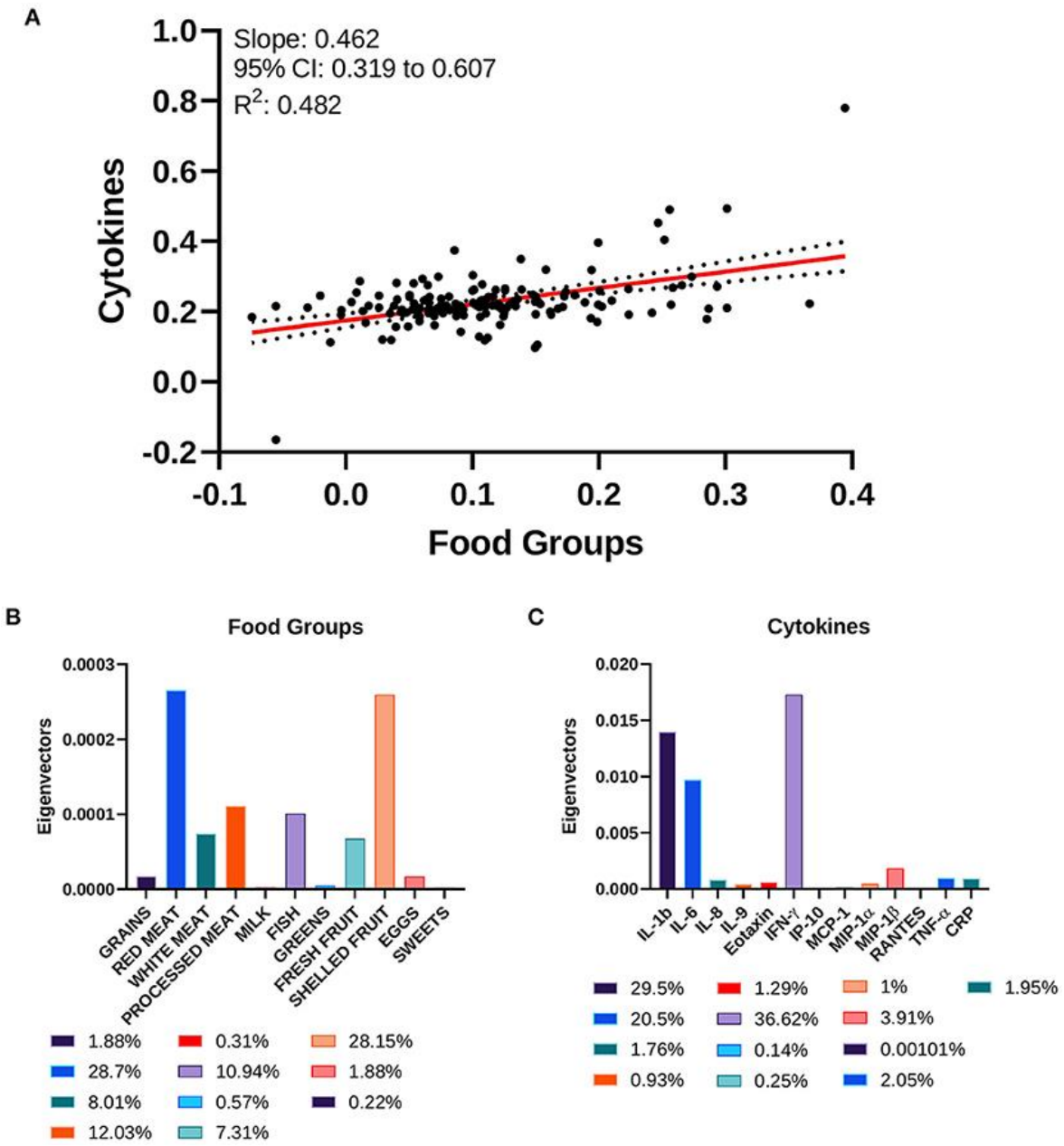

Figure 8 – Canonical Correlation Analysis of cytokines and food groups. (A) Two-way CCA setting: each subject is described by two canonical variates per mode, represented on a scatter plot. The two variate groups are maximally correlated, and their linear regression slope corresponds to the canonical correlation coefficient. (B) Food groups' and (C) cytokines' standardized weights are represented as absolute values and as percentage in the color legends.

At univariate analysis, individuals with a weekly grain intake higher than the 75th percentile displayed a significantly higher levels of circulating IFN-γ, MCP-1, and TNF-α. The intake of red meat was associated with a significant increase in IL-6, IL-8, and CRP. Moreover, subjects who ate more fruits showed higher levels of IL-8, IFN-γ, and IP-10. Increased levels of IL-8 were also detected in subjects consuming more sweets. A significant reduction in CRP was observed in individuals who ate more eggs, greens, or shelled fruits. In these latter subjects, a significant reduction of IL-1β and IL-6 was also found (Figure 9).

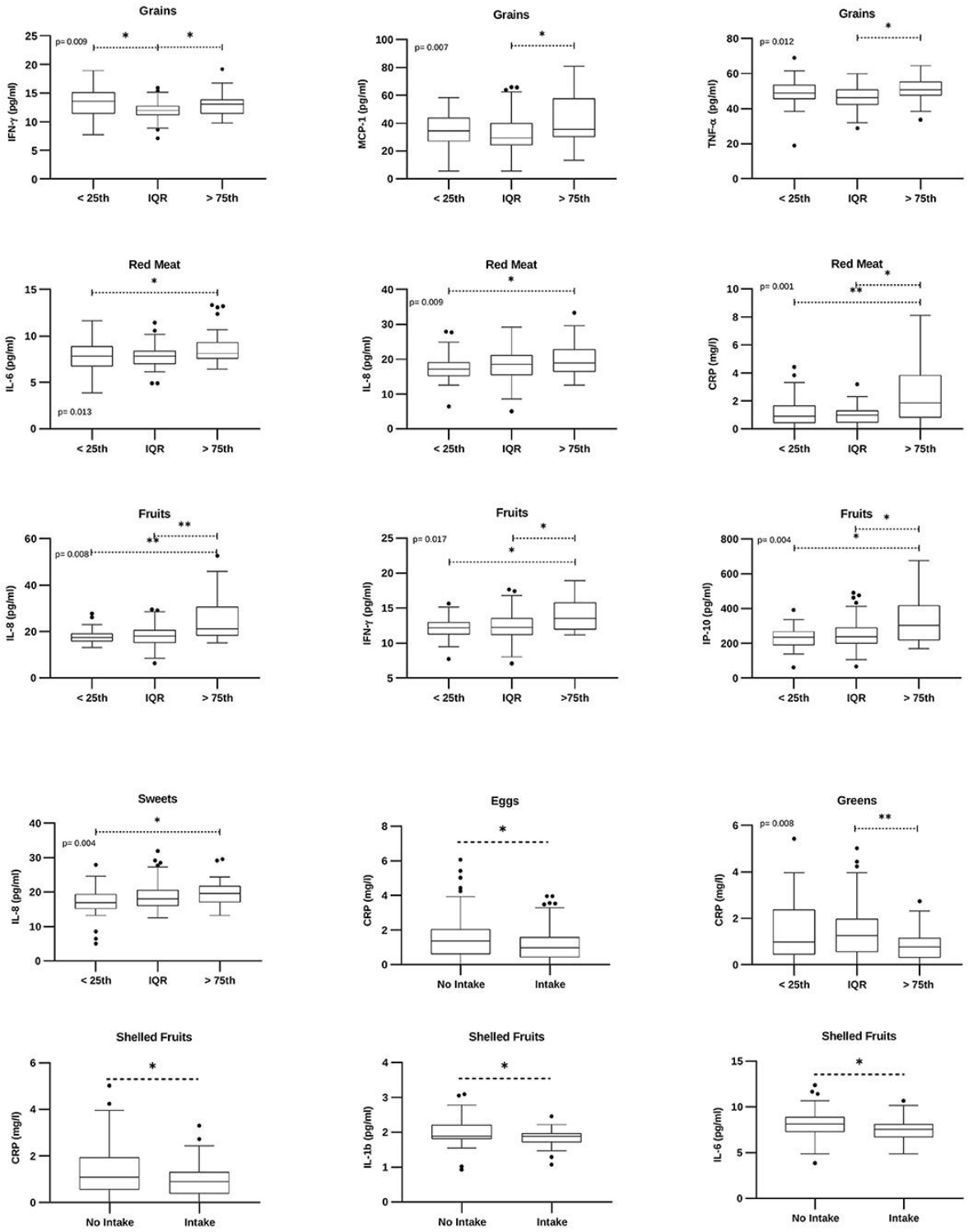

Figure 9 – Food Groups-related cytokines. Box Plots denote cytokine concentrations in subjects with low ("< 25th" – subjects with a weekly intake lower than the 25th percentile), medium ("IQR" – subjects with a weekly intake between the 25th and the 75th percentile), or higher (">75th" – subjects with a weekly intake higher than the 75th percentile) food intake; or intake/no intake of a specific food group. Cytokine concentrations are expressed as pg/ml. CRP concentration is expressed as mg/l. Figure reports only factors with statistically significant values. p = overall ANOVA test or Kruskal Wallis test; * $p < 0.05$; ** $p < 0.01$.

Multivariate Analysis and Cytokine Correlations in Healthy Subjects

In multivariable analysis, all significant associations among cytokines and lifestyle factors or food groups found at univariate analysis (Figure 3, 6) were still retained upon adjusting for BMI.

Among all cytokines, MCP-1, IL-1β, and CRP levels were significantly associated both with risk factors and with specific food groups (Figures 3, 6). As shown in Table 3, the association between MCP-1 and the intake of grains was not significant after adjusting for age; similarly, the association between IL-1β and shelled fruits was not significant when adjusted for age and smoke. Interestingly, CRP levels were not associated with the consumption of red meat, shelled fruits, eggs, and greens after adjusting for BMI (Table 3).

Table 3: Multivariate linear regression analyses between risk factors and food groups for MCP-1, IL-1β, and CRP. For each model, coefficients for risk factors, food groups and interactions among covariates are reported as estimate, 95% CI and p-value.

Marker	Risk Factor		Food Group		Interaction	
	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value	Estimate (95% CI)	p-value
MCP-1	**Age**		**Grains**		0.115	0.486
	0.356 (0.13 – 0.581)	**0.0022**	3.06 (-0.213 – 6.33)	0.07	(-0.209 – 0.439)	
IL-1β	**Age**		**Shelled Fruit**		-0.001	0.819
	0.007 (0.0023 – 0.012)	**0.0029**	3.06 (-0.219 – 6.34)	0.07	(-0.012 – 0.01)	
	Smoke				-0.289	0.248
	0.141 (0.03 – 0.252)	**0.014**			(-0.025 – 2.27)	
CRP	**BMI**		**Shelled Fruit**		-0.016	0.799
	0.356 (0.132 – 0.58)	**0.022**	0.32 (-1.07 – 1.05)	0.082	(-0.143 – 0.11)	
			Eggs		-0.055	0.411
			-0.054 (1.67 – 0.432)	0.77	(-0.187 – 0.077)	
			Red Meat		-0.122	0.115
			-0.12 (-0.263 – 2.65)	0.574	(-0.275 – 0.03)	
			Greens		-0.029	0.498
			-0.243 (-0.577 – 0.92)	0.061	(-0.117 – 0.057)	

Finally, a correlation matrix based on cytokines significantly associated with at least one LGCI-related risk factor (Age, BMI, Smoke, Physical inactivity, diet) indicated positive and significant correlations among all selected inflammatory cytokines, except for CRP, for which almost no correlations with cytokines were observed (Figure 10). Interestingly, IL-1β, TNF-α, IL-6 and IL-8 established a cluster of cytokines with r-values ranging from 0.88 (CI: 0.84 – 0.91) to 0.95 (CI: 0.93 – 0.96), thus representing a potential LGCI-related cytokine pattern biomarker (Figure 10).

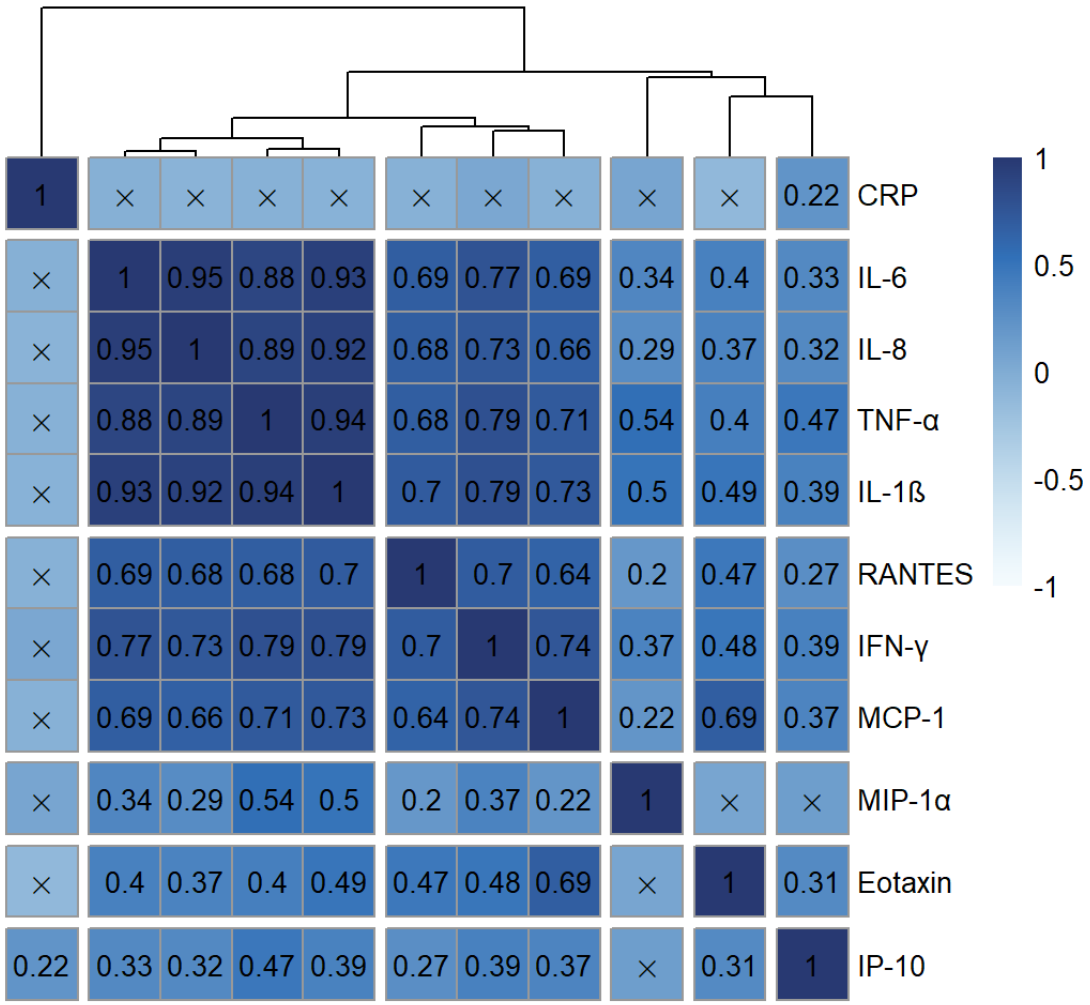

Figure 10 – Cytokine correlation matrix and hierarchical clustering. Pearson's correlation coefficients for cytokine's scaled measurements are visualized by both tile-color intensity (according to the legend on the right) and by the r-values inside the tiles. Coefficients closer to 1 show a positive correlation; coefficients closer to -1 show a negative correlation; coefficients cluser to 0 denote the absence of a correlation among the considered variables. all values not labeled with a black X are statistically significant (p-value <0.05). Dendrograms show the hierarchical clustering.

Neoplastic Population: Anthropometric and Clinical Features

The second enrolled population was represented by 132 patients with cancer, of which 64 (48.48%) had Breast Cancer, 52 (39.39%) had Colorectal Cancer, and 16 (12.12%) had Prostate Cancer.

The three patient subgroups displayed a well distinct characterization, with the Breast Cancer group being the youngest (mean: 55.88 years) but with the highest BMI values (median 29.56, IQR 26.81 to 33.98). Apart from a slightly lower alcohol exposure for Breast Cancer patients, no differences in behavioral data were noted.

Bioimpedance data reflected the marked anagraphical and physical differences among the three groups. Metastasis were reported mainly for patients with colorectal cancer (23.08%), while chemotherapy for patients with breast cancer (51.56%) (Table 4).

Table 4: Clinical phenotyping of the enrolled population. Age is expressed as mean ± standard deviation. Smokers are expressed as count and percentage. All other data are reported as median and range [IQR]. p-values in bold are considered statistically significant ($p < 0.05$).

Parameters [Unit]	Total (N: 132)	Breast (N: 64, 48%)	Colon (N: 52, 39%)	Prostate (N: 16, 12%)	p-value
Sex (Females, %)	48 (36.36%)	63 (98%)	21 (40.38%)	0 (0%)	<0.001
Age [Years]	60.95 ± 11.06	55.88 ± 11.06	63.37 ± 8.53	73.32 ± 7.73	<0.001
Height [m]	1.64 [1.57; 1.70]	1.61 [1.56; 1.65]	1.66 [1.59; 1.73]	1.7 [1.64; 1.75]	**<0.001**
Weight [Kg]	78.7 [67.77; 87.5]	78.9 [68.47; 88.4]	77.75 [63.08; 84.1]	81.5 [68.25; 88.95]	0.628
Phys. Act. (%)	54 (40.9%)	21 (32.81%)	24 (46.15%)	9 (56.25%)	0.143
Smokers (%)	28 (21.21%)	11 (17.19%)	12 (23.07%)	5 (31.25%)	0.429
Alcohol (%)	44 (33.3%)	14 (21.88%)	23 (44.23%)	7 (43.75%)	**0.025**
BMI (Kg/m²)	28.65 [25.41; 32.51]	29.56 [26.81; 33.98]	27.13 [24.37; 31.26]	26.76 [23.62; 30.71]	**0.012**
Waist (cm)	103.5 [95; 112.5]	103 [93.25; 112]	100.5 [97; 112.75]	106 [98.5; 112.5]	0.859
Hip (cm)	106 [101.25; 117.25]	111.5 [104; 119]	104 [99; 115]	104.5 [98.5; 106.38]	**0.018**
WHR	0.95 [0.89; 1.00]	0.9 [0.87; 0.96]	0.98 [0.94; 1.02]	1.01 [0.95; 1.03]	**<0.001**
FM (Kg)	24.3 [18.75; 32.85]	26.1 [22.3; 36.8]	19.95 [15.3; 28.28]	20.95 [16.97; 27.65]	**<0.001**
FFM (Kg)	51.3 [46.2; 56.2]	49.3 [45.1; 53]	53.4 [46.58; 58.83]	56.6 [52; 64.27]	**<0.001**
BMR (kcal)	1521 [1370; 1658]	1474 [1358; 1607]	1541 [1406; 1727]	1636 [1467; 1847]	**0.024**
TBW (L)	35 [31.7; 40.1]	33 [30.4; 35.6]	37.35 [32.73; 42.38]	42.15 [37.1; 45.2]	**<0.001**
ECW (L)	16.7 [14.85; 18.35]	16.1 [14.4; 17.8]	17.2 [15.83; 19]	18.55 [16.8; 20.08]	**0.002**
VAT (Kg)	10 [7; 12]	8 [7; 11]	11 [7.25; 13]	12.5 [11; 15.25]	**<0.001**
Bone (Kg)	2.6 [2.3; 2.8]	2.5 [2.3; 2.7]	2.7 [2.4; 2.95]	2.8 [2.58; 3.23]	**0.002**
Phase (°)	5.4 [5; 5.93]	5.4 [5.1; 5.8]	5.4 [4.95; 6.15]	5.35 [4.95; 5.5]	0.377
Metastasis	19 (14.39%)	7 (10.94%)	12 (23.08%)	0 (0%)	**0.039**
Chemoterapy	47 (35.61%)	33 (51.56%)	12 (23.08%)	2 (12.5%)	**<0.001**
METS	14 (10.61%)	5 (7.81%)	6 (11.53%)	3 (18.75%)	0.429
T2D	20 (15.15%)	6 (9.38%)	11 (21.15%)	3 (18.75%)	0.194
CVD	66 (50%)	29 (45.31%)	25 (48.07%)	12 (75%)	0.098
NAFLD	33 (25%)	17 (26.56%)	12 (23.08%)	4 (25%)	0.911
Hyperlipaemia	59 (44.7%)	30 (46.88%)	19 (36.53%)	10 (62.5%)	0.167
Anemia	7 (5.3%)	4 (6.25%)	2 (3.8%)	1 (6.25%)	0.834

BMI: Body Mass Index; WHR: Waist-to-Hip ratio; FM: Fat Mass; FFM: Fat-Free Mass; BMR: Basal Metabolic Rate; TBW: Total Body Water; ECW: Extracellular Water; VAT: Visceral Adipose Tissue; METS: Metabolic Syndrome; T2D: Type-2 Diabetes; CVD: Cardiovascular Disease; NAFLD: Non-Alcoholic Fatty Liver Disease.

Patients' serum was screened for the concentration of two panels of molecules: a 27-molecules array of cytokines, chemokines, and growth factors, and a 10-molecule array of metabolic markers. At the same time, C-Reactive Protein and Adiponectin were screened.

Overall, all factors were detectable in serum specimens, with a defined cytokine/chemokine profile for every individual (Table 5). Screened molecules displayed an overall balanced distribution among the three cancer subgroups, with the exceptions of a slight significant increase for IL-12 in Prostate Cancer patients, and a remarkably higher distribution for Leptin in Breast Cancer patients (Table 5).

Table 5: Serum concentration of cytokines, chemokines, growth factors and metabolic markers. CRP and Adiponectin values are expressed as mg/l, while every other concentration is reported as pg/ml. All values are reported as median and IQR [25th – 75th percentile].

Marker	Concentration (total)	Concentration (Breast)	Concentration (Colon)	Concentration (Prostate)	p-value
IL-1β	4.71 [3.74; 7.38]	4.39 [3.74; 6.95]	5.25 [3.96; 13.4]	5.58 [4.77; 7.9]	0.313
IL-1ra	184.9 [134.52; 230.8]	181.49 [121.1; 229.2]	184.91 [149.6; 237]	211.55 [130.78; 343.8]	0.704
IL-2	13.53 [11.51; 20.11]	13.41 [11.04; 20.24]	13.65 [11.51; 20.36]	13.89 [12.75; 20.24]	0.752
IL-4	4.49 [2.64; 8.87]	4 [2.45; 8.22]	4.84 [2.75; 10]	6.11 [3.96; 7.39]	0.395
IL-5	81.01 [62.74; 111.02]	77.95 [60.47; 112.95]	85.6 [65.77; 115.67]	87.9 [63.12; 97.89]	0.761
IL-6	7.03 [5.26; 17.07]	6.82 [5.18; 13.69]	12.66 [5.54; 50.59]	10.67 [6.62; 22.7]	0.802
IL-7	29.64 [19.66; 46.49]	31.04 [18.75; 48.67]	26.82 [19.66; 38.31]	35.91 [17.83; 63.79]	0.775
IL-8	21.55 [14.24; 52.92]	18.37 [13.54; 38.81]	53.23 [16.8; 789.8]	47.31 [23.29; 206.99]	0.721
IL-9	289 [200.26; 353.66]	303.15 [196.5; 362.5]	310.2 [200.85; 365.5]	273.2 [201.44; 308.34]	0.421
IL-10	8.33 [7.58; 9.84]	8.33 [7.58; 9.84]	8.7 [7.95; 10.22]	9.27 [8.24; 12.12]	0.417
IL-12	10.75 [10.23; 11.79]	10.75 [9.71; 11.86]	10.75 [10.23; 11.79]	12.05 [11.27; 24.03]	**0.035**
IL-13	2.83 [2.4; 3.6]	2.83 [2.4; 3.68]	2.83 [2.4; 3.25]	3.04 [2.34; 3.73]	0.841
IL-15	284.7 [233.1; 321.27]	287.4 [229.4; 327.6]	279.34 [224.2; 311.7]	264.3 [231.61; 288.07]	0.741
IL-17	40.23 [28.61; 62.39]	39.6 [28.21; 65]	41.26 [27.61; 63.92]	40.23 [32.63; 55.24]	0.837
Eotaxin	161.2 [112.1; 226.16]	165 [109.62; 231.84]	153 [116.34; 194.61]	186.6 [108.83; 334.26]	0.637
bFGF	78.74 [67.76; 108.3]	75.91 [59.58; 114.45]	80.97 [69.54; 122.54]	86.44 [71.63; 97.42]	0.357
G-CSF	148 [113.77; 407.9]	136.4 [116.2; 563.6]	191 [118.27; 2077]	230.12 [175; 627.21]	0.475
GM-CSF	6.81 [5.67; 8.7]	6.24 [5.62; 8.91]	7.38 [6; 9.38]	7.71 [6.62; 13.93]	0.468
IFN-γ	18.35 [13.59; 26.77]	18.1 [7.68; 24.76]	18.35 [15.7; 26.9]	20.8 [5.5; 27.27]	0.959
IP-10	1004 [608.7; 1439]	999.5 [526; 1432]	1007 [633.96; 1381]	1027 [595.72; 1897]	0.866
MCP-1	70.44 [50; 100.42]	70.52 [52.59; 105.92]	70.77 [50; 117]	75.26 [55.11; 93.32]	0.926
MIP-1α	4.69 [2.97; 44.63]	3.97 [2.72; 28.32]	6.62 [3.33; 250.59]	8.8 [4.56; 76.46]	0.576
MIP-1β	290.7 [235.75; 397.7]	289.4 [237.5; 360.6]	375.4 [252.2; 825.82]	320.56 [279.84; 407.4]	0.796
PDGF	1985 [1023; 2926]	1952 [878.9; 3004]	1979 [1170; 2711]	2311 [1129; 4226]	0.754
RANTES	20568 [16955; 26172]	21463 [17148; 26258]	20500 [17912; 27167]	18711 [16036; 23079]	0.316
TNF-α	125.49 [99.75; 200.5]	123.65 [96.38; 187]	147.4 [103.56; 259.1]	126.5 [116.56; 167.56]	0.592
VEGF	388.9 [279.3; 506.44]	354.4 [262; 526.14]	390.3 [294.7; 473.12]	396.9 [311.82; 473.12]	0.984
CRP	2.58 [1.14; 4.51]	2.59 [1.17; 4.63]	3.86 [1.44; 10.65]	2.44 [1.22; 5.21]	0.296
C-Peptide	1124 [873.52; 1556]	1155 [798; 1591]	1047 [878.37; 1636]	1252 [1061; 2233]	0.296
Ghrelin	5693 [3745; 9310]	5918 [3815; 9697]	6264 [3917; 9907]	6512 [4249; 13288]	0.748
GIP	310.38 [112.3; 539.3]	324.8 [110.15; 560.3]	308.9 [137.26; 529.5]	479.7 [191.2; 681.6]	0.456
GLP-1	864.5 [256.5; 975.96]	869.4 [249; 969.54]	854.47 [275.8; 935.9]	1040.7 [704.8; 1197.2]	0.107
Glucagon	3098 [1576; 3706]	3007 [1599; 3536]	3098 [1710; 3704]	3633 [1496; 5684]	0.284
Insulin	651.28 [358.28; 1067.98]	659.11 [354.33; 1161]	636.22 [328.16; 1108]	792.38 [516.14; 1089]	0.381
Leptin	12989 [6801; 18758]	17582 [10726; 24410]	9811 [4574; 15927]	9163 [4235; 16677]	**<0.001**
PAI-1	22173 [13,162; 55273]	20163 [12968; 38209]	25961 [13047; 57125]	27800 [12702; 57104]	0.559
Resistin	6655 [3720; 8725]	6686 [3820; 8473]	6225 [3636; 8767]	6946 [3846; 8473]	0.973
Visfatin	5706 [3450; 8614]	5694 [2743; 8883]	5822 [3901; 8439]	6714 [3789; 10038]	0.758
Adiponectin	4.2 [2.81; 5.78]	4.41 [3.2; 6.44]	3.9 [2.43; 5.77]	4.14 [3.57; 5.09]	0.357

The overlapping distribution for screened molecules among the three tumor subgroups is also emphasized after using of dimensional reduction techniques, such as t-Stochastic Neighbor Embedding (t-SNE) or Uniform Manifold Approximation and Projection (UMAP). As depicted in Figure 11, these techniques show both an homogeneous distribution for the cancer population, with the lack of a proper clustering for tumor types, as well as a well-defined distinction among healthy and cancer population (Figure 11).

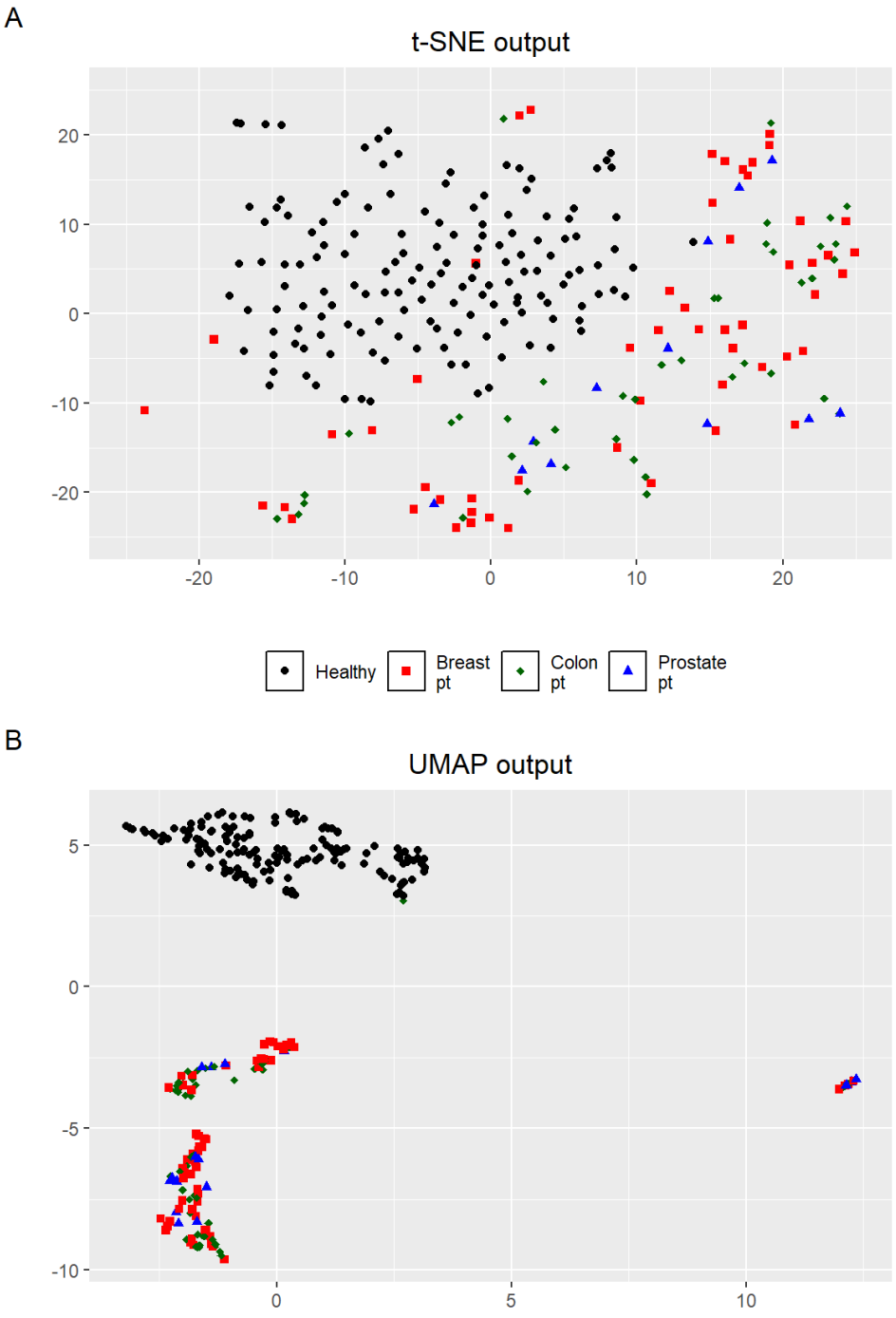

Figure 11 - Dimension Reduction Techniques applied to both Healthy and Tumor populations. (A) t-Stochastic Neighbor Embedding (t-SNE) and (B) Uniform Manifold Approximation and Projection (UMAP) graphical outputs are reported as dot-plots depicting all subjects clustered for their similarity score on the cytokines, chemokines, and growth factor levels. Different shapes depict the stratification for the Healthy (circle) from Tumor (triangle) populations, while colors depict the different tumor subgroups (Red – Breast, Green – Colon, Blue – Prostate).

In parallel, a Multi Factor Analysis (MFA) was conducted on the tumor population to rank all continuous variables and to depict the ones with the highest variance. As shown in Figure 12, BMI, as well as metabolic markers such as Visfatin, GLP-1 and GIP, resulted as the variables with the highest contribute for the tumor population variance (Figure 12).

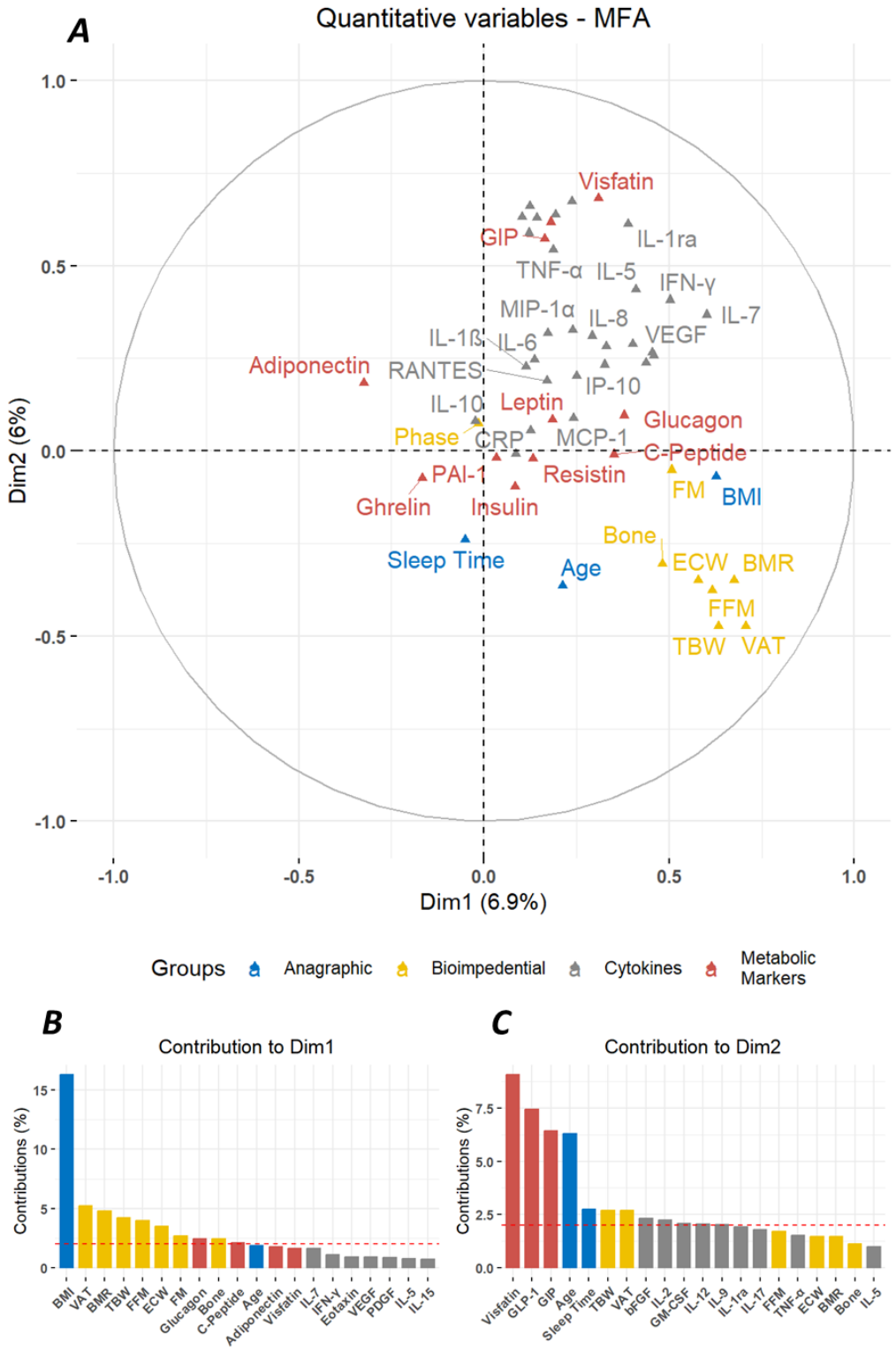

Figure 12 – Multiple Factor Analysis for all quantitative variables in Tumor Populations. (A) Biplot depicting all quantitative variables and their relative association with the first two dimensions obtained with MFA reduction. Higher relative positions on x or y axis depict a stronger correlation with the relative Dimension, thus thus reflecting a stronger contribute; as two variables get closer, their correlation gets higher, and viceversa. (B) Dimension 1 and (C) Dimension 2 contributions for all the screened variables, depicted as relative percentage. Red-dotted line indicates the average contribution value. Color codes depict the different kinds of variables (Blue: Anagraphic, Yellow: Bioimpedential; Gray: Cytokines, Chemokines, and growth factors; Red: Metabolic Markers).

This dimensionality reduction, however, confirmed the high variance homogeneity across the patients, with a lack of a proper effective discriminant clustering (Figure 13).

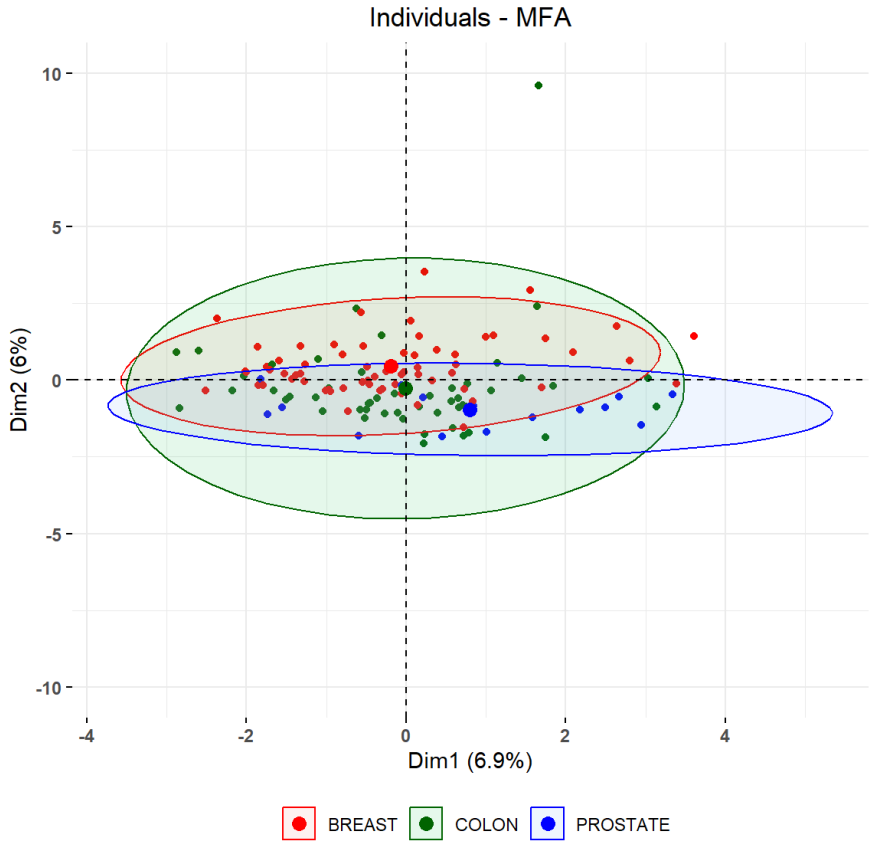

Figure 13 – Multi Factor Analysis clustering for individuals. Dot-plot depicting the two-dimensional reduction for Tumor Population data gathered from MFA. Patients are then clustered for Cancer Type. Ellipses depict the 95% Confidence Interval for the tumor-specific grouping. Color code depicts the different tumor subgroups (Red: Breast, Green: Colon, Blue: Prostate).

Molecular Markers and LGCI Risk Factors in neoplastic population

A Canonical Correlation Analysis (CCA) revealed that LGCI risk factors and molecular markers displayed a correlation coefficient of 0.836 (95% CI: 0.775 – 0.982) (Figure 14A). The standardized weights for the single risk factors indicated a stronger relevance of Metabolic Syndrome (Figure 14B). Among cytokines, IL-1β, IL-1ra, IL-6, IL-12, IL-17, IFN-γ, and MIP-1α displayed an impact above average for the contribution of correlation (Figure 14C).

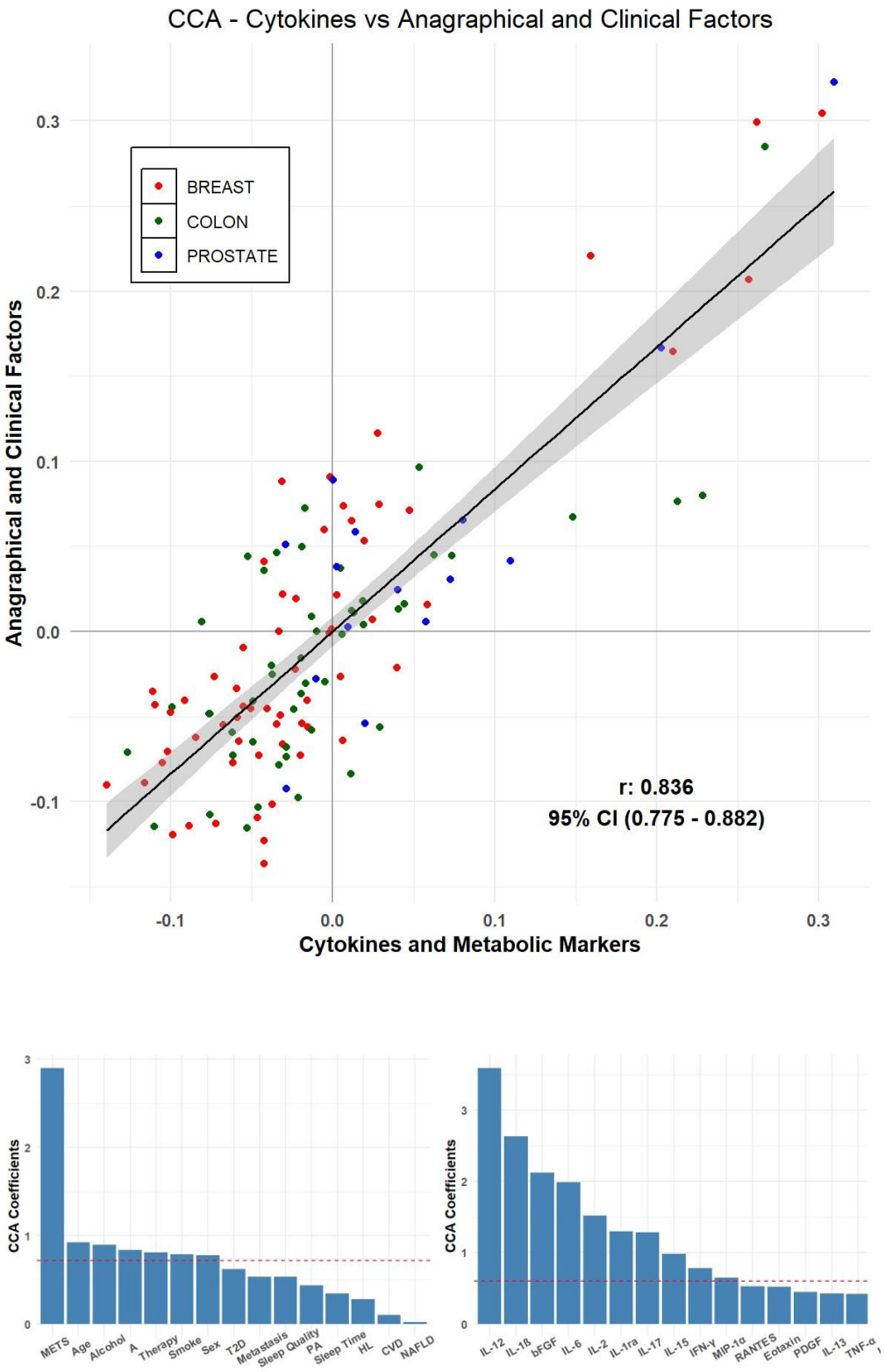

Figure 14 - Canonical Correlation Analysis of screened molecules and Risk factors. (A) Two away CCA setting; each subject is described by two canonical variates per mode, represented on a scatter plot. The two variate groups are maximally correlated, and their linear regression slope corresponds to the canonical correlation coefficient. (B) Risk factors' and (C) molecules' standardized weights are represented as absolute values; dotted red lines in (A) and (B) deploy the mean coefficient value.

Molecular Markers and Dietary Habits in the neoplastic population

Dietary information was collected with a weekly food-frequency questionnaire. Data relative to the weekly total consumption of each food item were collected. The population is characterized for a high consumption of Cereals (53.03%), Vegetables (53.03%), Fruits (55.3%), Cakes (68.18%), Pizza (48.48%), and Beverages (59.85%), with a and for a more moderate intake of Seafoods (71.21%), Meats (69.7%), Poultry (71.21%), and Processed Meats (57.58%) (Figure 15).

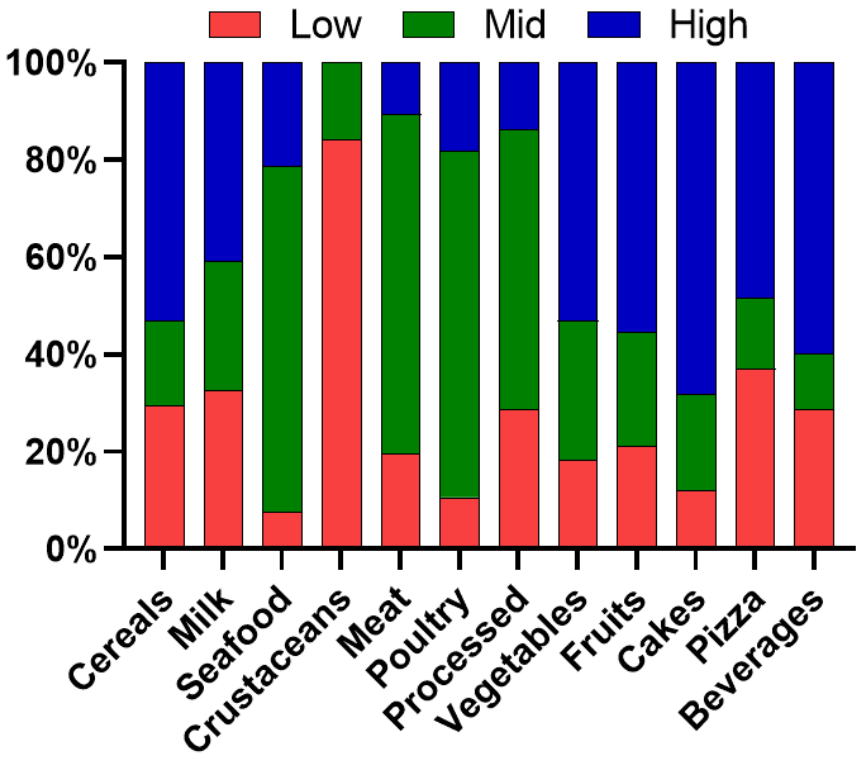

Figure 15 – Dietary habits of the enrolled volunteers. Histogram depicting weekly food habits for the Tumor population. Data are reported as relative percentage of the total population for every single group. Color code depicts the intake for single food groups (Red: low intake or no intake for Crustaceans; Green: Average intake or Intake for Crustaceans; Blue: high intake).

Food groups and inflammatory cytokines displayed a correlation coefficient of 0.821 (95% CI: 0.755 – 0.871, Figure 16A). The standardized weights for the single food items indicated a stronger relevance of Pizza, poultry, beverages, and Meats (Figure 16B). Among the screened molecules, the inflammation related pattern involving IL-8, IL-1ra, IL-6, IL-1β, and MIP-1β, as well as IL-12 and Visfatin, denoted a strong impact on the correlation among the two groups of variables (Figure 16C).

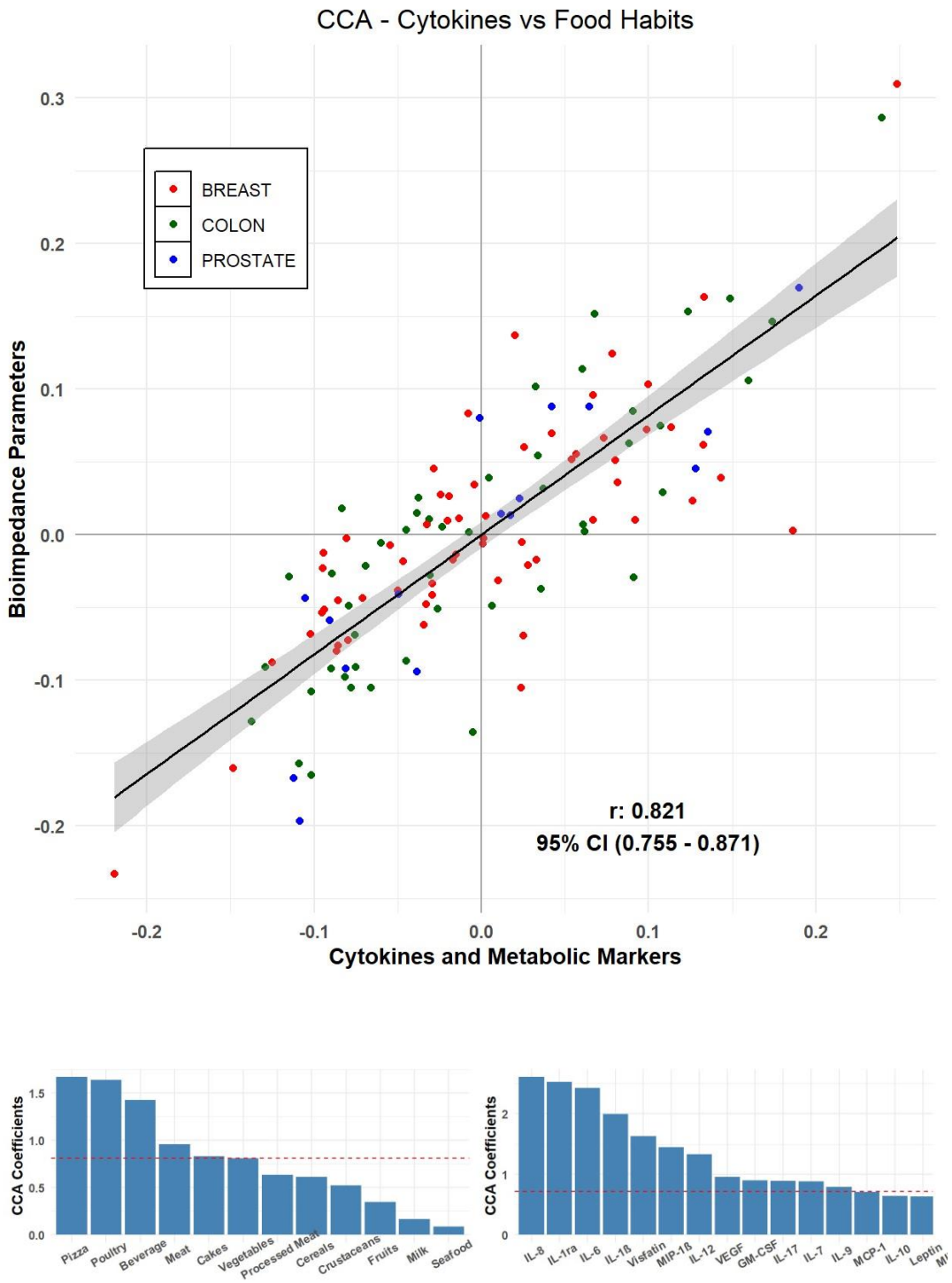

Figure 16 – Canonical Correlation Analysis of screened molecules and Risk factors. (A) Two away CCA setting; each subject is described by two canonical variates per mode, represented on a scatter plot. The two variate groups are maximally correlated, and their linear regression slope corresponds to the canonical correlation coefficient. (B) Risk factors' and (C) molecules' standardized weights are represented as absolute values; dotted red lines in (A) and (B) deploy the mean coefficient value.

Correlation and Inference Analysis for Screened Molecules

Next, a correlation analysis was performed for cytokines, chemokines, growth factors and metabolic markers. As reported in Figure 17, the 4-molecule cluster based on IL-1β, IL-6, IL-8, and TNF-α found in the Healthy population, was also displayed in the Tumor group, along with other inflammatory markers such as MIP-1α and MIP-1β, IL-4, and GM-CSF (Figure 17).

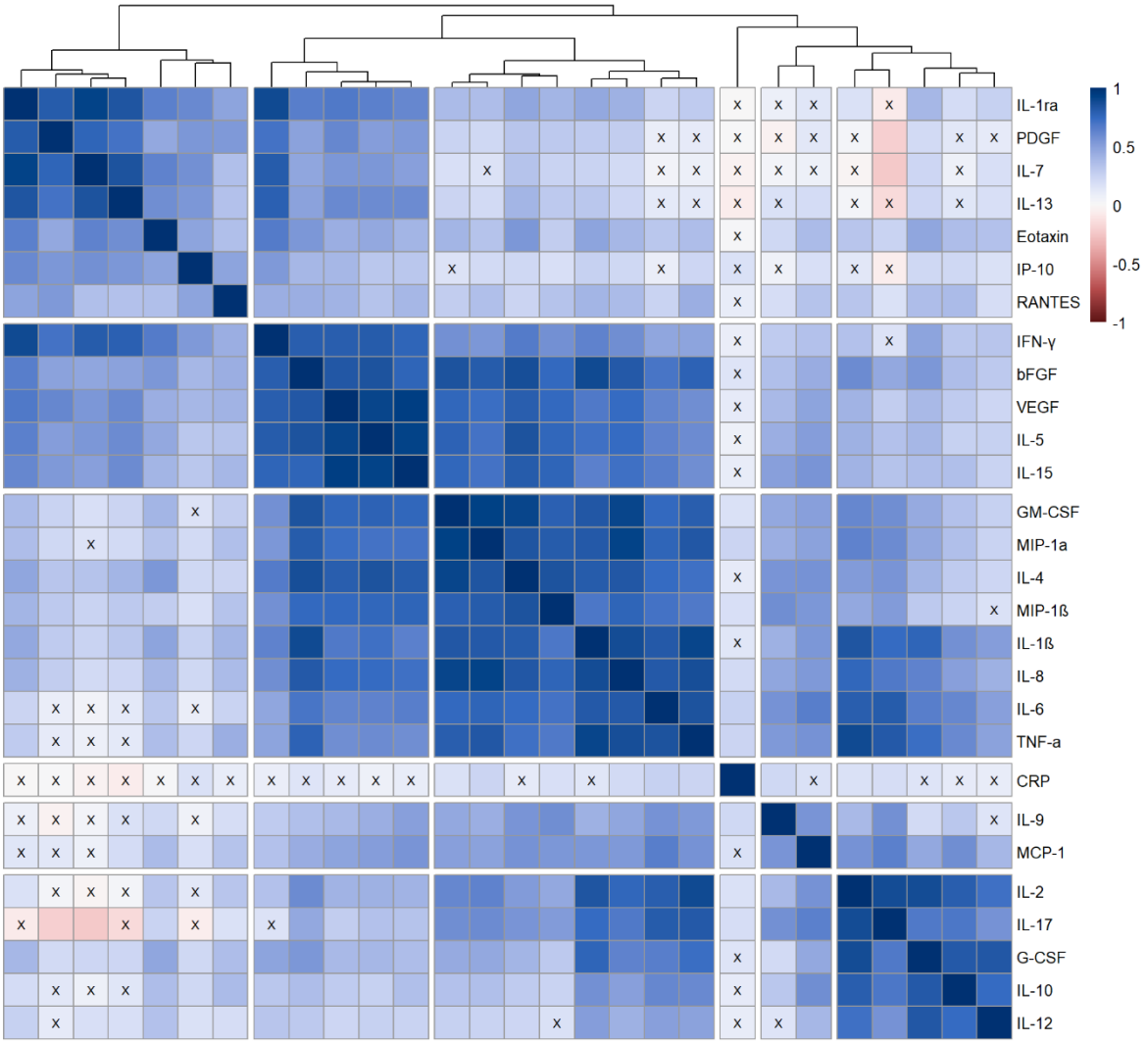

Figure 17 - Cytokine correlation matrix and hierarchical clustering. Spearman's rho coefficients for cytokine's scaled measurements are visualized by both tile-color intensity (according to the legend on the right) and by the r-values inside the tiles. Coefficients closer to 1 show a positive correlation; coefficients closer to -1 show a negative correlation; coefficients cluser to 0 denote the absence of a correlation among the considered variables. all values not labeled with a black X are statistically significant (p-value <0.05). Dendrograms show the hierarchical clustering.

The correlation analysis was then followed by a severe inference analysis for all the screened markers. As summarized in Figure 18, there were 36 significant interactions among screened markers and anagraphic parameters, 53 with Clinical parameters, and 55 with Dietary Habits.

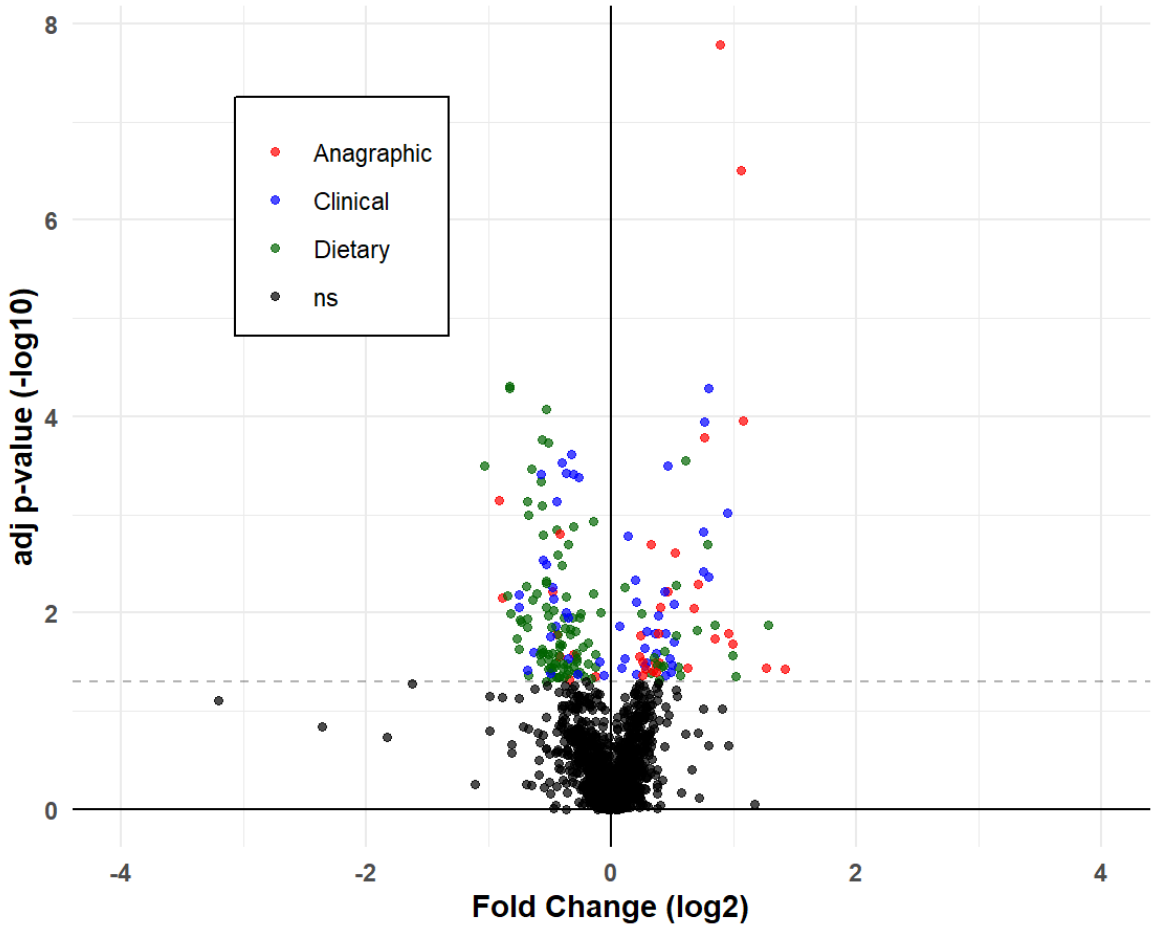

Figure 18 – Inference Analysis for screened cytokines and LGCI lifestyle factors. Volcano Plot summarizing univariate analyses between screened molecular markers and anagraphic, clinical, and dietary parameters. Each dot represents a single analysis, here represented as fold change (log2) and relative adjusted p-value (-log10). The dashed line represents the y-axis threshold for significance (p: 0.05). Black dots represent non-significant interactions, while colored dots represent significant associations between a marker and a lifestyle parameter (Red: Anagraphic, Blue: Clinical, Green: Dietary).

Among all the aforementioned univariate significant associations, IL-1β resulted significantly higher in males (p: 0.009), in patients with METS (p: 0.033), and with higher intake of Processed Meats (p: 0.022). It resulted downregulated in patients undergoing chemotherapy (p: 0.013), and in those with higher Vegetable intake

(0.018, Figure 19A). IL-6 resulted higher in patients performing regular Physical Activity (p: 0.039), and in those that consumed Crustaceans (p: 0.036) and Processed Meats (p: 0.024, Figure 19B). IL-8 levels were higher in Males (p: 0.037), Smokers (p: 0.037), and patients performing regular Physical Activity (p: 0.032, Figure 19C). Finally, TNF-α was strongly associated with Smoke (p: 0.043), and elevated Processed Meats intake (p: 0.038, Figure 19D).

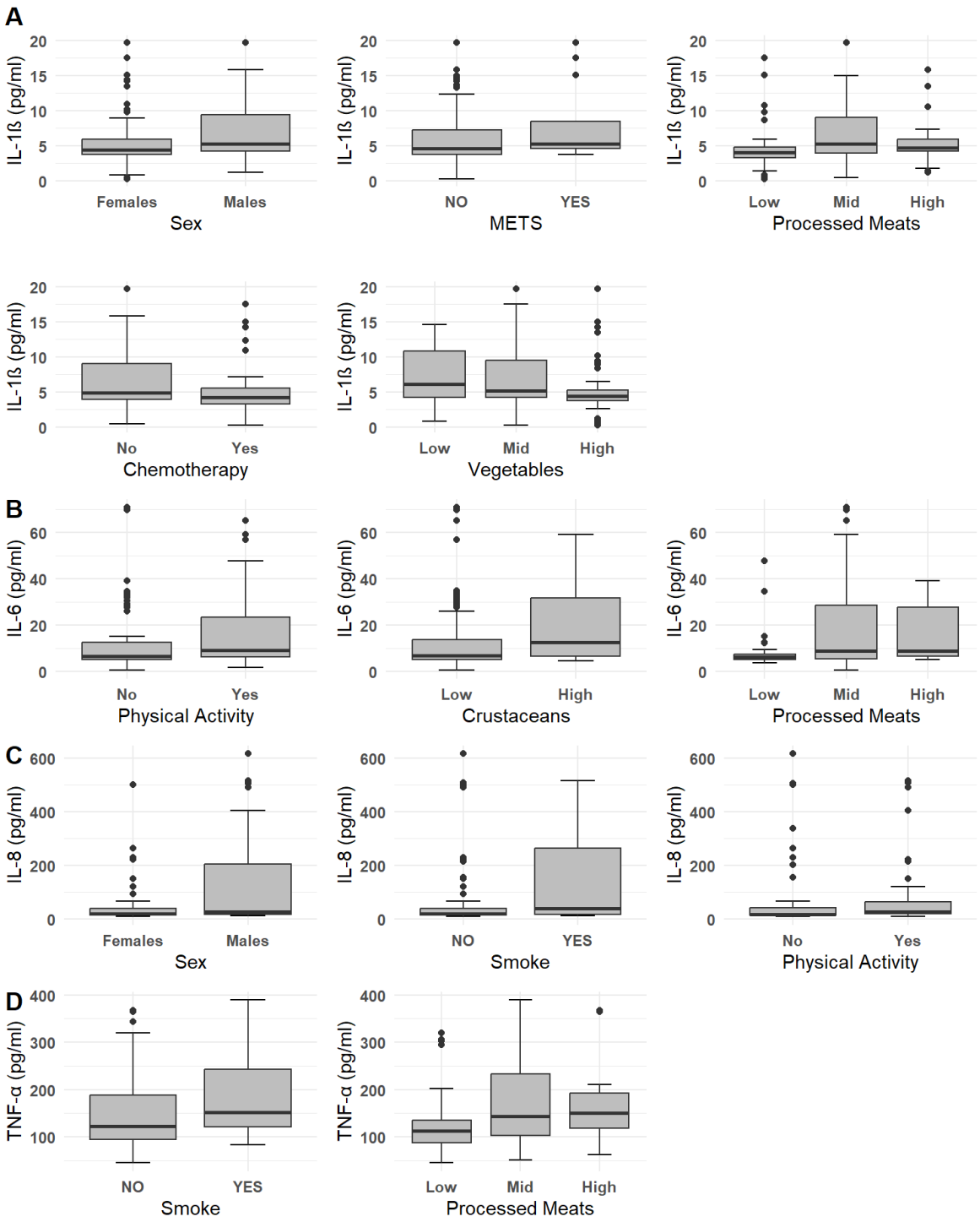

Figure 19 – Lifestyle, clinical and dietary factors related cytokines in tumor population. Box Plots denote cytokine concentrations in subjects with either no exposure or exposure to a specific lifestyle factor, or with a Low (<25th percentile), Mid (25th to 75th percentile) or High (>75th percentile) intake for a specific food group (Low or High for crustaceans). Cytokine concentrations are expressed as pg/ml. Figure reports only statistically significant interactions.

Notably, among the cytokine cluster, GM-CSF resulted positively associated with BMI (p: 0.006), and METS (p< 0.001), while it was reduced with Chemotherapy (p< 0.001), and MIP-1α was associated with Smoke (p: 0.016).

Regarding the screened metabolic markers, C-Peptide (p: 0.009) and Leptin (p< 0.001) displayed a significant association with BMI, while Adiponectin (p: 0.005) was negatively correlated. PAI-1 (p: 0.029) resulted higher in patients with NAFLD, which displayed lower levels of Ghrelin (p: 0.003). Finally, Patients with cardiovascular diseases displayed higher levels of Glucagon (p: 0.026).

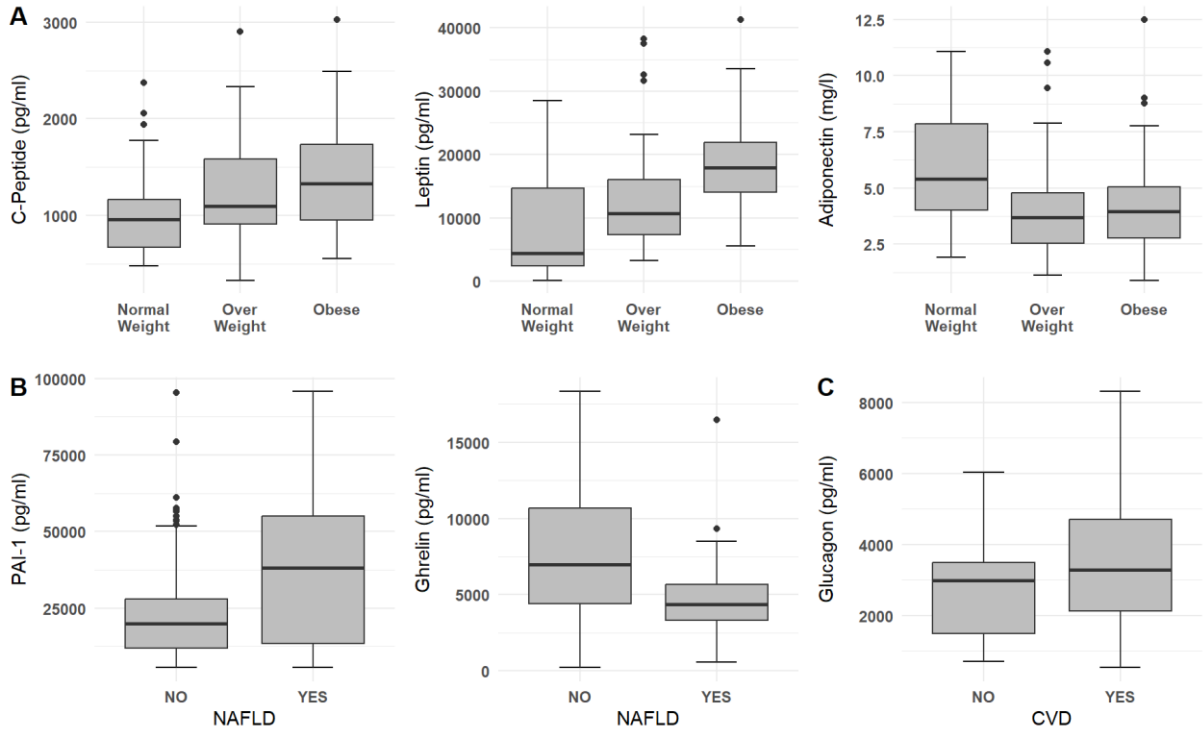

Figure 20 - Lifestyle, clinical and dietary factors related metabolic marker in oncological population. Box Plots denote cytokine concentrations in subjects with through (A) BMI levels, (B) NAFLD, and (C) cardiovascular diseases exposure. Marker concentrations are expressed as pg/ml. Figure reports only statistically significant interactions.

All univariate associations of different factors with the same molecular marker were then put in a multivariable linear regression model to assess their adjusted significance. According to the results shown in Table 6, IL-1β retained its significant association with Sex (adj-p: 0.021) and Vegetables intake (adj-p: 0.029), while IL-6 preserved a significant increase with Processed Meats intake (adj-p: 0.015), IL-8 levels remained significantly higher in Male (adj-p: 0.007) and smoker patients (adj-p: 0.015), and, finally, TNF-α was significantly associated with an higher intake of Processed Meats (adj-p: 0.048). Notably, GM-CSF associations with BMI, METS and Chemotherapy were all significant after multivariable adjustment (adj-p: 0.002, 0.011, and 0.01, respectively).

Table 6: Univariate and Multivariate linear regression analyses between risk factors and food groups for IL-1β, IL-6, IL-8, TNF-α, CRP, and GM-CSF. For each marker, both univariate and multivariate regression model outputs are reported as Estimate (95% CI), p-value and adjusted p-value.

Marker	Univariate Analysis		Multivariate Analysis	
	Estimate (95% CI)	**p-value**	**Estimate (95% CI)**	**Adj p-value**
IL-1β	**Sex**		**Sex**	
	1.75 (0.19 – 3.31)	**0.009**	**1.79 (0.27 – 3.31)**	**0.045**
	METS		**METS**	
	2.43 (0.001 – 4.86)	**0.033**	2.15 (-0.26 – 4.56)	0.08
	Chemotherapy		**Chemotherapy**	
	-1.85 (-3.42 – -0.28)	**0.013**	-1.56 (-3.15 – 0.029)	0.054
	Processed Meats		**Processed Meats**	
	2.12 (0.389 – 3.87)	**0.022**	0.4 (-0.61 – 1.4)	0.434
	Vegetables		**Vegetables**	
	-0.95 (-1.79 - -0.1)	**0.018**	**-0.92 (-1.75 – -0.09)**	**0.029**
IL-6	**Physical Activity**		**Physical Activity**	
	1.63 (3.05e-05 – 3.93)	**0.039**	2.81 (-2.91 – 8.52)	0.332
	Crustaceans		**Crustaceans**	
	2.84 (0.2 – 9.31)	**0.036**	5.28 (-2.48 – 13.05)	0.18
	Processed Meats		**Processed Meats**	
	8.57 (2.12 – 15.03)	**0.024**	**8.08 (1.62 – 14.53)**	**0.015**
IL-8	**Sex**		**Sex**	
	83.35 (29.35– 137.34)	**0.037**	**75.42 (20.97 – 129.94)**	**0.007**
	Smoke		**Smoke**	
	87.51 (22.1 – 152.92)	**0.037**	**80.02 (16.16 – 143.88)**	**0.015**
	Physical Activity		**Physical Activity**	
	5.99 (0.56 – 13.58)	**0.032**	8.37 (-44.73 – 61.47)	0.755
TNF-α	**Smoke**		**Smoke**	
	29.74 (0.61 – 59.74)	**0.043**	19.42 (-16.42 – 55.26)	0.286
	Processed Meats		**Processed Meats**	
	38.77 (6.03 – 71.51)	**0.038**	34.14 (0.31 – 67.96)	**0.048**
GM-CSF	**BMI**		**BMI**	
	0.134 (0.06 – 0.21)	**0.006**	**0.11 (0.04 – 0.18)**	**0.002**
	METS		**METS**	
	2.64 (1.39 – 3.90)	**<0.001**	**1.69 (0.4 – 2.97)**	**0.011**
	Chemotherapy		**Chemotherapy**	
	-1.33 (-2.26 – -0.42)	**<0.001**	**-1.15 (-2.01 - -0.27)**	**0.01**

Discussion and Conclusions

Cytokines play pivotal roles in inflammation, viral, autoimmune diseases, and in Low-Grade Chronic Inflammation (LGCI) (Rea et al. 2018; Monastero and Pentyala 2017; C. Liu et al. 2021; D'Esposito et al. 2021; Cabaro et al. 2021; Siano et al. 2021). However, the utilization of cytokines as diagnostic or prognostic tools encounters challenges in discerning "normal" from "abnormal" levels. Studies exploring cytokine concentrations in well selected populations are scarce, and often limited to a narrow number of examined variables.

In this investigation, we conducted a comprehensive analysis of cytokine profiles in a cohort comprising 150 healthy blood donors, and 132 patients with tumor remission. We explored how anagraphic, lifestyle and clinical factors, including age, BMI, smoke, physical activity, and dietary habits, might influence specific cytokines and modulate their concentrations. Our findings highlight the distinctive cytokine patterns, unique to each individual, influenced to different degrees by physiological and behavioral factors. Notably, in healthy patients, no discernible differences were observed between male and female participants, even considering BMI levels. These results align with previous other studies conducted across diverse populations (Forsey et al. 2003; Biancotto et al. 2013), suggesting that additional factors, such as hormonal influences, may counterbalance the BMI-elicited control. Furthermore, the study revealed lower concentrations of IL-1β and IL-8 in females with tumor remission compared to males, consistently with the known pro-initiation, maintenance, and recurrence roles of these molecules in breast cancer, the predominant cancer type in this cohort (J. K. Singh et al. 2013; Tulotta et al. 2021).

BMI emerges as the main determinant in the correlation between LGCI-realated risk factors and cytokines among healthy patients. Indeed, numerous studies have extensively characterized the cytokine profiles in subjects with obesity and severe obesity, revealing notable differences compared to those with normal weight (D'Esposito et al. 2021; Schmidt et al. 2015; van der Zalm et al. 2020). Despite

excluding subjects with obesity from our analysis, BMI continued to exhibit a significant association with CRP levels. Intriguingly, our observations revealed additional correlations between CRP levels and the consumption of specific foods, such as eggs, red meat, shelled fruits, and greens. However, when corrected for BMI, these associations dissipated, underscoring the profound impact of body mass on the observed trends. In the tumor patient's cohort, the BMI-CRP association persisted, accompanied by alterations in other well-established metabolic markers, including an increase in C-Peptide and a reduction in Adiponectin. Notably, higher BMI in this population was also linked to elevated levels of GM-CSF. While this factor plays a crucial role in modulating immune cells functions under pathological conditions, its dysregulation in various cancer microenvironments has been implicated as a key factor in cancer progression and aggressiveness (Kumar et al. 2022).

Tobacco use emerges as the second most influential factor shaping cytokine profiling, as evidenced by elevated levels of IL-1β and CCL5/RANTES among smokers. These findings align with *in vivo* studies demonstrating that cigarette smoke induces murine emphysema, lung inflammation, and DNA injury/apoptosis through IL-1β and CCL5-CCR5 signaling pathways (Ma et al. 2005; Churg et al. 2009). Notably, CCL5 has regenerative functions, primarily mediated by CCR1, suggesting the potential activation of tissue repair mechanisms (Kauts et al. 2013). This nuanced scenario is mirrored in cancer patients, where tobacco consumption correlates with increased levels of inflammatory cytokines, such as IL-8, MCP-1 and MIP-1α. Additionally, smokers show higher levels of bFGF, recognized for its pivotal role in cancer proliferation, fibrosis, angiogenesis, and metastasis, as extensively described (Ardizzone et al. 2023). Moreover, G-CSF, highly produced in various cancers, including breast (Fukui et al. 2018) and colon (Yang et al. 2005), demonstrates a noteworthy association with smoking in cancer patients.

Age accounts for 8.4% of the correlation observed between LGCI risk factors and inflammatory cytokines. The concept of "Inflammaging", a dysregulation of the

cytokine network well elucidated at cellular level, involves cellular senescence linked to a well-defined secretory phenotype (SASP), characterized by specific cytokines, including IL-6, IL-8, and VEGF (Laberge et al. 2015). While serum-levels data can be conflicting due to variations in the age groups considered, and health status in some studies, have enrolled different aged people, without considering health status, other studies have been performed on very old people, such as non-agenarians and centenarians (Minciullo et al. 2016). Our investigation revealed that 20-60 years old healthy subjects display an age-associated increase in IL-1β, Eotaxin, MCP-1, and MIP-1α. Interestingly, Eotaxin levels also show an increase in subjects aged 21-86 and in 7-17 when compared with 1-6 years old children (Biancotto et al. 2013).

We also examined the impact of physical activity on cytokine levels. Recent literature suggests that exercise induces both pro- and anti-inflammatory cytokines (IL-6, IL-8, IL-10, TNF-α), with levels returning to baseline within 5 to 24 hours post-exercise (Cerqueira et al. 2019). The dual response is also influenced by factors such as the type of exercise (aerobic or anaerobic) and whether it involves short-term or long-term physical activity (Scheffer and Latini 2020). Our findings indicate that individuals with higher Metabolic EquivalenT (MET) consumption in the 7 days preceding the questionnaire had elevated levels of MIP-1α, a protein primarily produced by macrophages and implicated in leucocyte recruitment (Maurer and von Stebut 2004). This increase may partially account for the mobilization of various leukocyte populations observed in studies investigating the effect of exercise on blood cells and molecules (Cerqueira et al. 2019). In tumor patients engaging physical activity, circulating IL-6 and IL-8 levels were higher. Previous research in B16F10 melanoma-induced murine models demonstrated that exercise-induced IL-6 mediates the inhibition of both tumor growth and NK cells infiltration, suggesting a role for IL-6 in enhancing intratumoral infiltration of cytotoxic immune cells (Ellingsgaard, Hojman, and Pedersen 2019).

Another interesting finding is the asscociation of metabolic markers with clinical comorbidities in the tumor population. More specifically, patients Non-Alcoholic Fatty Liver Disease displayed higher levels of PAI-1, an inflammatory marker that has been strongly associated with biopsy proven NAFLD severity, and with NASH, although for the latter there are some conflicting reports (Targher et al. 2007; Alsharoh et al. 2022). NAFLD was also associated with higher Ghrelin levels; while the role of ghrelin in hepatoprotective features, such as induction of FFA β-oxidation to prevent lipotoxicity, inhibition of JNK and NF-κB pathways, the ghrelin isoform alterations caused by chronical conditions (i.e. obesity, insulin resistance, T2D, cancer) could play a role in the initiation and advancement of NAFLD (Tuero et al. 2023). Higher levels of Glucagon have been detected in patients with cardiovascular diseases. Aside from its well-known action in regulating blood glucose levels, it is also known that Glucagon elicits an inotropic effect, mainly in healthy conditions, but this feature is severely abrupted in severe heart failure conditions. High circulating Glucagone levels may be the result of a homeostatic counteraction from the body against these chronic conditions (N. K. Singh et al. 2023). It is noteworthy that genetically predicted Glucagon levels seems to have an adverse impact on ischemic heart disease (Ng and Schooling 2020).

Healthy diets are widely recognized for their protective effects against LGCI. Various foods, including almonds, yogurt, nuts, dark chocolate, and extra virgin olive oil, as well as functional foods rich in omega-3 fatty acids, polyphenols, and fibers, have been attributed anti-inflammatory properties. Whole diets, such as Mediterranean diet has also demonstrated protective effects against LGCI (Luvián-Morales et al. 2022; Bonaccio et al. 2017; 2019; Bozzetto et al. 2019; 2020). Several dietary indices and scores have been developed to quantify dietary quality. Examples include the Dietary Inflammatory Index (DII®), Derived from literature and based on 45 food parameters (Shivappa et al. 2014); the Healthy Eating Index-2015 (HEI-2015), measuring dietary quality according to the Dietary Guidelines for Americans (DGA) (Millar et al. 2021); the Polyphenol Antioxidant Content (PAC)

score, assessing the total polyphenol content in the diet (Pounis et al. 2016); and the Empirical Dietary Inflammatory Pattern (EDIP), a score based on the reduced rank regression approach, developed in the United States and validated internationally (Norde et al. 2020). Additionally, specific dietary patterns, such as the Mediterranean diet and the Dietary Approaches to Stop Hypertension (DASH), have inspired the creation of dedicated indices (Alkerwi et al. 2015; Martínez-González et al. 2012).

Our primary finding underscores the association between the 7-day consumption of specific food items and elevated levels of particular cytokines subjects hailing from Southern Italy, where dietary habits align with the Mediterranean diet. The predominant components of their dietary intake included grains, greens, fresh fruit, milk and dairies, and white meat. Remarkably, in this population, we observed no discernible associations between specific food consumption and CRP levels, even when adjusted for BMI. At present, there are few studies and some discrepancies about the intake of grains. Some studies reported an anti-inflammatory effect of whole grains for their nutrients and fibers; other studies, performed with healthy subjects, displayed no effects (de Punder and Pruimboom 2013; Milesi, Rangan, and Grafenauer 2022). Moreover, the daily consumption of wheat products and cereals has been reported to contribute to chronic inflammation and autoimmune diseases (de Punder and Pruimboom 2013). Our data revealed that volunteers with grain consumption beyond the 75th percentile exhibited higher levels of TNF-α and IFN-γ, compared with those in the IQR range, representing 50% of the population. TNF-α increase may be potentially linked with carbohydrate-induced hyperglycemia, known to increase circulating cytokine concentrations by an oxidative mechanism (Esposito et al. 2002). Interestingly, IFN-γ was increased also in subjects with grain consumption under the 25th percentile. This low inflammatory profile may be attributable to the frequent consumption of cereals and whole-wheat pasta and bread. Red meat and shelled fruit, despite constituting a minor proportion of the overall dietary intake, exerted a profound influence on the correlation between

foods and inflammatory cytokines. Individuals with red meat consumption over the 75th percentile manifested the most pronounced inflammatory pattern, characterized by the increase in IL-6 and IL-8 levels. These data align with previous reports linking higher meat intake, as seen in Western diets, to inflammation and adverse health outcomes (Bonaccio et al. 2017). A strong association among the inflammatory molecules IL-6 and TNF-α with the elevated consumption of processed meats was also detected. While the association of processed meat consumption with IL-6 is well-documented, studies exploring these dietary factors and TNF-α and its soluble receptors are limited and often conflicting. Nonetheless, it is recognized that TNF-α is frequently positively correlated with other proinflammatory cytokines and portends a pathogenic prognosis in various chronic conditions (Schwedhelm et al. 2017).

The consumption of fruits and vegetables, known for their abundance of flavonoids and antioxidants, has been linked to a reduced risk of LGCI-related diseases and lower levels of certain inflammatory markers (Hosseini et al. 2018; Deledda et al. 2021). Notably, individuals with high consumption of fresh fruits surprisingly exhibited elevated circulating levels of IL-8, IFN-γ, and IP-10, compared to those with a moderate or low intake. While some studies suggest an anti-inflammatory role for polyphenols, primarily through the modulation of NF-κB and MAPK pathways at various levels (Deledda et al. 2021), clinical trials present contradictory findings. *In vitro* studies have even reported an induction of IL-8 expression by resveratrol, a polyphenol contained in many fruits, thus posing new questions (Deledda et al. 2021; Thiel et al. 2018). Moreover, the release of IL-8 and IL-10 may be induced by IFN-γ, suggesting a potential immuno-stimulatory effect of the fruit. Interestingly, the intake of shelled fruits was associated with a significant decrease of the levels of the highly pro-inflammatory molecules IL-1β and IL-6. These findings align with previous studies, demonstrating the anti-inflammatory and antioxidant properties of almonds and nuts (J.-F. Liu et al. 2013; Yu et al. 2016).

Thus, in our populations, specific cytokines are associated with age, BMI, smoking, physical activity, and certain dietary habits. These associations persisted even after adjusting for BMI. However, CRP, the classical marker of acute inflammation, was only linked to BMI and did not show correlations with the other cytokines. Thus, while CRP remains a valuable indicator of inflammation, the identification of other molecules as markers may be useful, especially in discerning the relative impact of specific factors in the progression of LGCI, becomes crucial. This holds particular significance when examining the influence of dietary habits.

In this investigation, we pinpointed IL-1β and IL-6 as the cytokines exhibiting the most robust correlation with the cytokinome and LGCI-risk factors both in healthy and in oncological populations. These molecules, recognized as inflammatory markers across various conditions (Cabaro et al. 2021; Parisi et al. 2020), and, together with TNF-α and IL-8, form a cohesive cluster that holds promise as a potential cytokine biomarker for LGCI. Notably, we confirmed the persistence of this cluster in the tumor population, underscoring its robustness.

In conclusion, this study furnishes compelling evidence for the assessment of multiple cytokines in meticulously characterized healthy and tumor populations. It sheds light on how lifestyle factors, aging, and clinical comorbidities exert distinct effects on specific cytokines.

Future research endeavors may consider broadening dietary analysis to incorporate the percentage of macronutrients, particularly the type of fats (i.e. omega 3, omega 6), which have demonstrated a significant influence on circulating cytokine concentrations (Kang and Weylandt 2008; Emanuel et al. 2022). Additionally, there is an opportunity to delve deeper into the impact of the specific tumor conditions, comparing nuances among different neoplastic scenarios. Furthermore, intervention studies could offer more comprehensive insights into the contributions of the examined parameters to cytokine levels. The outcomes of such investigations might pave the way for the development of new technological tools and digital

platforms aimed at early detection of mediators and risk factors associated with LGCI. These tools could, in turn, serve to promote healthy lifestyle behaviors, throughout various stages of chronic condition prevention.

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