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ORIGINAL ARTICLE

Toxicology of ageing in long-term dietary study on Sprague-Dawley rats

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ABSTRACT

The research in a newly developing field, toxicology of ageing, is highly relevant nowadays and very well interdisciplinary challenged. The improvement of eating habits might serve as an anti-ageing prevention and treatment strategy for metabolic disorders. The goal of our study was to contribute to the elucidation of the impact of ageing on immunological alterations along with prenatally conditioned metabolic syndrome in Sprague-Dawley (SD) female offspring rats. We have investigated the influence of guided two-generation nutritional intervention, specifically the effect of a high-fat diet (H) on fundamental metabolic parameters and cytokines. To mimic the dietary preferences of a western-style diet rich in high fat, we first fed the parental generation of SD rats either a control (C) standard diet or a high-fat diet (H, containing 1% cholesterol and 7.5% lard) three months prior to mating. Then, the type of diet either continued within the offspring´s generation (CC, HH) or changed (CH, HC). The non-invasive blood pressure measurement was carried out by tail-cuff plethysmography. The plasmatic levels of total cholesterol (TC), triacylglycerides (TAG), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), adipose triglyceride lipase (ATGL), and C-reactive protein (CRP) were assessed by ELISA kits. The serum levels of interleukins IL-1β, IL-4, IL-6, IL-10, IL-12, and tumor necrosis factor-α (TNF-α) were evaluated using magnetic bead–based immunoassays on Bio-Plex 200 systems (Bio-Rad, U.S.). Our data indicated that the inflammaging, which is age-related chronic low-grade inflammation, was present in diet-related disturbances, especially within the HH groups of SD female offspring rats. Therefore, epigenetic programming through parental diet appears to play a critical role in worsening inflammaging.

KEY WORDS: ageing; inflammation; high fat diet; Sprague-Dawley rats; interleukins

Introduction

The research field of ageing has been rapidly advancing in recent decades, and it has provided insight into the complexity of this phenomenon (Jolly, 2005; Rackova *et al.*, 2021). The biological age of an individual is relevantly influenced by the metabolic state of the body, which is, in turn, linked to a lifestyle and nutritional habits (Mattson *et al.*, 2017). Dietary interventions have been shown to provide health benefits, including the life-prolonging effect, in a range of experimental animals (Xu *et al.*, 2015). The results of recent human study show that, *e.g.*, mild caloric restrictions can have beneficial effects in humans (Varady *et al.*, 2022). Since nutrition is an essential and readily modifiable risk factor for disease prevention, many studies have consistently proven a steady relationship between diet and health, including in older adults (Stefler *et al.*, 2019; Salazar *et al.*, 2020; Malesza *et al.*, 2021). The fact that a maternal high-fat diet during pregnancy can cause the activation of proinflammatory cytokines is nowadays already a well-accepted certainty (Harmancıoğlu & Kabaran, 2023). Possible mechanisms that physiologically explain the link between maternal obesity and prenatal programming include epigenetic modifications such as DNA methylation, histone modification signatures, chromatin conformation, and microRNAs (non-coding RNAs) within some organs such as adipose tissue, liver, pancreas, and brain (Kitsiou-Tzeli & Tzetis, 2017). These epigenetic alterations may contribute to the development of obesity and other related metabolic disorders in a developing fetus as well as in childhood and adulthood (Montalvo-Martínez *et al.*, 2018).

Ageing is characterized by the accumulation of damage to macromolecules and cell architecture that triggers a proinflammatory state in blood and solid tissues, termed inflammageing (Walker *et al.*, 2022). Many specific interleukins IL-1β, IL-4, IL-6, IL-10, IL-12, and tumor necrosis factor α (TNF-α) play essential roles in inflammaging (Pansarasa *et al.*, 2022), *e.g.*, particularly IL-6 was signposted as a cytokine for gerontologists

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already decades ago (Ershler, 1993). Considerable research in both animal models and humans has examined the causes and consequences of inflammaging (Ferrucci and Fabbri, 2018). Although increased levels of inflammatory mediators (mostly IL-1, IL-6, TNF-α, and their receptors) are detected in all elderly individuals, higher levels of these biomarkers are associated with increased risk for many chronic conditions, including dementia, disability, and physical frailty (Zhao *et al.*, 2023).

Morris *et al.* (2019), in their review, very well summarized the data on genetic and epigenetic contributions to human ageing and longevity. While environmental and lifestyle factors are important at younger ages, the contribution of genetics appears to be more critical in reaching extreme old age. Thus, there is a number of factors that contribute to the complex process of ageing, which finally define whether someone will or not develop age-associated chronic diseases in late life. These determinants comprise genetic susceptibility as well as various behavioral, environmental, and dietary factors, all of which have been shown to influence specific pathways regulating the ageing process and the extension of life, making longevity a multidimensional phenomenon (Dominguez *et al.*, 2022).

The goal of our study was to contribute to the elucidation of the impact of ageing on immunological alterations, along with the prenatally conditioned pathophysiology of metabolic syndrome in Sprague-Dawley (SD) female offspring rats aged 15 months, corresponding to human middle age (Mena-Montes *et al.*, 2021). We have investigated the influence of guided two-generation nutritional intervention, specifically the effect of a high-fat diet (H) on fundamental metabolic parameters as well as the cytokines in offspring rats.

Material and methods

Animals and design of a long-term experiment

All experimental procedures involving animals were approved by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (permit number 3635/14-221). They conformed to Directive 2010/63/EU on the protection of animals used for scientific purposes.

The parental generation of SD rats, both males and females, aged 15 weeks, were from a certified provider (Velaz, CR). To mimic the nutritional preferences of a western-style diet rich in high fat, we first fed a parental generation of SD rats either a control (C) standard diet or a high-fat diet (H, 1% cholesterol and 7.5% lard) for three months before mating. Then, the type of diet either continued within the offspring´s generation or changed. Thus, we acquired the four combination experimental groups, marked by two letters, where the first letter indicates the parental diet type and the second letter indicates the offspring´s diet type: CC, CH, HC, or HH.

The standard rodent diet was produced by the certified producer of pellets at the Department of Toxicology and Breeding of Laboratory Animals, Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences, Dobra Voda, Slovakia, which is registered under the number α SK 100089, code 6147. Composition of the standard diet: wheat, processed animal protein, oat, barley-corn, extruded lucerne, soybean extracted grit, wheat bran, wheat germs, mineral mix, vegetable oil, natrium chloride. Added vitamins *per* 1 kg: E 672 vitamin A – 20,000 IU; E 671 vitamin D3 – 2,000 IU; vitamin E – 70 mg; added amino acids *per* 1 kg: DL-methionine – 1.2 g; L-lysine – 0.8 g. Analytical components: 19.10% nitrogen substances; 3.60% fiber; 5.10% oil and fat; 5.85% ash; 9.10% humidity. The rat's body weight was monitored once a week.

The rats had free access to water and food, were kept on a 12-hour/12-hour light/dark cycle, and were housed five animals per cage. Animals were organized in experimental groups (n=10 rats/group).

Blood pressure measurement

The blood pressure (BP) and heart rate (HR) of the experimental animals were measured using non-invasive tail-cuff plethysmography (PowerLab 4/30, AD Instruments, USA) at the beginning of the long-term experiment (3rd month of age) and at the end (15th month of age). Before the measurement itself, the rats were warmed up for 8–10 minutes by an infrared lamp to increase body temperature and dilate the vessels. A rat-friendly modification of blood pressure measurement, as described by Liptak *et al.* (2017), was employed to minimize the stress of the animals. Systolic BP and HR were calculated as the average of five consecutive measurements.

Determination of metabolic parameters by ELISA

The blood was collected from the *plexus chorioideus* of female SD rats at specific time points during the long-term experiment (3rd and 15th months of age in SD rats). We evaluated the plasmatic levels of metabolic parameters as follows: total cholesterol (TC), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), triacylglycerols (TAG), adipose triglyceride lipase (ATGL), and C-reactive protein (CRP), using a commercial colorimetric ELISA kit following the manufacturer's protocol. The absorbances of the resulting colored compound were measured spectrophotometrically according to the ELISA diagnostics kits manuals (Erba Lachema, CR) on LabSystems 352 Multiscan MS Microplate Reader (ThermoFisher Scientific, U.S.).

Determination of interleukins by multiplex immunoassay

The serum levels of specific interleukins, including IL-1β, IL-4, IL-6, IL-10, IL-12, and tumor necrosis factor α (TNF-α), were comprehensively assessed using magnetic bead–based immunoassays on Bio-Plex 200 systems (Bio-Rad, U.S.) at the 3rd and 15th months of the experiment. Analyses were performed in duplicate and analyzed using the Bio-Plex Manager Software version 6.2. Out-of-range

values OOR< were set to zero. Values that were out of the standard range but postulated from the standard curve were included.

Statistical evaluation

All data were statistically evaluated using Past 4.03 Software, originated from the IT department of the University of Oslo, Norway (Hammer *et al.*, 2001). To assess differences among all experimental groups, a Two-Way ANOVA (age × diet) was used, as well as post hoc ANOVA Tukey´s test for interactions (age × diet). Data were expressed as means ± SEM. The level of $p<0.05$ was considered a statistically significant difference. The particular symbols were used to mark significancies as follows: *15th month *vs* 3rd month (when comparing particular experimental groups, *e.g.*, CC *vs* CC; CH *vs* CH; HC *vs* HC; HH *vs* HH); # when comparing CH *vs* CC, respectively & HH *vs* HC at a particular time point of 15th month. If the level of a specific measured parameter was not detected, it means the value was below the detection limit of the analytical method, and this is indicated within the table using the shortcut ND. NS means a non-significant outcome from the used statistical analysis.

Results

In our long-term study on SD female offspring rats, the initial body weight of animals at the beginning of the experiment did not differ between individual groups: it ranged from 298±6 g to 312±10 g (Table 1). Rats of all groups consumed similar amounts of pellets, 25±3 g/rat/day. The weight of all rats increased with age. The body weight of animals at the end of the dietary experiment differed significantly between the 15th month and the 3rd month. Moreover, at the 15th month of age, there was also a statistically significant variance in body weight, particularly between the HH group and CC group: 531±36 g *vs* 429±24 g, ^^$p<0.01$, respectively, and as also shown by &$p<0.05$ for HH *vs* HC diet and/or ᵘ$p<0.05$ for HH *vs* CH diet. However, the most pronounced weight difference corresponded to only a 24% increase in body weight, which aligns with reports of an inverse correlation between levels of adipose ATGL protein (elevated in our H diet groups) and body mass index in humans (Yao-Borengasser *et al.*, 2011). Since ATGL performs the first step of TAG hydrolysis and thus it is rate-limiting, we refrained from measuring the secondary-step enzyme HSL. Similarly, the BP and HR levels of the experimental animals, measured at the beginning and at the end of the experiment, are listed in Table 1. In general, BP levels remained steady when compared between the 15th month and the 3rd month, except in the CH group, in which we registered a significant diminution: 111±3 mmHg *vs* 131±1 mmHg, **$p<0.01$. Concerning HR parameter, by comparing 15th month *vs* 3rd month time points, a significant enhancement was recorded in CH and CC groups, **$p<0.01$.

The basal biochemical parameters of lipid profile and CRP levels within experimental animals, measured at the 3rd and 15th months of their age, are listed in Figure 1. The H diet entirely affected the levels of TC, TAG, HDL-C, and ATGL in CH and HH groups, partially also LDL-C and CRP levels in the CH group, when comparing the 15th month vs the 3rd month. Furthermore, there was a proven H diet impact on the lipid profile of ageing SD female offspring generation of rats, when comparing CH *vs* CC, respectively HH *vs* HC groups, since the levels of TC, HDL-C, LDL-C, ATGL, and CRP were significantly increased.

The overall overview of various serum levels of particular interleukins IL-1β, IL-4, IL-6, IL-10, IL-12, and tumor necrosis factor α (TNF-α), evaluated within this study, is shown in Figure 2. Universally, in all parameters, there is an evident predominant trend in raising the levels of measured interleukins when comparing the 15th month vs the 3rd month time points. Regrettably, we encountered significant biological variability within particular experimental groups, which is reflected in the relatively large error bars of the SEM. This may be the reason why almost no significant changes were observed using the employed

Table 1. The physiological parameters of body weight (BW), blood pressure (BP) and heart rate (HR) measured within Sprague Dawley´s rats with different dietary regimens at their age of 3rd and 15th month.

		BW (g)	BP (mmHg)	HR (BPM)
3rd month	CC	311±8	127±1	282±26
	CH	298±6	131±1	294±22
	HC	311±14	130±4	372±9
	HH	311±10	137±4	379±19
15th month	CC	429±24 **	125±2	417±13 **
	CH	446±17 ***	111±3 **	409±10 **
	HC	446±27 ***	119±4	388±15
	HH	531±36 *** & ᵘ ^^	124±4.18	421±24
Two-way ANOVA	age	$p<0.001$	$p<0.001$	$p<0.001$
	diet	$p<0.05$	NS	NS
	age x diet	NS	NS	$p<0.05$

The experimental groups are marked by two letters while 1st letter means parental diet type, and 2nd letter is the indication of offspring´s diet type: CC, CH, HC, or HH, while C is the control diet and H means high-fat diet. NS – non significant. Statistical analysis was done by two-way ANOVA (age; diet) followed by a *post-hoc* Tukey´s test. **$p<0.01$, ***$p<0.001$ for 15th *vs* 3rd month in particular exp. group; &$p<0.05$ for HH *vs* HC diet in 15th month; ᵘ$p<0.05$ for HH *vs* CH diet in 15th month; ^^$p<0.01$ for HH *vs* CC diet in 15th month.

statistical method. Nevertheless, there are important consequences of both the effect of age (p<0.001) and the diet (p< 0.001), as well as their interaction (p<0.05), in IL-12 level, when comparing all the groups by ANOVA. The levels of IL-6 and TNF-α were significantly affected (increased) by diet (p<0.01) and age (p<0.05), respectively. Lastly, post hoc analysis revealed that the level of interleukin IL-6 was also significantly elevated (p<0.01) in HH vs HC experimental groups in the 15th month.

Discussion

Along with the extension of life expectancy and the rising percentage of older individuals in the general population, understanding why ageing results in progressively higher susceptibility to chronic morbidity, disability, and frailty has become a public health priority (Bektas *et al.*, 2018). Moreover, Ferrucci & Fabbri (2018) summarized the understanding of inflammaging as a risk

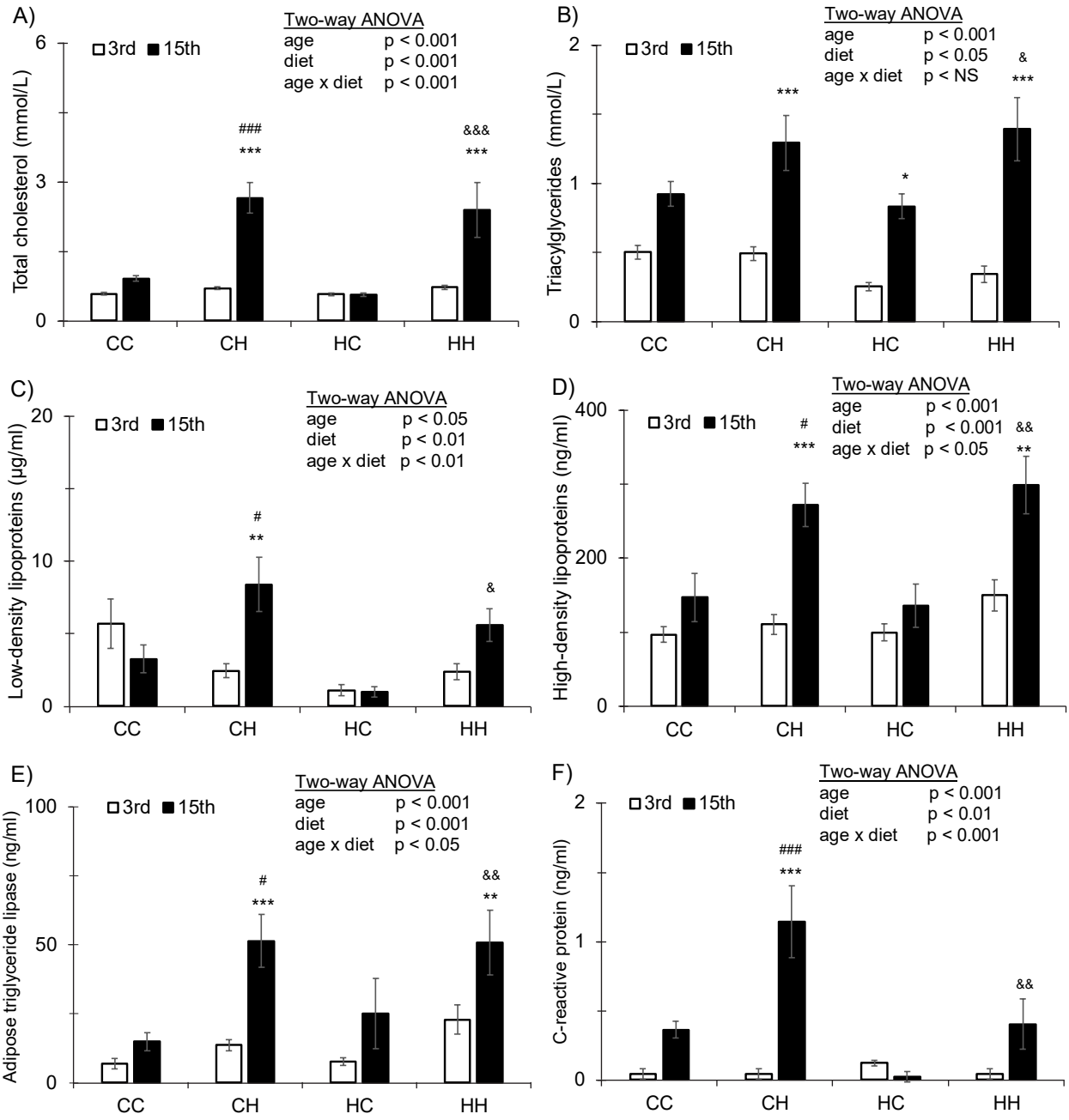

Figure 1. The biochemical parameters of lipid profile and CRP levels measured within Sprague Dawley´s rats with different dietary regimens at their age of 3rd and 15th month: **A)** Total cholesterol (TC); **B)** Triacylglycerides (TAG); **C)** Low-density lipoproteins (LDL-C); **D)** High-density lipoproteins (HDL-C); **E)** Adipose triglyceride lipase (ATGL); **F)** C-reactive protein (CRP). The experimental groups are marked by two letters, while 1st letter means the parental diet type and 2nd letter indicates the offspring´s diet type: CC, CH, HC, or HH, where C is the control diet and H means high-fat diet. NS – non-significant. Statistical analysis was done by two-way ANOVA (age; diet) followed by a post-hoc Tukey´s test. *p<0.05, **p<0.01, ***p<0.001 for 15th *vs* 3rd month in particular exp. group (either CH or HH); #p<0.05, ##p<0.01, ###p<0.001 for CH *vs* CC diet in 15th month; &p<0.05, &&p<0.01, &&&p<0.001 HH *vs* HC diet in 15th month.

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factor for cardiovascular disease, in addition to many age-associated chronic diseases and other adverse health outcomes. Progress has been made in understanding the complex relationship between ageing and acute and chronic inflammation, primarily due to the expansion of tools available to measure and quantify immune function and biomarkers. Walker *et al.* (2022), in their recent review, apart from connecting cellular senescence and mitochondrial dysfunction to impaired mitophagy and DNA damage, have explored the emerging multi-omics efforts that aspire to define the complexity of inflammaging: i) to identify molecular signatures and novel targets for interventions; ii) to counteract the excessive inflammation and its deleterious consequences; iii) to preserve the physiological immune response. No one can ignore the ageing process, but there is evidence that suggests exercise, a healthy diet, and sound routines may significantly delay this complex and irreversible process

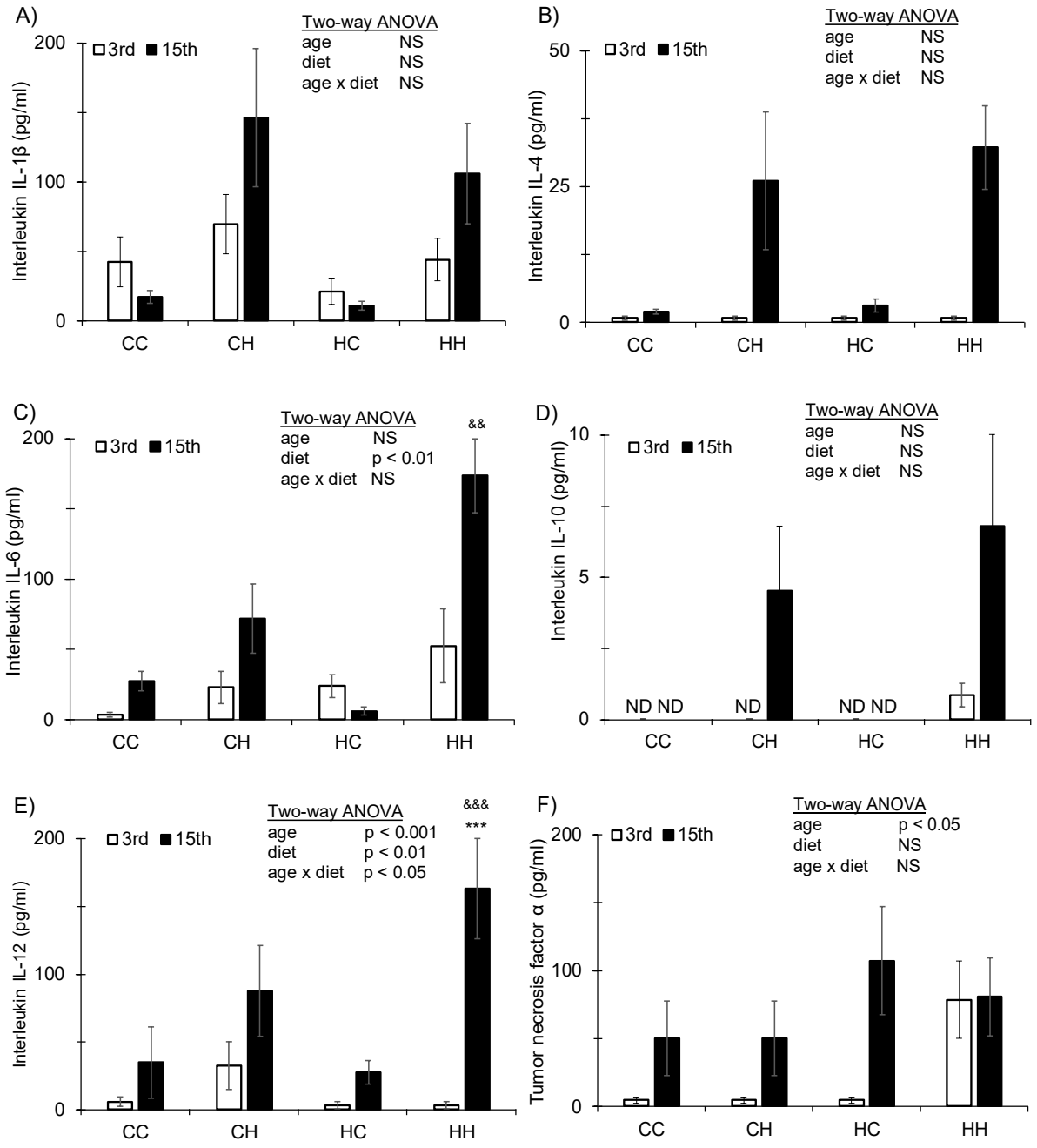

Figure 2. The levels of cytokines measured within Sprague Dawley´s rats with different dietary regimens at their age of 3rd and 15th month: **A)** Interleukin IL-1β; **B)** Interleukin IL-4; **C)** Interleukin IL-6; **D)** Interleukin IL-10; **E)** Interleukin IL-12; **F)** Tumor necrosis factor (TNF-α). The experimental groups are marked by two letters, while 1st letter means the parental diet type, and 2nd letter indicates the offspring´s diet type: CC, CH, HC, or HH, where C is the control diet, and H means high-fat diet. ND - not detected. NS – non-significant. Statistical analysis was done by two-way ANOVA (age; diet) followed by a post-hoc Tukey´s test. ***p<0.001 for 15th *vs* 3rd month in HH group; &&p<0.01, &&&p<0.001 HH *vs* HC diet in 15th month.

(Mishra *et al.*, 2023). Bojang *and* Manchana (2023) also highlighted various dietary approaches for healthy ageing in their review. Making dietary changes can promote healthy ageing, which may be an essential strategy for maintaining physical and cognitive function and preventing age-related diseases.

In this study, we investigated the impact of guided nutritional intervention, specifically the effect of a high-fat diet (H) on the offspring generation of female SD rats, which may also be influenced by parental high-fat diet. Since it is clear that lipid metabolism plays a crucial role in regulating the ageing process (Johnson and Stolzing, 2019), we investigated the cluster of metabolic parameters (Figure 1). Our data confirm their aggravation, correlating with both age and the influence of the H diet. The intrauterine and early-life epigenetic programming can also play a critical role, as the most profound worsening of inflammatory markers was observed in the 15-month-old HH group.

Except for TAG, using two-way ANOVA analysis, we confirmed an interaction between the factors of age and diet for all metabolic parameters measured, suggesting that age is a significant determinant of the diet's effect and vice versa. Interestingly, a decrease in CRP in the 15-month-old HH group was observed compared to the CH group of the same age. Consistently, a higher intake of fat tended to be associated with lower CRP values among women (Fredriksson *et al.*, 2004).

Since our other goal was also to contribute to the elucidation of the impact of ageing on immunological alterations, along with the pathophysiology of metabolic syndrome in SD female offspring rats, we focused on the most abundant pro-inflammatory (IL-1β, IL-6, IL-12, and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10). Our findings (Figure 2) are in good agreement with studies that support the importance of the evaluation of inflammatory cytokine levels within the pathophysiology of ageing (Leonardo *et al.*, 2023; Wei *et al.*, 2023), primarily through phospholipid oxidation in vascular cells and immune cells (Wang *et al.*, 2023). These data from a long-term *in vivo* experiment on ageing offspring of SD rats might shed light on metabolism variations related to nutritional preferences. Moreover, further insights into the mechanisms of ageing might be gained by considering the research of various civilizations and metabolic diseases. Additional impact on clinical study trends might also be considered.

Efforts to characterize inflammation in humans have relied on one or a few inflammatory proteins, with IL-6, TNF-α, and CRP being the most widely used (Furman *et al.*, 2019). Cytokines produced by lymphocytes are classified into two types depending on their pattern of immune response regulation. Type 1 cytokines include interleukin (IL)-2, interferon-γ, and tumor necrosis factor-β, which mediate immune responses against intracellular pathogens and promote delayed-type hypersensitivity. Type 2 cytokines, including IL-4, IL-5, IL-10, and IL-13, mediate immune responses against extracellular pathogens and regulate allergic inflammation by promoting IgE class-switching and eosinophil activation (Romagnani, 2001). Wang *et al.* (2021), in their study on SD rats, also found that maternal high-fat-diet-induced IL-4 upregulation in liver cells of male offspring. In general, a low-grade pro-inflammatory state is associated with ageing (inflammaging), characterised by a "2-fold-to-4-fold" increase in proinflammatory cytokines in the plasma level of healthy older people (Giunta *et al.*, 2022).

Interleukin-6 (IL-6) is a multifunctional cytokine that presumably plays a primary role as a mediator of several acute-phase inflammatory responses. While IL-6 expression is typically low, and serum levels are usually non-detectable in the absence of inflammation, with advancing age, serum levels become detectable, and it is proposed that this reflects an age-associated loss in the normal regulation of gene expression for this molecule. The cause of this is most likely multi-factorial (Eshler, 1993; Maggio *et al.*, 2006). It has been shown that mediators such as IL1-β, TNF-α, and IL-6 depend on body weight and age, are influenced by the dietary regimen, and might affect B cell function (Gupta *et al.*, 2013). Pongratz *et al.* (2015) demonstrated in their study that a high-fat diet has immunosuppressive effects on old male Wistar rats, as all analyzed immunoglobulin isotypes, except for IgA, increased with age, while the high-fat diet attenuated this increase. In our study, IL-6 was significantly affected (increased, $p<0.01$) by a high-fat diet. However, post hoc analysis showed a significant increase in IL-6 only in the prenatally programmed 15-month-old HH group compared to the 15-month-old HC group (&& $p<0.01$). Unlike other cytokines tested, only IL-1β and TNF-α did not appear to maintain the trend of the highest increases in the HH rats. It can be speculated that these cytokines may decrease even more profoundly with age, thereby mediating immunosuppression.

Interleukin-12, a pro-inflammatory member of the IL-6 family, plays a crucial role in the development of age-related cardiovascular diseases, such as atherosclerosis, myocardial infarction, and stroke, and is increased in aged subjects (Rea *et al.*, 2000; Rea *et al.*, 2018). Interestingly, our study suggests that, in addition to age, a long-term high-fat diet combined with prenatal dietary programming can be a critical pro-inflammatory and gerontogenic factor, elevating blood IL-12 levels in middle-aged animals. In addition, age and H can amplify each other, as we found a significant effect also for their interaction.

This experimental study may suggest the potential of modulating inflammaging as a promising strategy to prevent health decline associated with aging and prenatally programmed age-related metabolic diseases. Such conscious and proactive approaches, including dietary regimen precautions, are likely to be the most effective at the early stage of health decline, when the compensatory capacity of the organism is not yet completely exhausted and may still counteract physiological and functional declines. Moreover, our study suggests that a balanced diet might be an effective way to prevent age-related disorders, especially in descendants of parents who kept unhealthy dietary habits. New pharmacological interventions that

would selectively affect some of the signaling pathways regulating inflammation might be taken into consideration as well. Indeed, there is an apparent urge to balance the relationship between risks and benefits.

Conclusions

Our data indicate that the inflammaging, age-related chronic low-grade inflammation, was present within this study performed on 15-month-old (middle-aged) female offspring of SD rats. Moreover, that was also enhanced by diet-related disturbances, which were associated with significant alterations in metabolic parameters. Notably, epigenetic programming through parental diet appeared to play a critical role in worsening inflammaging. Although various interacting mechanisms can be proposed, the underlying molecular pathways are not well understood. Prenatally conditioned immunological interactions with metabolism, along with their pharmacological modulation, could be a focus for future detailed research.

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Conflict of interest

The authors declare no conflict of interest.

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