IMPACT OF TRAINING FREQUENCY ON BIOLOGICAL AGE GLYCAN BIOMARKERS AND INJURY PREDISPOSITION

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Gordan Lauc
PhD, professor

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Sveučilište u Zagrebu
PRIRODOSLOVNO-MATEMATIČKI FAKULTET
BIOLOŠKI ODSJEK

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UTJECAJ UČESTALOSTI TRENIRANJA NA GLIKANSKE BIOMARKERE BIOLOŠKE DOBI I PREDISPOZICIJE ZA NASTANAK OZLJEDA

DOKTORSKI RAD

Prof.dr.sc. Gordan Lauc

Zagreb, 2024. godina

The work presented in this doctoral thesis was performed at Genos Ltd., Zagreb, Croatia under the supervision of professor Gordan Lauc, PhD, as a part of the postgraduate doctoral program in Biology at the Department of Biology, Faculty of Science, University of Zagreb.

Ovaj doktorski rad je izrađen u Genos d.o.o., Zagreb, Hrvatska, pod vodstvom prof. dr. sc. Gordana Lauca, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu.

ABOUT MENTOR

Dr. Gordan Lauc is Professor of Molecular Biology at the University of Zagreb and the Director of the Human Glycome Project and National Centre of Excellence for Personalized Healthcare. He was born in Osijek (Croatia) in 1970, graduated molecular biology at the University of Zagreb Faculty of Science in 1992, and obtained PhD in Biochemistry at University of Zagreb in 1995 under supervision of Professor Mirna Flögel. He got his postdoctoral training at the Institute for Medical Physics and Biophysics in Münster (under supervision of Professor Jasna Peter-Katalinid) and Johns Hopkins University in Baltimore (under supervision of Professor Yuan C Lee).

Dr. Lauc is the author of over 300 research papers published in international journals and 11 national and international patents. His laboratory performed the first large scale studies of the human plasma glycome (in 2009) and human IgG glycome (in 2011), which were the basis for the subsequent first genome-wide association studies (GWAS) of the human plasma and IgG glycomes. He was invited to lecture at numerous international conferences and universities, and was elected visiting professor at Johns Hopkins University, University of Edinburgh, and Kings College London. In 2011 he was inducted in the prestigious Johns Hopkins Society of Scholars.

In 2007 Dr. Lauc established Genos Ltd, a private research organization accredited by the Croatian Ministry of Science, Education and Sport that is currently global leader in providing high-throughput glycomic analysis for the research community. Genos was ranked as #1 in the 2013 global survey of The Scientist magazine for “The best place to work for researchers” in the category “Industry” and has received more than 30 research grants over the years across various research areas in Glycobiology. He is currently coordinating the ERC Synergy project “GlycanSwitch”.

Najljepše zahvaljujem svom mentoru, prof. dr. sc. Gordanu Laucu, na kompletno otvorenim mogućnostima u odabiru tema istraživanja u sklopu doktorskog rada, na uvijek otvorenim vratima prilikom bilo kakvih problema i na smirenosti i optimizmu kada bi negdje zapelo.

Svim suradnicima i kolegama na Kineziološkom fakultetu Sveučilišta u Zagrebu, Kineziološkom fakultetu Sveučilišta u Splitu, Kliničkoj bolnici Sveti Duh u Zagrebu i Poliklinici Vaš pregled u Zagrebu želim zahvaliti na sudjelovanju u projektima koji su omogućili izradu ovog rada.

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Zadnje, no ne i manje važno, zahvaljujem svojoj mami na mnogim znanstvenim razgovorima i što mi je bila veliki uzor kao pionir u ekoinženjerstvu i industrijskoj mikrobiologiji u Hrvatskoj, te na pokazivanju što znači nikad ne odustati, kao i na uvijek dostupnom i vrlo ključnom baka-servisu, jer bi bez potpore obitelji sve bilo znanto teže. Istovremeno zahvaljujem mojoj najužoj obitelji: ocu, suprugu, kćeri i sinu na trpljenju svih mušica, kao i na potpori kada je vrijeme bilo najkritičniji faktor.

University of Zagreb
Faculty of Science
Department of Biology

Doctoral thesis

IMPACT OF TRAINING FREQUENCY ON BIOLOGICAL AGE GLYCAN BIOMARKERS AND INJURY PREDISPOSITION

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Regular moderate exercise has multiple beneficial effects, yet very little is known about the impact of exercise on immunoglobulin G (IgG) glycosylation. In this thesis, the aim was to analyze the impact of various training frequencies on glycan biomarkers of biological age and to investigate currently related gene variants associated with soft-tissue injuries. The results demonstrated that glycan profiles of regularly moderately active population had the lowest GlycanAge index, 7.5 years less when compared to competing athletes, and 7.4 years less when compared to the inactive population. Furthermore, results supported the association of the *COL12A1, COL27A1, Tenascin C* and *matrix-metalloproteinase 3 (MMP3)* gene variants with either increased or reduced risk of developing anterior cruciate ligament ruptures or Achilles tendinopathies, types of injuries that are common in sport, impacting both professional competing athletes and recreational exercisers.

(89 pages, 5 figures, 11 tables, 271 references, original in English)

Keywords: Biological Age, Glycosylation, Immunoglobulin G, Single Nucleotide Polymorphism, Sport Injury

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Doktorski rad

UTJECAJ UČESTALOSTI TRENIRANJA NA GLIKANSKE BIOMARKERE BIOLOŠKE DOBI I PREDISPOZICIJE ZA NASTANAK OZLJEDA

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Redovna umjerena tjelesna aktivnost blagotvorno utječe na cjelokupno zdravlje. Trenutno se još uvijek zna jako malo o utjecaju vježbanja na glikozilaciju imunoglobulina G (IgG). U ovom doktorskom radu cilj je bio istražiti utjecaj učestalosti treniranja na glikanske biomarkere biološke dobi, kao i povezanost trenutno poznatih varijanti gena s ozljedama mekog vezivnog tkiva kod sportaša. Značajno niži GlycanAge indeks potvrđen je u grupi redovnih umjerenih vježbača, dok su profesionalni sportaši i neaktivna popoulacija imali značajno viši GlycanAge indeks. Osim toga, postoji značajna povezanost genskih varijanti unutar *COL12A1, COL27A1, Tenascin C (TNC)* i *matriks-metaloproteinaza 3 (MMP3)* gena s povišenim ili smanjenim rizikom od nastanka rupture prednjeg križnog ligamenta i tendinopatija u Ahilovoj tetivi, kod profesionalnih sportaša u odnosu na kontrolnu skupinu. Ozljede ligamenata i tetiva su česte sportske ozljede koje pogađaju i rekreativne i profesionalne sportaše.

(89 stranica, 5 slika, 11 tablica, 271 referenca, jezik izvornika: engleski)

Ključne riječi: biološka dob, glikozilacija, immunoglobulin G, polimorfizam jednog nukleotida, sportska ozljeda

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1.INTRODUCTION

We are living in an era where an epidemic of sedentary lifestyle has led to ever-increasing levels of obesity, in children as well as in adults. In the last thirty five years obesity levels have doubled in more than 70 countries [1]. As it is well known, low levels of physical activity are associated with the increased risk of cardiovascular diseases (CVDs), diabetes type 2 (D2B) and different types of cancer, put together under the umbrella of non-communicable diseases (NCDs) [2]. Chronic diseases are in their nature a heavy burden even for the more developed countries, as they encounter for both costs and illness related inabilities [3], while being categorized as one of the major mortality factors in the modern era [4,5]. Furthermore, lack of physical activity is considered as one of the primary causes of many of chronic diseases [2–5]. So, it came as no surprise that humans are currently fighting another silent war – one against obesity. Bennjamin Caballero asked that exact question. Humans against Obesity: who will win [6]? In 1998 the World Health Organization (WHO) issued one of the first reports where obesity was referred to as an epidemic [7].

Twenty years later we are witnessing a relentless global rise in both obesity and NCDs. The definition of obesity is explained through the excess of adipose tissue that affects health, usually accumulated over many years of constant chronic positive caloric intake, coupled with having very little to no physical activity [8]. In the United States, almost 40% of the population is obese (a little more percentage of women than men), while severe obesity accounts for over 7.5% of population [9]. In Europe, WHO has reported that obesity affects nearly 60% of adults and almost every third child, showing an alarming trend. Recent assessments carried out by WHO show that obesity and overweight account for the high fourth risk factor in the European region, just after high blood pressure, poor dietary choices and tobacco use [10]. Crude measurement of Body Mass Index (BMI) is used to define weight categories. BMI under 19.5 is considered underweight, 19.5-24.9 is considered normal, 25-30 is considered overweight, while those over 30 are considered obese [11]. It is important to underline the limitations of BMI estimates, as lean muscle weighs more than fat. There are sub-populations where simple BMI calculations information simply doesn’t apply, for example, professional athletes with high muscle mass to body fat ratio.

It is well known that the introduction of physical activity into everyday life leads to improved cardiorespiratory fitness (CRF), which is in turn associated with less risk of CVDs and overall mortality rates [8,12,13]. Even though it is common knowledge, it is practiced poorly, and a

sedentary lifestyle prevails, leading to the global burden of illnesses and lower quality of life, in both high-income and middle-income countries [13,14].

1.1. PHYSICAL ACTIVITY AND EXERCISE TRAINING

In 2020 WHO issued guidelines for physical activity and sedentary behavior [15], stating that all adults should try to reach the goal of 150 to 300 minutes of moderate physical activity or 75 to 150 minutes of vigorous physical activity; while children and adolescents should aim for 60 minutes daily of moderate to vigorous physical activity to achieve health benefits. Unfortunately, recent estimates on the global level indicate that one-quarter of adults and even more problematic, three-quarters of adolescents do not reach weekly recommendations [16], even though there is an immense amount of evidence supporting the idea of lifelong exercise and its association with healthier and longer lifespan, delaying the onset of more than forty NCDs [17]. In the past decades, exercise capabilities and activity status have become trusted predictors of cardiovascular and overall mortality. Each metabolic equivalent (MET) increase in exercise capacity has led to a 12% increase in survival rate among men [18].

To reach optimal cardiometabolic health, the Maastricht study showed once again that one should practice medium to higher intensity physical activity and reduce sedentary time in order to increase CRF, which directly leads to improved cardiometabolic health [19]. Cardio-respiratory fitness is to this timepoint considered as the single most important predictor of overall health, especially in the obese population, where it was proven to be a better predictor when compared to the weight loss effect in relation to CVD outcomes [20]. Even a single measurement of fitness status was shown to significantly improve the classification of risk for development of CVDs related mortality risks in both 25-year span and 10-year span [21]. The latter were indications of the long-term exercise-induced protection of cardiovascular health and functions, but short-term benefits of exercise have also been investigated. Just one single bout of exercise can diminish, to a level, the magnitude of previous cardiac and vascular damage. Interestingly, even a short-term exercise can offer immediate protection to the aging population or those suffering from co-morbidities [22]. When it comes to intensity levels, all variants of intensity protocols (low-, medium- and high-) have been shown to initiate positive changes in the obese male population, with greater effects in medium- and high-intensity level protocols than low-intensity ones [23]. Exercise in general improves the metabolic function of

the white adipose tissue, while stimulating angiogenesis and improving adipose tissue cellularity, and improves cardiac autonomic function over time in populations suffering from both obesity and diabetes [24,25], states which have been previously linked with the low levels of chronic inflammation [26].

1.2. AGING, INFLAMMATION, AND INFLAMM-AGING

The low-grade systemic inflammation is the hallmark of many NCDs, with the increased risk in obese adults, who have increased visceral fat mass that stimulates innate immunity, and when the inflammatory state continues over time, adipocytes, together with the recruited immune cells will release proinflammatory cytokines [27,28]. Furthermore, the same low-grade systemic inflammation is one of the important hallmarks of aging, together with several others such as genomic instability, telomere shortening, changes in epigenetic modifications, issues with macroautophagy, loss of mitochondrial functions and dysbiosis [29].

Aging can be considered a risk factor for developing many chronic diseases, as they have been positively associated. Those chronic diseases vary from CVDs, T2D, pulmonary, kidney and neurodegenerative diseases, sarcopenia, osteoporosis, osteoarthritis and different types of cancer, which paints a dark picture for the aging population [29–31]. First proposed by Claudio Francesci in 2000, the term inflamm-aging has come to life [32]. Inflamm-aging was proposed as the imbalance between two opposite sides of inflammation – between pro-inflammatory and anti-inflammatory networks, leading to the constant low-grade chronic inflammatory state [33–35].

A high level of pro-inflammatory factors, such as C-reactive protein (CRP), different interleukins (IL-1, IL-1RN, IL-6, IL-8, IL-13, IL-18), interferons (IFNα, IFNβ, IFN-γ) and tumor necrosis factor alpha (TNFα) are detected in the pro-inflammatory state [36–39]. On the other hand, the anti-inflammatory state is characterized by the presence of anti-inflammatory cytokines interleukins (IL-1Ra, IL-4, IL-6, IL-10) and transforming growth factor beta (TGF-β1), as well as heat shock proteins (HSPs) and lipoxin [36–39].

1.3. PHYSICAL ACTIVITY, AGING, AND REGULATION OF THE INFLAMMATORY RESPONSE

1.3.1. Physical activity and aging

The loss of muscle strength and volume, the condition called sarcopenia, as well as decreased levels of regeneration and impaired physical capabilities, are the main hallmarks of aging skeletal muscle [40]. Lack of physical activity will further impact increasing frailty in the aging population. Resistance exercise is one of the currently recommended types of exercise when battling sarcopenia and frailty [41,42]. The low-level systemic inflammation, that is characteristic for the aging organism, can be countered by introducing physical activity and exercise, as it results in the decrease of age-related muscle loss and the increase of regeneration capabilities, muscle strength and muscle mass, directly improving quality of life, while mitigating effects of aging related to physiological changes and the onset of NCDs [40,43–46].

The aging population has shown positive outcomes in interventions related to metabolic health through dietary interventions and caloric restrictions, through the increase of physical activity [47], as well as when moderate- and high-intensity aerobic exercise was introduced to a previously sedentary older male population [48]. Hayes et al. [48] demonstrated that a short-term aerobic exercise intervention lasting 6 weeks has lowered interleukin 6 (IL-6) levels in lifelong sedentary older adults, while age-matched life-long exercisers have had even lower levels of circulating IL-6. Contracting skeletal working muscle releases IL-6 into circulation after every bout of exercise, with the magnitude depending on intensity and duration of exercise. Continuous physical activity will lead to a reduction in basal IL-6 production, leading to the adaptation of the working muscle [49]. Even though IL-6 is considered as a pro-inflammatory cytokine, in the cascade induced by a bout of exercise, IL-6 will initiate the release of anti-inflammatory cytokines further along in the cascade [50,51].

1.3.2. Physical activity, overtraining syndrome, and immuno-suppression

Treading carefully down this path, on the matter of exercise-induced immuno-suppression, two opposed factions are strongly holding their ground on whether exercise suppresses immune competency or not [51–60]. Insufficient physical activity can cause an equal amount of damage as can overtraining. Overtraining syndrome (OTS) is described as the result of continuously

applying excessive amounts of training stress through exercise, in monotonous volume or difficulty levels. OTS usually occurs in periods of high physical and psychological stress, when there is not enough recovery time between high-stress events and when the dietary regimen doesn't adequately support the physiological needs of the individual. It is hypothesized that proinflammatory cytokine response could lead to the development of immunosuppression, resulting in overall lower performances and susceptibility to injuries and infections [51,61–65].

1.3.3 Anti-inflammatory effects of exercise

During exercise, working skeletal muscle produces and releases interleukin-6 (IL-6). IL-6 is considered as one of the first classified myokines, cytokines produced by the working skeletal muscles [66,67]. As skeletal muscles produce and release IL-6, an anti-inflammatory cascade is triggered, increasing the release of other anti-inflammatory cytokines, such as IL-1Ra and IL-10, supporting the anti-inflammatory effects of exercise [39,56,66]. Exercise of higher intensity will trigger higher cytokine response, leading to the conclusion that anti-inflammatory response is physical-fitness dependent, which is in accordance with previously mentioned beneficial changes in myokine levels, where greater effects were observed at medium-intensity and high-intensity exercise protocols [23,68].

For patients suffering from cardiometabolic diseases, such as CVDs or T2D, physical exercise has an exceptionally beneficial anti-inflammatory effect. When IL-6 is produced, it triggers the IL-10, which leads to inhibition of TNFα, further stimulating IL-1Ra and limiting IL-1β signaling (Figure 1) [69]. Apart from the short-term anti-inflammatory effect, long-term exercise leads to metabolic remodeling, creating a caloric deficit when dieting properly, which causes mobilization of fat from adipose tissue, leading to the reduction of adipocytes, which in turn reduces inflammation [70,71]. Physical exercise alters the advancement of atherosclerosis, one of the leading mortality causes, by triggering metabolic and anti-inflammatory pathways [72]. Furthermore, exercise induces an increase in insulin sensitivity of the working muscles during exercise bouts [73], additionally supporting an anti-inflammatory environment, simultaneously reducing chronic low-level inflammation, detected in the aging population and in populations suffering from different cardiometabolic diseases [69,74,75].

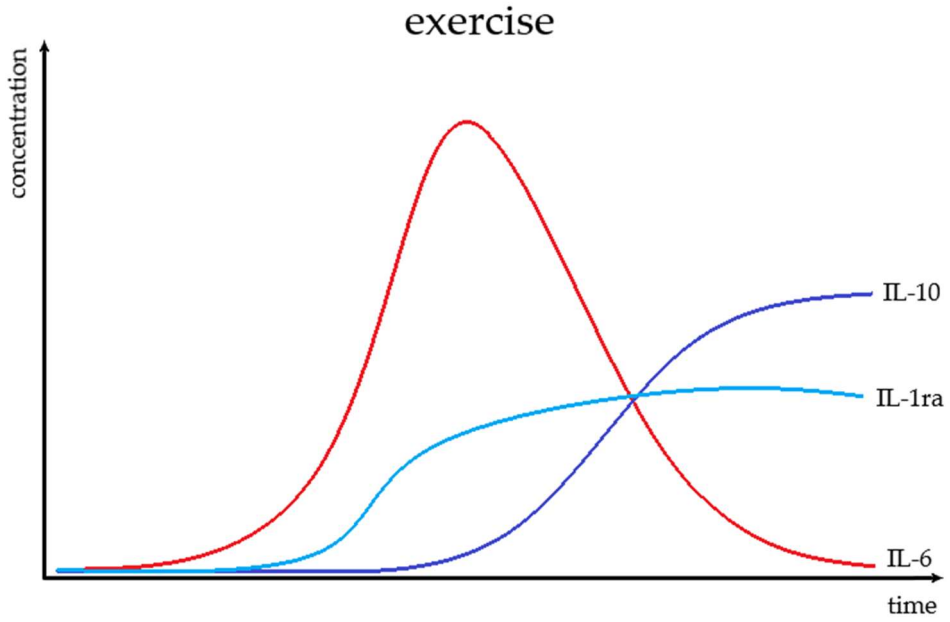

Figure 1. Anti-inflammatory effect of exercise.
During physical activity, cytokine response to exercise involves a marked increase in IL-6 production, followed by Il-1Ra and Il-10 increase.

While the awareness of increased inflammation with aging rises, an interesting question was asked – whether it is an inflamm-aging or actually an inflamm-inactivity [76]. 'A diseasome of physical inactivity' is defined as a cluster of different diseases: neurodegenerative, metabolic, cardiovascular diseases, and different types of cancer.

Rest et al. [47] investigated the metabolic effects of 13-week lifestyle intervention in older adults, targeting a 12.5% caloric reduction and 12.5% increase in energy expenditure through physical activity in 164 older adults (mean age = 63.2 years; BMI = 23-35 kg/m^2). Results showed a 4.2% weight loss, comparable to a study in young adults, but fasting insulin levels were much less decreased with a more heterogeneous response. Blood pressure, thyroid function, glucose, and lipid metabolism were significantly improved, demonstrating overall improvement in metabolic health.

Physical inactivity is considered as a high-risk factor for the accumulation of visceral fat, which through long-term accumulation leads to abdominal adiposity, further leading to chronic inflammation [77].

1.4. IMMUNOGLOBULIN G AND IgG GLYCOSYLATION

1.4.1. Immunoglobulin G (IgG)

During a secondary immune response, plasma cells, differentiated and maturated from B lymphocytes, secrete the immunoglobulin gamma (IgG) (Figure 2), one of the most prevalent antibodies in the human body, with a concentration around 10 mg/mL [78–80]. IgG weighs close to 150 kDs, has four subclasses (IgG1, IgG2, IgG3, IgG4) and is made of two functional fragments (Figure 2) [79,81,82], antigen-binding fragment (Fab) and crystallizable fragment (Fc). The IgG glycoprotein has a conserved N-galactosylation site in the Fc region, and variable O- or N-linked glycosylation sites in the Fab region [83]. While Fab recognizes and binds different antigens (bacteria, toxins, viruses etc.), Fc will bind different molecules, therefore initiating pro- and anti-inflammatory responses [84]. Apart from the functional aspects, IgG is built out of two heavy and two light chains. The light chain contains a variable region domain as well as a constant domain, while the heavy chain has four domains; one variable, followed by three constant domains [85].

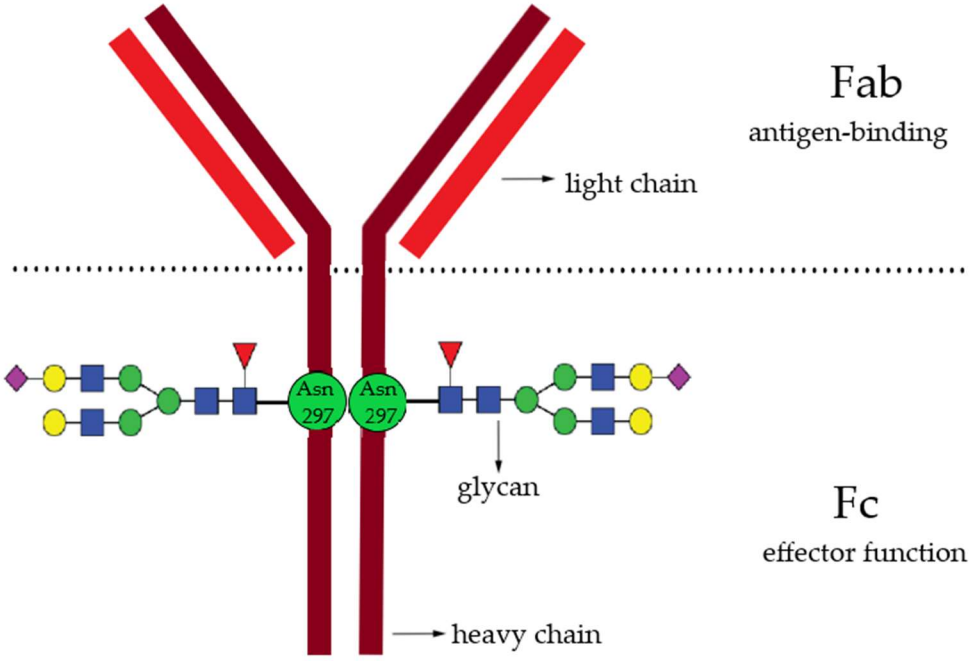

Figure 2. Immunoglobulin G – structure of IgG.
The IgG molecule is composed of two heavy and two light chains. If looking at function, IgG has two functional fragments: the effector function of the crystallizable fragment (Fc) and the antigen-binding domain function of the antigen-binding domain fragment (Fab). To each heavy chain of the Fc fragment, there is an N-glycan covalently attached to a highly conserved N-glycosylation site at the asparagine (Asn) 297 position.

The IgG is classified as highly stable, with a 12 days half-life [86], and is involved in several humoral immune processes: antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement activation and complement-dependent cytotoxicity (CDC), antigen neutralization, hypersensitivity reactions, targeting and recognizing invading particles for phagocytosis [81,82,84,87–92]. Furthermore, as IgG is involved in many processes of the immune system, it links IgG directly to numerous pathological states, ranging from inflammatory and autoimmune disorders, cardiometabolic and neurodegenerative conditions, as well as different types of cancer [87,88,93–97].

1.4.2. Immunoglobulin G glycosylation

Glycosylation is the post-translational modification that results in complex structural changes of the protein. The process of glycosylation is very complex, it involves close to two hundred glycosyltransferase enzymes. During that process, it is determined which proteins will end up being glycoproteins, placement of glycans on those proteins and final glycan structures that will provide complete glycoprotein functions [98]. Synthesis of the activated nucleotide monosaccharides starts in the cytosol or the nucleus and is transported into the lumen of the endoplasmic reticulum (ER), by adding glycan precursor to the elongating IgG polypeptide chain. Remodeling of glycans continues in the Golgi apparatus, where different glycosidases and glycosyltransferases are shaping the final IgG glycoform [94,99–101]. Different glycan structures on different proteins will result in a number of functions, such as protein folding, and regulation of protein backbone functions via additional glycosylation, or due to their size, they can provide protease cleavage protection without interfering with the protein function [102].

The glycan biosynthesis is not directed by a genetic template, contrary to proteins, they are synthesized without a template. Many environmental factors as well as over 700 genes control glycan biosynthesis, creating the intertwined effect of the environment and one's genetic template [98,103], in a similar manner as it is with aging [30]. In order to bind to all Fcγ receptors (FcγR), glycosylation of IgG is crucial, by maintaining an open conformation of heavy chains [104], while deglycosylated IgG can't mediate the inflammatory response triggered *in vivo* [105]. The IgG Fc domain interacts with inhibitory or activating FcγR, expressed in different effector cells, in order to initiate the immune response that is needed,

therefore Fc glycans act in a fine-tuning manner by modulating FcγR on different immune cells. On the other hand, Fab glycans modulate IgG's antigen-binding affinity and can affect its half-life [106,107]. While Fab glycosylation is less researched and understood, Fc glycosylation has been researched in great detail, marking the Fc glycans as the main regulatory components of IgG-mediated immunity [88].

1.4.3. IgG glycans and derived traits

Glycans are complex oligosaccharide molecules, synthesized from up to 15 monosaccharide residues (Figure 3).

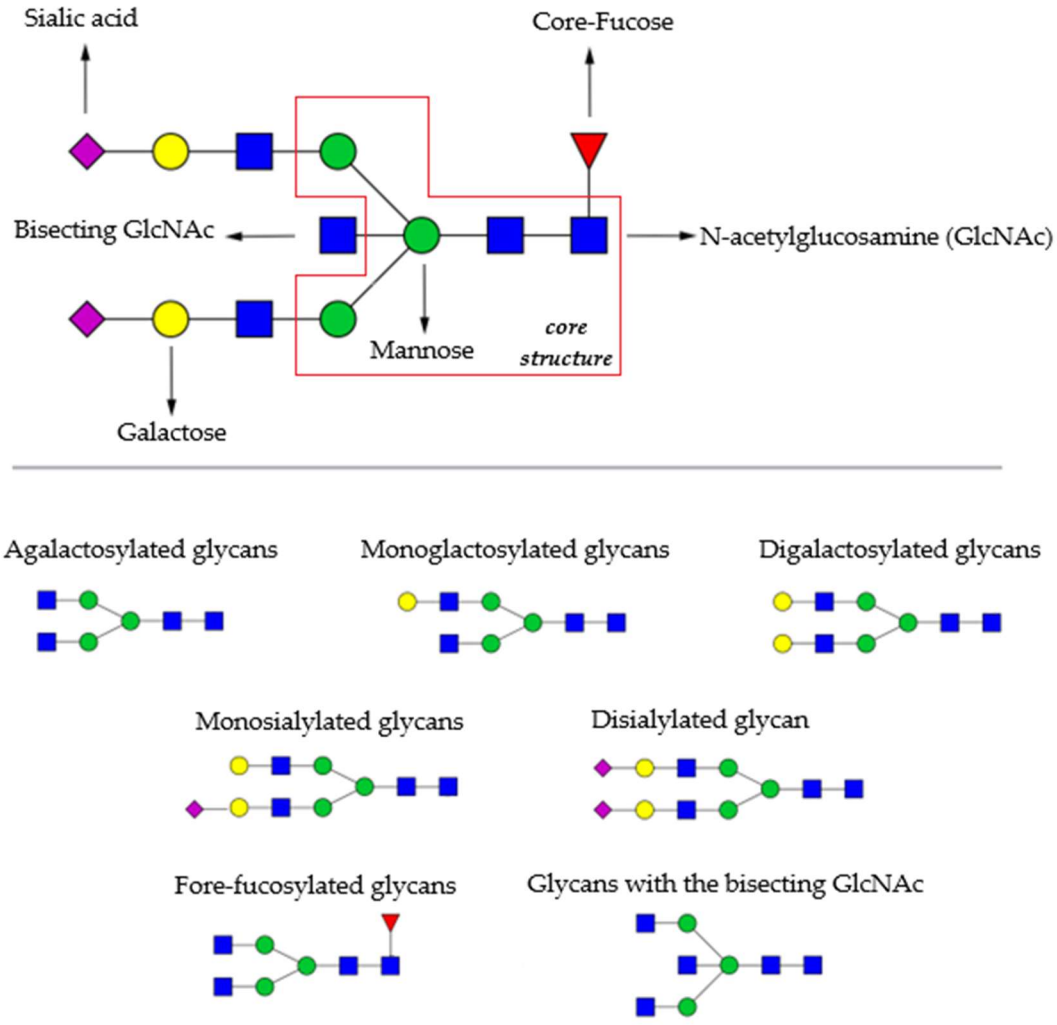

Figure 3. Structures of the main IgG glycans

A conserved pentasaccharide core is composed of two of the N-acetylglucosamines (GlcNAcs) and three mannoses (Man), with different compositions and additions of galactoses and sialic acids, and further bisected with the additional GlcNAc, or with the addition of fucose at the pentasaccharide core.

Glycans are defined as N-linked or O-linked glycans, being covalently attached to the polypeptide backbone, via amide link to asparagine (Asn) for N-linked glycans and to the oxygen atom of serine (Ser) or threonine (Thr) for O-linked glycans [87]. Together with nucleic acids, lipids and proteins, glycans have the key role in cell communication and adhesion, functional protein and macromolecule formation, as well as ligand binding to specific receptors, resulting in signaling pathways activation [99,108]. The IgG effector functions are highly related to the attached N-glycans, directing IgG's potential from anti- to pro-inflammatory and back (Figure 4).

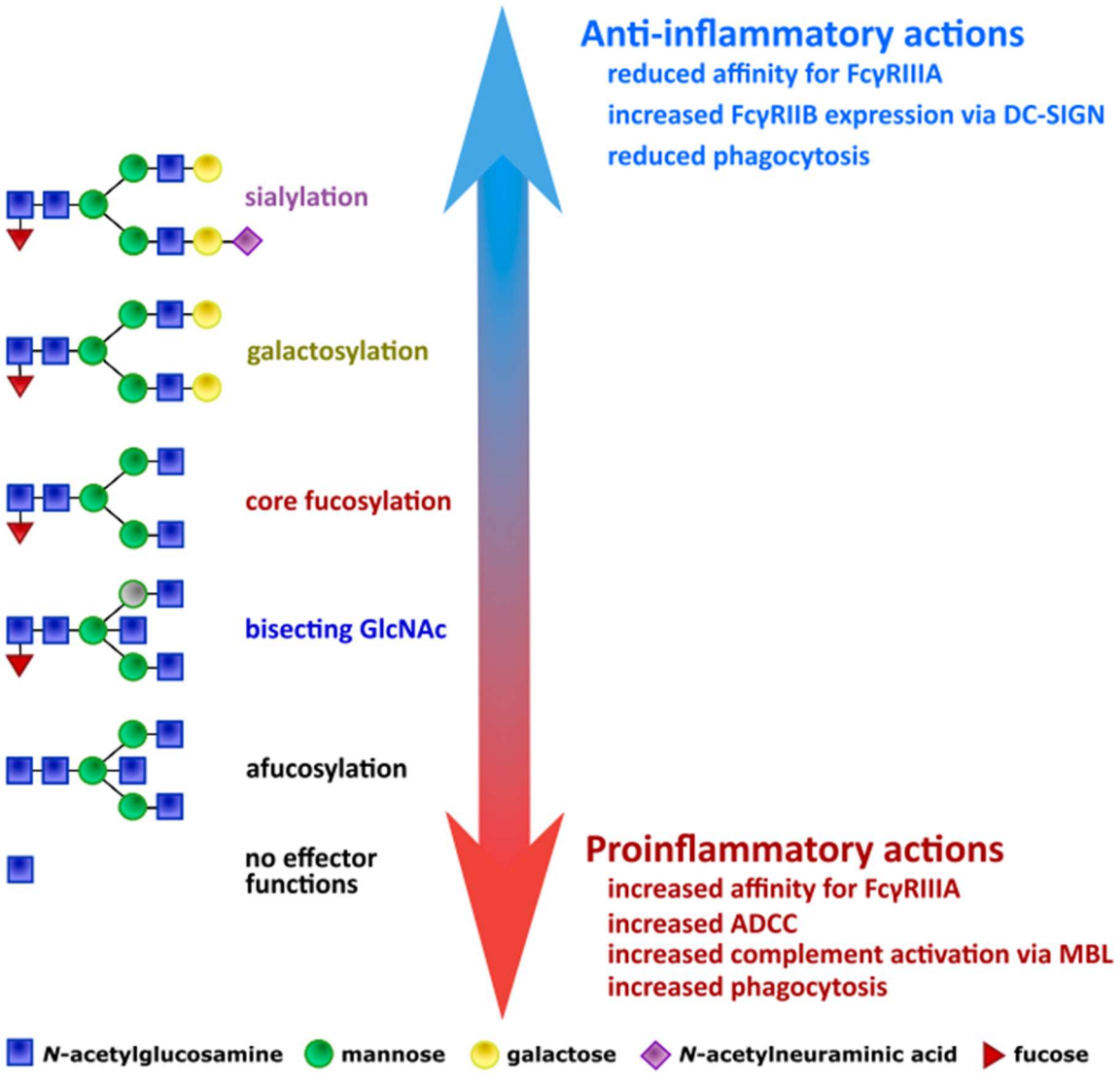

Figure 4. Immunoglobulin G effector functions in relation to its N-glycosylation
The N-glycans direct the effector functions of IgG, changing its potential from anti-inflammatory to pro-inflammatory and back, depending on the type of glycans attached to the IgG molecule. Reprinted from Mol Aspects Med, N-glycans as functional effectors of genetic and epigenetic disease risk by Štambuk T, Klasić M, Zoldoš V, Lauc G. [109]

The core structure of all IgG glycans is the same: a conserved pentasaccharide core is composed of two GlcNAcs and three mannoses (Man), but what sets them apart is the extension of the core structure by additional GlcNAcs that can be added to the core structure (number of antennae that can be one or two), additional galactoses added to the core structure (zero, one or two), additional sialic acids added to the galactose residues (zero, one or two) and the presence or lack thereof bisecting GlcNAc and/or core-fucose [110].

IgG isolated from the healthy human plasma is partially galactosylated (45%), while sialylated and bisected glycans appear in a much less extent (10% and 15% for the latter), though core-fucose levels are high (up to 90%) [111,112]. When it comes to regions, the Fc region is highly core-fucosylated, with mostly asialylated glycans, while the Fab region has higher levels of glycans containing bisected GlcNAc, sialylated and galactosylated glycans [113,114].

Galactosylation (G)

IgG glycosylation is strongly associated with age, in a negative correlation between galactosylation level and age [87,115,116]. Galactosylation levels represent an interface between pathological and physiological processes occurring in our bodies. In healthy adults agalactosylated (G0) and monogalactosylated (G1) structures take up to 35% of total IgG Fc glycans, while there is only around 15% of digalactosylated glycans (G2) [117,118]. On an individual level, the percentage of galactosylated structures is fairly constant, unlike being the most variable trait when the population is observed [119,120]. IgG galactosylation is partially driven by estrogen, with raised levels in pregnancy, fluctuating levels during the menstrual cycle and with decreased levels after menopause [116,121–126]. Moreover, increased levels of agalactosylated IgG glycan structures have been found in different diseases and various states that have underlying inflammatory conditions, associating agalactosylation with poor metabolic health, considering it as a marker of inflammation [85,95,116,127–133].

Additionally, several studies have revealed that terminal galactose residue removal will significantly reduce complement-dependent cytotoxicity (CDC) [134,135], while on the other hand presence of galactose will increase IgG affinity by Fcγ receptor activation, directly increasing antibody-dependent cell-mediated cytotoxicity (ADCC) [136]. Lastly, as galactose residue is a prerequisite for sialylation, and sialylation acts as a pro- to anti-inflammatory switch, it can be concluded that agalactosylated glycans can lead to a pro-inflammatory state.

Sialylation (S)

Terminal sialic acids are considered to act as a switch from the anti- to the pro-inflammatory state of IgG when homeostasis is interrupted. 10-15% of total IgG Fc glycans are sialylated, including both mono- (S1) and disialylated (S2) glycan structures [117,118]. If sialylation levels are elevated, it will lead to an increase in complement deposition and CDC [136]. Sialic acid reduces the ability of IgG's binding capacity to the Fcγ receptor IIIa, decreasing the ADCC activity, while enhancing anti-inflammatory effects [137–140]. Anti-inflammatory properties of intravenous IG (IVIG) are likely linked to sialic acid residues on Fc N-glycans only, as sialic acid-rich glycans of the Fab fragment did not increase IVIG therapy properties [141,142]. When neuraminidase was introduced to IVIG treatment, its activity was diminished, matching levels of deglycosylated IVIG. Through the interaction with Fcγ receptors, asialylated IgG N-glycans will direct the immune response towards the pro-inflammatory state [91,143], similarly to sialylated glycans often being decreased in autoimmunity, increasing the pro-inflammatory state [138].

Core fucosylation (CF)

In the absence of fucose attached to the first N-acetylglucosamine (GlcNAc) at the IgG N-glycan core structure, the ADCC exhibits almost a 100-fold increase in its activity, resulting in cytotoxicity of targets, or in the case of antibody-dependent cellular phagocytosis (ADCP), phagocytosis of targets [144–149]. Afucosylation of the Fc fragment N-glycans of IgG represents a switch for strong binding to Fcγ receptors III, as IgG is usually almost completely fucosylated (90%) [118], while afucosylation of IgG represents a fine-tune mechanism by improving IgG binding to FcγRIIIa on NK-cells [150] and FcγRIIIb on neutrophils [151]. A decrease of IgG core-fucosylation was observed in autoimmune thyroid disease [152]. On the other hand, an increase in IgG fucosylation was found in individuals with a higher level of inflammation [153].

Bisecting GlcNAc (B)

The presence of a bisecting GlcNAc increases ADCC, by binding of the IgG antibody to the Fcγ receptor [149,154,155], although it was proved that the level of increase is very small, even with a high proportion of bisected GlcNAc glycans, when compared to the effect of

afucosylated glycans on ADCC [148]. If the bisection is already present, it will inhibit the addition of core-fucose, and as afucosylated IgG glycans increase Fc binding and ADCC levels, bisection could cause an indirect ADCC increase. Additionally, the addition of the bisecting GlcNAc to glycans was proposed to suppress immune functions [155,156].

1.5. CHANGES IN IgG N-GLYCANS

1.5.1. Aging

Glycosylation and aging are delicately interlaced, as it is the process involving many different enzymes, substrate availability, status of the cell metabolism and disruption of homeostasis [157]. It was established rather early that human N-glycan profiles are age and sex dependent [158]. One of the first published studies revealed that levels of agalactosylated glycans change with age and that the ratio of agalactosylated IgG glycoforms starts to rise after the age of 25. At the same time, levels of IgG digalactosylation will start to decrease, while monogalactosylated glycoforms remain constant [112,115,116,153,159,160]. In older population, a typical N-glycan profile would include a decrease in galactosylation and sialylation levels, as galactose is a substrate for sialylation, as well as an increase in the amount of bisected glycans [35,131,153]. When related to age, decreased levels of bisecting GlcNAc in agalactosylated glycans were associated with familial longevity in younger participants [159], while relative levels of GlcNAc increase with age. Agalactosylated glycans with the bisecting GlcNAc are considered to increase IgG's pro-inflammatory activity by modulating Fcγ and other receptors [110]. As glycan structures are altered during the process of aging, as well as in age-related diseases, glycans represent useful diagnostic biomarkers [157].

1.5.2. Diseases and inflammation

Inflammation is typical for aging and poor metabolic health, low galactosylation and sialylation levels, and high fucosylation levels are related to a low-grade inflammation state [153]. Chronic higher levels of inflammation is a well-established risk factor for CVD development [94,161,162]. Changes in IgG N-glycosylation have been found in atherosclerosis, with a higher abundance of bisected GlcNAc. On the contrary, the absence of GlcNAc and sialylation increase was associated with less risk of atherosclerosis development [95]. Changes in IgG N-

glycosylation have also been observed in hypertension, where bisected N-glycans were increased in individuals who developed hypertension during the follow-up [97]. Changes in IgG N-glycan composition have been observed in all major diabetes subtypes [96], in acute systemic inflammation [120], in inflammation and poor metabolic health [153], in different types of cancers, neurodegenerative diseases, chronic inflammatory gastrointestinal and liver disease, kidney diseases [87,88,163,164].

1.5.3. Obesity

Greto et al. [165] have demonstrated that in the obese population low-calorie diet has induced a significant decrease of bisected IgG N-glycans, which was followed with bariatric surgery, resulting in further weight loss during the one-year period, finally resulting in extensive alterations of IgG glycome. Digalactosylated and sialylated glycans were significantly increased, with a substantial decrease in agalactosylated and core-fucosylated IgG N-glycans.

Another study done by Deriš et al. [166] investigated glycome compositions of 1850 individuals, undergoing a dietary intervention in the DIOGenes study. The most significant changes in the IgG glycome occurred in the first 8 weeks of intervention, in a low-calorie diet restriction period. Changes in the glycome included a statistically significant decrease of agalactosylated and an increase of sialylated N-glycans, while the follow-up period following one of five weight maintenance diets during the next six months, revealed an increase in bisected glycans and a decrease in sialylated glycans, without significant changes between different diet groups.

Following the previous study, they analyzed plasma N-glycans in the same cohort [167], with the results that demonstrated a significant increase in low-branched glycan structures and a significant increase in high-branched glycan structures, while after the low-calorie diet period, in the weight maintenance period, the significant rise in N-glycans with the antennary fucose was observed.

Štambuk et al. [168] followed up with another study on two separate cohorts undergoing both caloric restriction diet and bariatric surgery. A low-calorie diet resulted in a decrease in high-branched trigalactosylated and trisialylated plasma N-glycans, followed by an increase in low-branched N-glycans in both cohorts. After bariatric surgery, observation in the follow-up

showed an increase in complex, high-branched, antennary fucosylated, extensively galactosylated and sialylated N-glycans and a significant decline in a simple low-branched, core fucosylated, bisected, agalactosylated, and asialylated glycans. One year follow-up indicated the N-glycan complexity decreases, accompanied by the increase of low-branching glycans.

A study done by Russel et al. [169] demonstrated the association of increased central adiposity with the pro-inflammatory IgG N-glycans, showing that 22 glycan peaks were associated with at least one of the fat measures, replicating previously associated BMI with agalactosylated IgG N-glycans, while measures of central adiposity explained variations in the IgG glycome.

Back in 2013 Nikolac Perkovic et al. [133] found a small, but positive correlation between BMI and levels of agalactosylated glycans, demonstrating that obesity induces an inflammatory state during the development of obesity.

Liu et al. [170] researched the difference in IgG N-glycans between four different groups. The group with normal BMI and normal waist-to-hip (WHR) ratio, normal BMI with central adiposity, obesity with normal WHR and obesity with central adiposity. Their results showed that central obesity generated more changes in IgG N-glycans than regular obesity with normal WHR, bringing central adiposity further into focus, as IgG N-glycans showed stronger inflammation in central adiposity with obesity group than in a group of only obese participants with normal WHR, indicating that population with the normal BMI, but with central adiposity is also at greater risk of mortality and CVDs.

1.5.4. Exercise intervention

Hulmi et al. [171] tackled the effects of intensive weight reduction in relation to body composition and serum hormones in a group of female fitness competitors (age 27.5 ± 4.0 years, body mass index 23.4 ± 1.7 kg/m^2). Four months of a fat-loss diet coupled with increased aerobic exercise in normal-weight females competing in fitness sport led to a 12% decrease in body weight and a 30-50% decrease in fat mass, while the control group maintained the initial body composition. Two years later, continuing the research on the same cohort, Sarin et al. [172] proved the occurrence of immunosuppression in response to prolonged intensive physical training, coupled with a caloric deficient diet that has led to an intensive weight loss. The

intervention group (N = 42) showed an extensive alteration of all measured immune function parameters, including IgG glycome and cytokine profile. Effects included, among others, suppressed immune cell proliferation, attenuated systemic inflammation, and loss of immune cell function, due to the reduced antibody secretion. Digalactosylated and sialylated N-glycans without bisecting GlcNAc were significantly decreased in the end of the weight-loss period. Agalactosylated, monogalactosylated glycans and glycans with the bisecting GlcNAc were significantly increased, demonstrating a pro-inflammatory IgG activity. Following with the weight-regain period and recovery from the intervention, measured parameters have returned to baseline. The lingering effect of diet and exercise was observed in a reduced form of TNFα, suggesting a prolonged effect of attenuation in levels of systemic inflammation.

Tijardović et al. [173] conducted a small study on male kinesiology students, where 15 of them entered a 6-week repeated sprint training exercise protocol, followed by a 6-week recovery period. After the intervention no statistically significant results were observed, though the trends were pointing towards the pro-inflammatory IgG activity, while after the recovery period significant decrease in agalactosylated N-glycans, and a significant increase in digalactosylated and sialylated N-glycans was observed, suggesting that intense physical activity could have a long-lasting effect, by reducing levels of systemic basal inflammation, similar to what was observed in Sarin et al. study [172], where they reported reduced levels of TNFα, implicating it as the prolonged effect of the intervention.

Considering previous studies, Šimunić-Briški et al. [174] merged the issues of physical inactivity, obesity and the aging population and researched the effects of physical activity in a previously inactive, obese, middle-aged population. 397 participants (50.30 ± 9.23 years, BMI 30.57 ± 4.81) underwent 3 months of three different exercise protocols, with their parameters measured at the beginning and at the end of the intervention. Though their morphological properties have improved, as well as their motor functions, at the end of the intervention IgG glycan profile showed a significant increase in agalactosylated, monogalctosylated, asialylated and core-fucosylated N-glycans, and a significant decrease in digalactosylated, mono- and di-sialylated N-glycans. Even though IgG N-glycan derived traits demonstrated a pro-inflammatory IgG potential, a statistically significant cardio-protective GP9 (FA2[3]G1) [162,175] increase was also noted, possibly an indication of the importance of regular physical activity in female population.

1.6. GLYCANAGE, THE GLYCAN BIOMARKER OF BIOLOGICAL AGE

The glycan clock of age, was first proposed by Krištić et al in 2014. [116], commercially referred to as GlycanAge, can currently predict chronological age with an error of 9.7 years, while associated with multiple physiological and biochemical traits. A prediction model for biological age is based on three different IgG N-glycans [176]. In the last several years, studies have demonstrated that lifestyle changes can reverse the glycan clock of age, highlighting it as one of the promising biomarkers of biological age. One of the first studies measuring the intervention effect on biological age using the Glycan Age index was done by Jurić et al. [122], measuring the effects of estradiol, followed by Šimunić-Briški et al. [177] in a study determining the effects of exercise frequency, intensity and the duration period on biological age.

As the Glycan clock of age is the first available biological clock where reversal was proven possible through lifestyle interventions, Mijakovac et al. [178] performed a study where they checked for heritability. Results showed a heritability estimate of 39% on average, remaining stable in all time points longitudinally, suggesting just a moderate contribution of additive genetic factors to a glycan clock variation, proving that environmental factors, one of which is lifestyle choices, have an important impact on biological age. Glycomic biomarkers are of key importance in suboptimal health status management, as they allow for the prediction, prevention and personalized medicine [179,180]. Recently CellPress has enlisted GlycanAge as a biomarker of aging, associated with multiple diseases, one of the first commercially used tests used to track individual responses to lifestyle changes [181].

1.7. SPORT-RELATED SOFT TISSUE INJURIES

Regular exercise has been proven beneficial for those who exercise regularly, but it does increase the risk of injuries. Recreational athletes, especially those who have returned to sports activities after several years of inactivity, often lead a sedentary lifestyle, with long sitting working hours, making them prone to overuse injuries, affecting tendons a bit more than ligaments. On the other hand, in a similar, yet different manner, competing professional athletes are at risk of overuse injuries due to the high frequency and intensity of their training [182,183]. Most sport-related injuries are non-contact, affecting soft tissues and they occur in ligaments,

tendons, or muscles, common both in competitive sports as well as in physical recreation. Skeletal ligaments are composed of collagenous fibers, forming dense bands that are anchored to the bones at both ends, varying in size, location and shape and performing different functions [184]. Tendons connect muscle to bone and are responsible for force transmission from muscle to bone, enabling joint movement. Physical demands of sports activities may account for tendinopathy incidence in professional and recreational athletes.

1.7.1. Anterior cruciate ligament injuries

A non-contact injury of the anterior cruciate ligament (ACL) occurs when force is generated by the person themselves and excessive loading is applied to the ACL [185]. As sports performance depends on both biological and mechanical properties of the connective tissue, high levels of competing and excessive training lead to overload, thus increasing injury risks [186]. ACL rupture is a very common and severe knee injury, which affects professional as well as recreational athletes. Surgical reconstruction is currently the most widespread method for the treatment of ACL injuries in active patients [187]. Some estimations show that in the United States, there are between 100,000 and 250,000 ACL injuries yearly, costing 17,000-25,000$ per procedure. In Scandinavian countries, reports show 32-38 ACL injuries per 100 000 people, 32 per 100 000 in Germany and 37 per 100 000 in New Zealand [188–190].

Over 60% of ACL injuries are non-contact, women are more prone to non-contact injuries than men under similar training conditions. ACL injury occurs when an excessive force is applied to the ACL, if there was no physical contact between athletes, the injury will be characterized as a non-contact injury [191]. Ligament rupture risk depends on both extrinsic and intrinsic factors [192]. Extrinsic factors include different types and intensities of physical activity, playing surface and footwear, supplements use, nutrition and other physiological factors. Intrinsic risk factors for ACL injury, apart from age and gender are anatomical risk factors that include femoral notch, tibial plateau and ACL geometry, the quadriceps angle, foot pronation, pelvic tilt, body mass index, general joint laxity, and anterior knee laxity. The only hormonal risk factor identified by the literature is the menstrual cycle.

Neuromuscular risk factors are dynamic knee valgus, knee flexors and extensor pre-activation and finally, genetic risk factors include familial predisposition and specific sequence variants within genes [193]. There is a 3 to 6 times higher chance for women to suffer ACL rupture and

one with the ACL rupture will twice more likely have ACL rupture occurrence in close family [194].

1.7.2. Tendon injuries

Tendinitis is a term used to describe chronic pain in the tendon. Achilles tendinopathy (AT) is one of the most common overuse tendon injuries associated with running or jumping and can be acute or chronic [195]. In the general population, AT has an incidence of 1.85 per 1000 [196]. About 30% of runners have exhibited Achilles tendinopathy (AT), with an annual incidence of 7% to 9% [197]. Repetitive movements and overuse are associated with an increased risk of injury. Long-distance runners compared with age-matched controls exhibit an increased incidence of tendinopathy. Patellar tendinopathy is more common in volleyball, basketball, and handball as well as in football [197].

It is still not quite understood the exact underlying causes and intrinsic pathogenic mechanisms of tendinopathies. Both inflammation and degradation are the assumed leading causes, and once again overuse is underlined. When the line between physiology and pathology is crossed, micro-ruptures of tendon fibers no longer promote healing, but with the involvement of inflammatory cytokines, chronic inflammation takes place [198].

Tendons are predominantly composed of water (55-70%). Dry mass consists of 65-80% of collagen fibers (primary, secondary, and tertiary), and structural proteins that provide tissues with tensile strength. The rest of the dry mass is composed of proteoglycans, elastin and other proteins and glycoproteins. Type I collagen makes approximately 85-90% of the total collagen content, type III is the second most abundant collagen, with up to 10% and the rest is made of type V, XI and XIV [199]. Several genes that affect tendon fiber formation have been identified and associated with tendinopathy [200,201].

1.8. Genetic predispositions for occurrence of sport-related soft tissue injuries

1.8.1. Collagens

Mutations within genes that encode collagens cause soft tissue disorders. Several studies have already suggested that genetic predisposition is a significant factor in its etiology. The process is controlled at the transcriptional level. Single nucleotide polymorphisms (SNPs) are a very common variation within a population, each represents one nucleotide difference. SNPs occur every 100-300 base pairs and account for up to 90% of the changes within the genome.

COL1A1

Collagen type I fibrils are a major component of ligaments and form strong parallel bundles of fibers. Two genes that regulate the production of collagen are *Collagen1α1 (COL1A1)* encoding collagen I α1 polypeptide and *Collagen 1α2 (COL1A2)* encoding for collagen I α2 polypeptide, in the 2:1 ratio for A1 collagen. It has been reported that mutations and SNPs located within the *COL1A1* gene are associated with ACL tears [202–205], shoulder dislocations [206], joint laxity [207], bone mineral density [208,209] as well as osteogenesis imperfecta [210]. No associations were found in AT injuries [211]. The polymorphism within intron 1 of *COL1A1*, the Sp1 binding site, is the most widely investigated genetic variant within the *COL1A1* gene. A G>T substitution (SNP rs1800012) occurs at nucleotide 1023 of intron 1.

COL3A1

Collagen Type III, a product of the *COL3A1* gene, encodes pro-α1 chains of type III collagen [212]. The role of Collagen type III is to adjust the flexibility and strength of tissues when expressed [213]. Abnormalities in the *COL3A1* gene and its product, type III collagen, cause various disorders. *COL3A1* SNP rs1800255 (Ala531Thr, A531T, 2009G>A) allele A conferred a 1.71-fold risk for intracranial aneurysms in the Chinese Han population [214], as well as pelvic organ prolapse [215], where AA genotype was significantly associated with the condition. Stepien-Slodowska et al. [216] performed a study on a population of Polish skiers who had anterior cruciate ligament rupture, where the AA genotype was overrepresented. When replicated in the Norwegian and Finnish populations, in a study by Sivertsen et al. [217],

no associations between the risk of ACL ruptures and rs1800255 were observed in female elite athletes.

COL5A1

Collagen Type V has several isoforms, most of them containing α chain encoded by the *COL5A1* gene [218]. Collagen type V, though minor in total fibril percentage (5%), has an important role in the formation of tiny diameter fibrils, expressed in tissues together with the type I collagen. A *COL5A1* gene variant was associated with chronic AT in the South African population in a study done by Mokone et al. [219], replicated in the Australian population by September et al. [220], but in the British population El Khoury et al. [221] noted the association with Achilles tendinopathy only in the male population. Alvarez-Romero et al. [222] showed that the *COL5A1* rs12722 CC genotype was associated with an increased risk of ACL rupture and of ligament injuries in the Japan cohort.

COL12A1

The *Collagen12A1* gene encodes α1 chains of different long (XIIA) and short (XIIB) homotrimeric isoforms of type XII collagen [223]. Posthumus et al. [224] showed the association between the *COL12A1* gene (rs970547, S3058G) and ACL ruptures, but only among female participants. In the following study by Ficek et al. [225] results couldn't be replicated, but when they joined forces, inferred T-A pseudo-haplotype was significantly over-represented in female ACL groups in both South African and Polish cohorts, as well as when combined [226]. Furthermore, the COL12A1 rs970547 was associated with the ACL tear in the Indian population in the study by John et al. [227]. When checked for Achilles tendinopathy, the *COL12A1* gene variants haven't shown statistical significance within the South African population, in a study by September et al. [228].

COL27A1

The *COL27A1* gene encodes the homotrimeric α1 chain of type XXVII fibrillar collagen, located on chromosome 9q32-33, 708 kbp upstream of *Tenascin C (TNC)* gene, similar to minor fibrillar collagens COL5A1, COL5A3, COL11A1 and COL11A2 [229]. Fibrillar collagens are responsible for the structural framework and tensile strength. Saunders et al. [230]

investigated the impact of the *COL27A1* gene variants on the occurrence of Achilles tendinopathy in South African and Australian populations, and while there was no association of single variants with AT in the two populations, the GCA haplotype (rs946053-rs13321-rs2104772) occurred significantly more frequently in TEN participants compared to CON.

1.8.2. Tenascin C (TNC)

Tenascin C (TNC) is an oligomeric, multifunctional, modular glycoprotein of the extracellular matrix (ECM) [231], located on the long arm of chromosome 9 (9q32–q34). The *TNC* gene encodes the tenascin-C glycoprotein, expressed during wound healing and tissue remodeling in adults [232]. High rates of expression are observed in the ECM of musculoskeletal tissues that are under compressive stress during mechanical forces transmission [233]. The *TNC* gene has been significantly overexpressed in Achilles tendinopathy [234], while polymorphisms within the *TNC* gene have been associated with asthma and allergic diseases [235,236]. Gene variants in the *TNC* gene showed an association with AT in the Australian and South African populations in studies done by Mokone at al. and Saunders et al. [237,238].

1.8.3. Matrix metalloproteinase 3 (MMP3)

Matrix metalloproteinases (MMPs) are zinc endopeptidases that degrade connective tissue and the ECM [239]. The stromelysin-I, or matrix metalloproteinase 3 (MMP3) is a broad specter of proteoglycanase, with a key role in the maintenance or remodeling of the soft connective tissues, such as ligaments and tendons [240]. In complete tendon ruptures, as well as in tendinopathies, the *MMP3* gene was often downregulated. *MMP3* is located on chromosome 11 (11q22.3) [241], with several sequence variants that have been associated with different conditions. Chen et al. [242] reported associations with osteoarthritis, as did Barlas et al. [243] in the Turkish population. Ye et al. [244] associated *MMP3* variants with the functional status of rheumatoid arthritis, and Kalichman et al. [245] reported associations with intervertebral disc degeneration. Furthermore, several studies have reported associations between *MMP3* gene variants, in both the ACL rupture category as well as in the category of Achilles tendinopathy. Tendinopathies were associated with polymorphisms within the *MMP3* gene in South African, British, Australian, Chinese Han ancestry and Croatian populations [246–250]. When it comes to ACL ruptures, *MMP3* gene variants were associated in Polish, Thailand and Croatian populations [251–253].

1.9. RESEARCH PROBLEM AND SCOPE OF THE THESIS

Many studies have shown the beneficial impact of exercise on overall health. However, very little is known about the impact of exercise on IgG glycosylation. The only two studies done before this one involved young healthy adult population, in a very small sample size and in the short duration of exercise interventions. One of the hypotheses of this thesis is that training frequency and intensity have an impact on glycan biomarkers of biological age and that professional competing athletes as well as sedentary population will have a higher discrepancy between biological and chronological age.

Every physical activity carries a risk of injuries, both extrinsic and intrinsic. In the last two decades, several studies have associated candidate gene variants with different types of soft tissue injuries. Another hypothesis of this thesis is that there will be a statistically significant correlation between genetic polymorphisms related to tendon and ligament injuries in the population of professional competing athletes that have suffered from soft tissue injuries when compared to the unaffected control group.

The aim of this thesis and the presented scientific papers was to analyze genotype correlation with soft tissue injury occurrence and the discrepancy between biological and chronological age between athletes and controls. Genetic predispositions for soft tissue injuries that often occur in professional, as well as in recreational sports were analyzed in professional competing athletes and in the unaffected control group, by investigating whether there is a statistically significant correlation between genotypes and injury occurrences. The glycan biomarkers of biological age were analyzed in groups of athletes with different training frequencies and in inactive sedentary population to investigate whether there is a discrepancy between biological and chronological age in the aforementioned groups.

2. Regular moderate physical exercise decreases Glycan Age index of biological age and reduces inflammatory potential of Immunoglobulin G

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RESEARCH

Regular moderate physical exercise decreases Glycan Age index of biological age and reduces inflammatory potential of Immunoglobulin G

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Abstract

Physical inactivity and obesity are growing concerns, negatively impacting the general population. Moderate physical activity is known to have a beneficial anti-inflammatory effect. N-glycosylation of immunoglobulin G (IgG) reflects changes in the inflammatory potential of IgG. In this study, GlycanAge index of biological age (GlycanAge), one of the first commercially used biomarkers of aging, was employed to assess effects of exercise intensity in three different groups of athletes: professional competing athletes, regularly moderate active individuals and newly involved recreational individuals, compared to the group of inactive individuals. GlycanAge was significantly lower in the active group compared to the inactive group (β = -7.437, p.adj = 7.85E-03), and nominally significant and increased in professional athletes compared to the active group (β = 7.546, p = 3.20E-02). Competing female athletes had significantly higher GlycanAge comparing to active females exercising moderately (β = 20.206, p.adj = 2.71E-02), while the latter had significantly lower GlycanAge when compared with the inactive counterparts (β = -9.762, p.adj = 4.68E-02). Regular, life-long moderate exercise has an anti-inflammatory effect in both female and male population, demonstrated by lower GlycanAge index, and it has great potential to mitigate growing issues related to obesity and a sedentary lifestyle, which are relentlessly increasing world-wide.

Keywords Glycosylation · GlycanAge index · Biological age · Physical activity · Aging biomarkers

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Introduction

Physical inactivity and obesity are closely intertwined, representing two issues that heavily burden the health care systems in developed countries. Coupled with the increase in life expectancy, there is a growth in both the proportion and number of older adults in total world's population. Currently, the population aged over 60 years is close to a billion, expected to double by 2050 [1].

Obesity is considered one of the modern age epidemics, advancing relentlessly and currently affecting over 2 billion worldwide [2]. Excessive nutrient intake, combined with insufficient physical activity, leads to a chronic positive caloric balance, resulting in increased adiposity that leads to obesity. This condition is directly linked to an increased risk of cardiovascular diseases (CVDs), type 2 diabetes (T2D) and other metabolic disorders. Moreover, it is linked with a reduction in aerobic capacity - cardiorespiratory fitness (CRF) [3]. The definition of CRF is described as the capacity of cardiovascular and respiratory systems to provide oxygen-enriched blood to working skeletal muscles and the muscles' capacity to use oxygen for energy production to produce movement. CRF is used to determine cardiometabolic health, implying that interventions associated with increased physical activity, exercise training and the reduction of sedentary lifestyle would lead to its improvement. Physical inactivity is estimated to be the cause of 6–10% of premature mortality cases, related to CVD, T2D, breast and colon cancers. The global burden of physical inactivity is substantial, increasing rapidly, with the highest relative burden expected in the high-income countries [4].

Physical activity is a bodily movement produced by the contraction of the skeletal muscle that increases energy expenditure above the basal level, as defined by US Center for Disease Control and Prevention (CDC). Physical exercise triggers a cascade of inflammatory events, as every exercise bout leads to micro-damage in the working muscle. During and directly after exercise, a single bout of exercise will trigger an acute inflammatory response in the skeletal muscle, essential for muscle repair and regeneration. However, chronic systemic inflammation may affect and impair the acute anabolic exercise response [5].

The main hallmarks of aging skeletal muscle include the loss of strength and muscle mass, lessened regeneration, and weaker physical performance, along with insulin resistance, mitochondrial dysfunction, and impaired muscle metabolism. Sarcopenia, the decrease in muscle mass and strength, begins in the later stages of the third decade, continuing to the fourth decade of life. By the fifth decade, up to 10% of muscle mass can be lost due to atrophy, caused by a lack of physical activity. A significant decrease in muscle mass occurs after the fifth decade [6].

Aging is characterized by various processes, including muscle loss, a decrease in functional fitness, often followed by changes in body composition. As aging is associated with increased pro-inflammatory signaling, anti-inflammatory processes become impaired with aging. Macrophages within a skeletal muscle have an important role in repair and regeneration. The phenotypic transition of these macrophages from pro-inflammatory M1 to anti-inflammatory M2 is vital for attenuating the inflammatory response, leading to muscle regeneration, promoting myogenesis, and facilitating fiber fusion and growth. Older adults exhibit elevated basal levels of numerous pro-inflammatory cytokines, including IL-6. Immediately post exercise, older adults show greater levels of pro-inflammatory cytokine. In the 24–72 h post exercise period, macrophage infiltration is impaired, failing to recruit M1 macrophages. However, in the following 4–7 days, macrophage levels continuously increase [5]. Furthermore, inflammaging is an age-related process characterized by an increase in pro-inflammatory markers occurring in later stages of life. This is related to higher susceptibility to chronic morbidity, frailty, disability, and premature death. Two of the main treatment options, widely available yet underused, remain caloric restriction and physical activity [7]. Muscle contraction during exercise training induces the production and release of myokines (cytokines produced by muscle cells), including the proinflammatory interleukin 6 (IL-6), which can increase up to 100-fold after exercise [8]. Subsequently, IL-6 induces an increase in the production of the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1ra) and IL-10, stimulating the occurrence of anti-inflammatory cytokines. IL-6 is involved in mediating the pro-inflammatory effects of both the innate and adaptive immune responses, attracting neutrophiles to the site of damage, while being involved in B and T-cell differentiation [9, 10]. Regular exercise training reduces basal IL-6 production, leading to decreased IL-6 plasma levels at rest and in response to exercise, considered to be a training adaptation [11]. Consequently, the introduction of aerobic training in a previously sedentary male population aged 60 and above resulted in significantly lower levels of circulating IL-6 after 3 months of exercise. Moreover, the levels of circulating Il-6 were even lower in age-matched lifelong male exercisers [12]. In professional athletes, cytokine production at severe intensities leads to changes in inflammatory parameters, including increased lactate, endotoxins and TNFα immediately after exercise, followed by higher concentrations of glucose and a trend for increased anti-inflammatory IL-10 one hour post exercise [8]. In moderate exercise, a marked increase in IL-6 and IL-10 occurs, leading to an anti-inflammatory effect by inhibiting TNFα and stimulating IL-1a. Additionally, muscle derived IL-6 produced by exercising muscles promotes

improved glucose tolerance, ultimately having indirect long term anti-inflammatory effects through body composition improvement [9].

Immunoglobulin G (IgG), the most abundant antibody, has a central role in the adaptive immune system. IgG is secreted by plasma B cells and is found in all body fluids. It contains a fragment antigen-binding (Fab) and a fragment crystallizable (Fc) region, with the latter containing a conserved N-glycosylation site. Glycosylation is a ubiquitous posttranslational modification in which glycans are enzymatically added to various proteins and other molecules [13]. The addition of various IgG glycans acts as a switch between pro- and anti-inflammatory status of IgG. The IgG glycome accounts for all types of glycans present in one's IgG molecules, and while it remains stable in homeostasis, it is very responsive to intrinsic and extrinsic environmental factors, aging, or disease development. In aging, the IgG glycome changes distinctively, which led to the development of Glycan Clock, the first biological age biomarker based solely on IgG glycans [14]. Following that discovery and further research into IgG glycosylation in health and disease, IgG glycans were proposed as novel biomarkers in personalized medicine. Many studies since revealed dynamic switches in the composition of IgG glycans, alternation from homeostasis to a proinflammatory in the early onset of various diseases. These conditions include cardiometabolic, autoimmune, neurological diseases; cancers, and infection related inflammation [13]. Additionally, changes related to sex hormones have been observed, with these hormones driving significant shifts during the regular menstrual cycle in women, as well as in menopause, where it was shown that menopause increases biological age, while hormone supplementation reduces it back to a premenopausal state [15]. Further-more, it has been demonstrated that a reduction in body fat in overweight population leads from pro-inflammatory to anti-inflammatory shift [16–18]. Exercise training has a similar anti-inflammatory effect, which is more pronounced in the young population. Tijardović et al. showed that intense physical exercise induces an anti-inflammatory shift in IgG glycans in young male population [19], while Sarin et al. demonstrated that intense physical exercise coupled with caloric restriction in normal BMI female population led to a pro-inflammatory shift, that returned to baseline values in the recovery period [20]. Even though exercise is considered to evoke anti-inflammatory effects, in the middle-aged, sedentary, overweight population, three months of moderate physical activity didn't have the desired effects in that short period, as metabolic changes take longer [21]. In this cross-sectional study, we investigated the impact of exercise duration and intensity on GlycanAge index of biological age (GlycanAge), one of the first commercially used biomarkers of aging, used to track responses to lifestyle interventions [22].

Materials and methods

Participants

The study population included a total of 276 apparently healthy participants, divided into four different groups, depending on their physical activity level. In the inactive group (INACT) there were 99 participants (F = 42 (42.4%), M = 57 (57.6%); average age 44.9 ± 8.65; average height 174.8 ± 9.8 cm, average weight 82.4 ± 10.6 kg; average BMI 27.0 ± 1.95 kg/m2). In the newly involved recreational group (REC) there were 77 participants (F = 67 (87%), M = 10 (13%), average age 50.6 ± 6.0; average height 168.0 ± 7.4 cm, average weight 75.1 ± 9.4 kg; average BMI 26.6 ± 2.1 kg/m2). In the professional competing athletes group (PRO), there were 38 participants (F = 5 (13.2%), M = 33 (86.8%), average age 31.2 ± 8.6; average height 185.8 ± 7.0 cm, average weight 85.5 ± 12.2 kg; average BMI 24.7 ± 2.7 kg/m2). In the regularly moderate active group (ACT) there were 62 participants (F = 24 (38.7%), M = 38 (61.3%), average age 40.9 ± 8.4; average height 176.7 ± 10.1 cm, average weight 76.3 ± 16.2 kg; average BMI 24.2 ± 3.3 kg/m2). All participants reported their prior and current activity level, training intensity where it applies, possible prior injuries and competing status for competing group, as well as their competing sport. Minimal requirements for each group were as follows: professional athletes were either single or team categorized competing athletes in their respective sports (athletics, martial arts, football, basketball, handball, water polo, rowing, hockey), with structured and organized trainings on average one training per day. In the regularly moderate active group participants have had mostly supervised trainings 2–3 times per week regularly, and for minimum of three years prior to entering this study, but most of them were life-long exercisers. In the newly involved recreational group participants have started with regular, supervised moderate physical activity 2 times per week, and they were included in this study after the first exercise season (9 months of moderate, regular physical activity with 1 month of winter break and two months of summer break), after being sedentary and inactive for at least 5 years prior to starting with moderate physical activity. Lastly, the inactive group consisted of sedentary population that has no regular physical activity in their everyday lives, for at least last five years, most of them even longer.

Experimental protocol

All participants underwent the same initial protocol during their yearly check-ups with their medical doctor, voluntarily entering the study, filling in the questionnaire, signing the informed consent and having their morphological characteristics determined. Morphological characteristics included body mass, height, and body mass index (BMI). Following the initial questionnaire, an informed consent was signed, and morphological characteristics were determined, following with collection of dry blood spots (DBS) from each participant. Following the DBC collection protocol, selected finger was wiped with ethanol and air dried. MD performed skin puncturing with a 21 G BD contact-activated lancet (BD, Franklin Lakes, NJ, USA). After the blood drop was formed, it was transferred to form a blood spot on Whatman Human ID Bloodstain Cards, Cat. No. WB100014 (GE Healthcare, Anaheim, CA, USA). The blood spots on collection cards were left to air dry at room temperature for next two hours, afterwards being stored in air-tight zipped bags, containing desiccant at -20 °C [23].

IgG N-glycan analysis was conducted in accordance with the protocol outlined by Šimunović et al. [23], incorporating slight adjustments. Initially, DBS samples were cut into smaller fragments, placed in a 96-well collection plate, and gently shaken for three hours at room temperature with 800 µL 1x phosphate buffer saline (1× PBS, prepared in-house). The isolation of IgG from DBS was performed using CIM® r-Protein G LLD 0.2 mL Monolithic 96-well Plate (2 µm channels) (BIA separations, Ajdovščina, Slovenia) following an optimized high-throughput method [24]. In summary, IgG was eluted with 0.1 M formic acid (prepared in-house) and promptly neutralized with 1 M ammonium bicarbonate (prepared in-house). The resulting IgG eluate (600 µL) underwent overnight drying in a vacuum concentrator. The dried IgG was subsequently desalted with 800 µL methanol (Honeywell, Charlotte, NC, USA) cooled to − 20 ◦C. After resuspension and centrifugation at 2000× g for the duration of 15 min, 78 µL of the supernatant was carefully extracted. This process was repeated with an additional 800 µL of cold methanol, and the protein precipitate was left to dry in a vacuum concentrator for two hours. The enzymatic release of N-glycans from the dried and desalted IgG employed the specific enzyme PNGase F, and the resulting free IgG N-glycans were subsequently labelled with 2-aminobenzamide (2-AB). The fluorescently labelled IgG N-glycans were then subjected to analysis using hydrophilic interaction liquid chromatography (HILIC) ultraperformance liquid chromatography (UPLC) [25]. The obtained chromatograms were manually integrated and categorized into 24 glycan peaks (GP). A comprehensive list of IgG N-glycan structures corresponding to individual glycan peaks is available in Supplementary Fig. 1.

Statistical analysis

To make the glycan measurements comparable across the samples, normalization was performed by expressing the amount of glycans in each peak as % of total integrated area. Next, glycan measurements were log-transformed and batch correction was applied using the "ComBat()" function within the "sva" package in R [26], where the plate was modelled as a batch covariate. Once the estimated batch effects were subtracted from log-transformed values, the values were exponentiated to obtain corrected measurements on the original scale and GlycanAge was derived [14, 15]. Differences in glycan measurements (GP1-GP24) and GlycanAge between groups were determined in pairwise manner (Inactive vs. Active; Inactive vs. Recreational; Inactive vs. Professional; Recreational vs. Active; Active vs. Professional) using regression model where glycan values were included as dependent variable and group was modelled as independent variable with age and sex included as additional covariates. An inverse transformation of ranks to normality was applied to glycan values prior to statistical testing. Differences in GlycanAge were also determined in sex-stratified manner. To control for multiple testing, we performed a correction using the "p.adjust()" function in R, which implements the Benjamini–Hochberg procedure for controlling the false discovery rate (method = "fdr"). An adjusted p-value of < 0.05 was considered as significant. Data analysis and visualization were carried out using R software (version 4.3.0) [27].

Results

The glycosylation of immunoglobulin G was analyzed in 276 healthy participants with different intensity level of physical activity to investigate the impact of exercise intensity on the composition of IgG N-glycans. The N-glycome composition was used to derive the GlycanAge index for each participant.

Participants in the active group, exercising regularly for at least three years, most of them even longer, had significantly lower GlycanAge when compared to the participants in the inactive group (β = -7.437, p.adj = 7.85E-03). Furthermore, differences were even more pronounced when stratified for sex, females in the active group had even lower GlycanAge when compared to the females in the inactive group (β = -9.762, p.adj = 4.68E-02), while the male counterparts had nominally significant effect with same trends, when compared to the male participants in the inactive

Table 1 Pairwise comparisons and effect estimate of exercise on GlycanAge between different intensity groups, after adjusting for chronological age and sex (ALL), and pairwise comparison and effect estimate of exercise on GlycanAge between different intensity groups in two gender groups (F/M) after adjusting for chronological age. Effect size, standard error (SE), *p* value (p). P-values are adjusted for multiple testing using Benjamini-Hochberg procedure to control for false discovery rate (FDR)

Pairwise comparison	ALL/sex-specific	Effect	SE	p	p.adjusted
Inactive vs. active	ALL	-7.44	2.24	1.10E-03	**7.85E-03**
Inactive vs. active	F	-9.76	3.70	1.04E-02	**4.68E-02**
Inactive vs. active	M	-5.91	2.82	**3.86E-02**	1.24E-01
Active vs. professional	ALL	7.55	3.47	**3.20E-02**	1.08E-01
Active vs. professional	F	20.21	6.66	5.41E-03	**2.71E-02**
Active vs. professional	M	4.39	4.07	2.85E-01	4.63E-01
Recreational vs. active	ALL	-8.28	3.27	**1.25E-02**	5.46E-02
Recreational vs. active	F	-8.24	4.13	**4.92E-02**	1.54E-01
Recreational vs. active	M	-6.14	5.52	2.72E-01	4.48E-01
Inactive vs. recreational	ALL	2.82	2.52	2.66E-01	4.46E-01
Inactive vs. recreational	F	2.92	3.10	3.48E-01	5.28E-01
Inactive vs. recreational	M	0.88	4.65	8.50E-01	9.18E-01
Inactive vs. professional	ALL	-0.69	3.31	8.35E-01	9.09E-01
Inactive vs. professional	F	7.59	7.40	3.11E-01	4.94E-01
Inactive vs. professional	M	-2.04	3.78	5.91E-01	7.18E-01

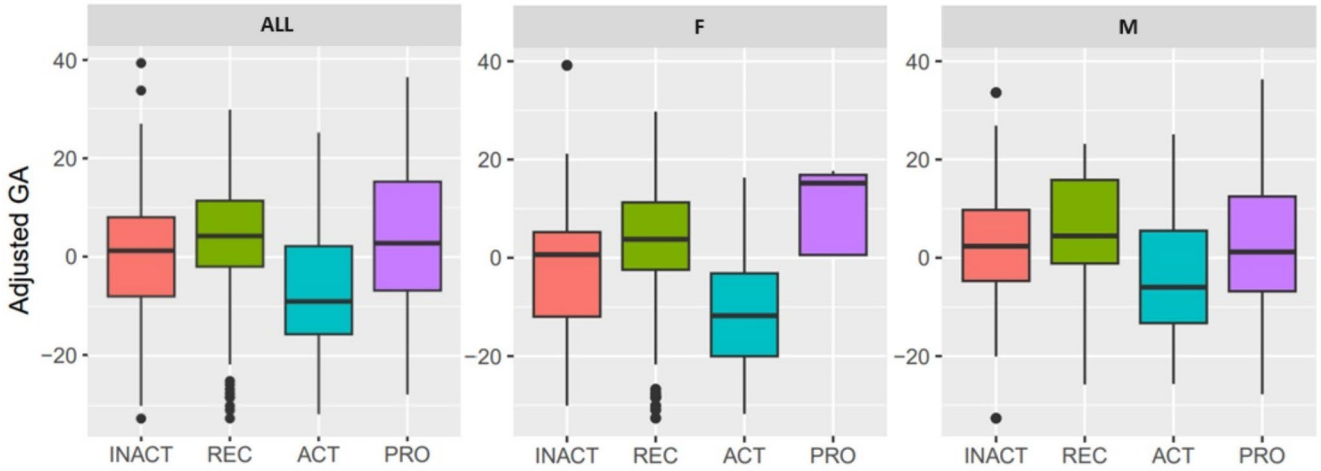

Fig. 1 Box plots showing differences in age adjusted GlycanAge between different intensity groups for all and between genders (F/M). Residual GlycanAge values are plotted after adjusting GlycanAge values for chronological age (red box: inactive group, green box: recreational group, blue box: active group, purple box: professional group)

group (β = -5.911, p = 3.86E-02). Participants in the professional group had a nominally significant effect in the opposite direction, having higher GlycanAge, when compared to the participants in the active group, (β = 7.546, p = 3.20E-02). Following the same trend as mentioned in the pairwise comparison between the active and inactive group; females in the professional group had higher GlycanAge when compared to the females in the active group (β = 20.206, p.adj = 2.71E-02), while male counterparts in the professional group lacked the significant results, when compared to the male participants in the active group (β = 4.388, p = 4.63E-01). Lastly, participants in the active group had nominally lower GlycanAge when compared to the participants in the inactive group (β = -8.278, p = 1.25E-02, p.adj = 5.46E-02). Following the trends mentioned above, females in the active group had nominally lower GlycanAge when compared to the females in the recreational group (β = -8.239, p = 4.92E-02), while male counterparts in the active group lacked the significant results, when compared to the male participants in the recreational group (β = -6.138, p = 2.72E-01) (Table 1; Fig. 1).

Changes in the abundance of specific glycan peaks are observed between different intensity exercise groups and inactive group. When comparing active and inactive groups, GP3, GP5, GP11, GP17, GP19, GP20, GP21 and GP24 were significantly decreased, while GP14 was significantly increased; GP4 was nominally decreased, while GP8 was nominally increased. When comparing active

and professional groups, GP4 was significantly increased, while GP15 was significantly decreased; additionally, GP10, GP11, GP14 and GP16 were nominally decreased. In the comparison of active and recreational group, GP3, GP5 and GP11 were significantly decreased, while GP21 was nominally increased. Comparing the recreational group to the inactive group, GP5, GP17, GP20 and GP21 were significantly decreased. Lastly, comparing professionals to the inactive group we have observed following differences: GP5, GP11, GP13, GP17, GP19, GP20, GP21 and GP24 were significantly decreased, GP8 was significantly increased; while GP3 and GP16 were nominally decreased (effect estimates and p.adj values are presented in Table 2, list of IgG N-glycan structures corresponding to individual glycan peaks is available in Supplementary Fig. 1).

Discussion

In this study, we investigated the effects of exercise intensity on the GlycanAge index, biomarker of aging, in several groups exercising in different manners. The IgG N-glycans reflect pro- and anti-inflammatory events happening throughout one's organism, making them very responsive markers of different types of interventions. The main result of our study shows that the active group has 7.4 GlycanAge years less when compared to the inactive group, tremendously underlying the importance of regular physical activity throughout one's lifetime. When stratifying by sex, the results are even more prominent. Females in the active group exhibit a younger GlycanAge profile, almost for a full decade younger compared to their counterparts in the inactive group, while males are nominally younger by almost 6 GlycanAge years compared to their respective counterparts.

Table 2 Pairwise comparisons and effect estimate of exercise on GP1-GP24 between groups, after adjusting for age and sex. Effect size, standard error (SE), p value (p). P-values are adjusted for multiple testing using Benjamini–Hochberg procedure to control for false discovery rate (FDR). Only data with nominally significant and significant p values is presented. Full data set is available in Supplementary Table 1

Pairwise comparison	trait	Effect	SE	p	p.adjusted
Inactive vs. active	GP5	-1.25	0.14	3.39E-16	**4.58E-14**
Inactive vs. active	GP20	-0.75	0.15	2.23E-06	**5.01E-05**
Inactive vs. active	GP3	-0.69	0.15	5.00E-06	**7.50E-05**
Inactive vs. active	GP24	-0.73	0.15	4.72E-06	**7.50E-05**
Inactive vs. active	GP11	-0.66	0.14	5.77E-06	**7.79E-05**
Inactive vs. active	GP21	-0.65	0.14	1.16E-05	**1.42E-04**
Inactive vs. active	GP17	-0.66	0.15	2.07E-05	**2.33E-04**
Inactive vs. active	GP19	-0.56	0.15	3.62E-04	**3.05E-03**
Inactive vs. active	GP14	0.39	0.12	2.24E-03	**1.26E-02**
Inactive vs. active	GP4	-0.33	0.14	**1.70E-02**	6.95E-02
Inactive vs. active	GP8	0.35	0.16	**2.69E-02**	1.01E-01
Active vs. professional	GP4	0.55	0.17	2.02E-03	**1.23E-02**
Active vs. professional	GP15	-0.59	0.19	2.10E-03	**1.23E-02**
Active vs. professional	GP11	-0.45	0.19	**1.68E-02**	6.95E-02
Active vs. professional	GP16	-0.51	0.22	**2.45E-02**	9.47E-02
Active vs. professional	GP10	-0.48	0.22	**3.13E-02**	1.08E-01
Active vs. professional	GP14	-0.35	0.16	**3.00E-02**	1.08E-01
Recreational vs. active	GP5	-0.65	0.17	2.57E-04	**2.32E-03**
Recreational vs. active	GP3	-0.55	0.20	7.83E-03	**3.77E-02**
Recreational vs. active	GP11	-0.51	0.19	9.56E-03	**4.45E-02**
Recreational vs. active	GP21	0.32	0.15	**3.73E-02**	1.23E-01
Inactive vs. recreational	GP21	-0.77	0.15	6.69E-07	**1.81E-05**
Inactive vs. recreational	GP17	-0.76	0.16	3.00E-06	**5.79E-05**
Inactive vs. recreational	GP5	-0.49	0.16	1.87E-03	**1.20E-02**
Inactive vs. recreational	GP20	-0.48	0.17	5.30E-03	**2.71E-02**
Inactive vs. professional	GP5	-1.32	0.21	4.78E-09	**3.23E-07**
Inactive vs. professional	GP11	-1.21	0.20	1.88E-08	**8.44E-07**
Inactive vs. professional	GP20	-1.32	0.25	3.58E-07	**1.21E-05**
Inactive vs. professional	GP21	-0.96	0.24	7.31E-05	**7.59E-04**
Inactive vs. professional	GP17	-0.98	0.24	9.54E-05	**9.19E-04**
Inactive vs. professional	GP24	-0.85	0.23	4.31E-04	**3.42E-03**
Inactive vs. professional	GP8	0.79	0.22	4.91E-04	**3.68E-03**
Inactive vs. professional	GP13	-0.77	0.24	1.63E-03	**1.10E-02**
Inactive vs. professional	GP19	-0.75	0.25	2.71E-03	**1.46E-02**
Inactive vs. professional	GP3	-0.50	0.21	**2.14E-02**	8.48E-02
Inactive vs. professional	GP16	-0.51	0.24	**3.16E-02**	1.08E-01

It is known that regular physical activity has the beneficial effect on chronic inflammation, as cross-sectional study has reported an inverse association between exercise and inflammatory blood biomarkers [28].

According to data from the European Health Interview Study (EHIS) in Croatia in 2019, almost three quarters of the general population is overweight (BMI > 25) or obese (BMI > 30), accounting for 49.5% and 23.7%, respectively. Only a little over one quarter falls into the normal and underweight categories, comprising 26.6% and 0.3%, respectively, painting a gloomy picture. Furthermore, 2010 data shows that 66% male and 75% female population with a stroke related diagnosis are overweight. Similarly, 78% of males and 74% of females with a hypertension related diagnosis are overweight, as well as 79% of male and 84% of female population with a T2D diagnosis are overweight. Additionally, only 19.5% of the population is physically active for at least 150 min weekly, as recommended by the WHO, while 36% of the population spends 7 or more hours daily in a sedentary manner, excluding sleeping [29]. Metabolic syndrome, a major public health concern, is defined by intertwined risk factors, including central obesity, hypertension, abnormalities in blood sugar stability, as well as an imbalance of lipids. Visceral adipose tissue, with its significant metabolic activity and secretion of pro-inflammatory cytokines and adipokines, plays a crucial role in the pro-inflammatory insulin resistance state. Therefore, changing body composition is of utmost importance. Regular moderate exercise training over time induces changes in body composition, and reduced body fat, ultimately diminishing the pro-inflammatory state, shifting it towards an anti-inflammatory status [30].

The reduction of glycans with the bisecting N-acetyl glucosamine (GlcNAc) is associated with decreased inflammatory potential of IgG, while their increased abundance is associated with T2D and with a higher cardiovascular risk. In the active group, four out of nine glycan peaks containing bisected GlcNAc structures are significantly lower than in the inactive group. An increase in digalactosylated and sialylated glycans is associated with anti-inflammatory IgG potential, while an increase in agalactosylated and asialylated structures is associated with a pro-inflammatory status.

Professional competing athletes train at higher intensities, with higher training frequency, resulting in less time in between to recover. Compared to the active group that practices moderate exercise, our results show that the group of professional athletes has 7.6 more GlycanAge years. Similarly, as mentioned above, when stratified by sex, females in the professional group show a dramatically older GlycanAge profile, two full decades older, compared to their counterparts in the active group. It is important to underline that there is a very limited number of professional female athletes in this study (N = 5), but somewhat similar results were reported in the study performed by Sarin et al. [20]. In the aforementioned study, Sarin et al. investigated the impact of prolonged intensive exercise training, coupled with a low-calorie diet in 42 healthy women (age 27.5 ± 4.0 years, body mass index 23.4 ± 1.7 kg/m2). After 4 months of intervention, resulting in intense weight loss, the test group had a significant pro-inflammatory IgG N-glycan profile. This was characterized by a decrease in glycan peaks containing digalactosylated and sialylated glycans, while glycans with bisecting GlcNAc, agalactosylated, and monogalactosylated glycans were significantly increased when compared to the control group.

Jurić et al. reported a significant effect of estradiol on biological age in females, which could be one of the reasons why professional female athletes have a significantly higher GlycanAge than their counterparts, as professional athletes often have a very low percentage of body fat, which is known to potentially interfere with the menstrual cycle [15]. They showed that the deprivation of gonadal hormones results in a median increase in GlycanAge for 9.1 years, suggesting a significant influence of hormone levels on IgG glycans. On the other hand, in the male population, this trend of a significantly increased GlycanAge between professional athletes and the active male population was not observed. Tijardović et al. did not report any significant pro-inflammatory increase in IgG N-glycans in their repeated sprint training male cohort (age 20.0 ± 1.0 years, height 181 ± 4.0 cm, weight 77.7 ± 6.0 kg) [19]. In our study, male professional athletes did not have such a prominent effect, showing no significant differences compared to their respective counterparts in the active group.

These results, combined with ours, imply that intense physical activity has the adverse effects on both male and female population. Unlike regular moderate physical activity, exercising at high intensities causes oxidative damage, triggering an immediate immunological response. High intensities stimulate oxygen consumption, leading to the formation of reactive oxygen species (ROS) produced by mitochondrial activity and increased aerobic respiration. Oxidative stress occurs when there is an imbalance between ROS formation and antioxidant response [30]. Furthermore, chronic exhaustive training is considered the cause of an imbalance between Th1 and Th2 profiles, with Th2 becoming predominant, resulting in cellular immunosuppression, inflammation, and increased susceptibility to infections [31, 32]. Overtraining syndrome (OTS) in professional athletes leads to an overall degradation of performance and results, with the immune system being particularly compromised. To prevent OTS, monitoring of exercise training load and

other stressors is necessary to detect the development of OTS in a timely manner [33].

Regular moderate exercise training has beneficial effects over time. When comparing a group of recreational exercisers to the inactive group, we did not observe any significant results, mainly to the underlying effect of obesity in the beforementioned group. The lack of physical activity, coupled with an unhealthy diet and a sedentary lifestyle, is likely to increase bodyweight. Almost three quarters of our general population are overweight and obese [29], so many individuals introduce moderate exercise at the latter stages of body weight gain. Higher levels of body fat are associated with a pro-inflammatory IgG N-glycan profile, while weight loss and the reduction of body fat substantially affect IgG N-glycosylation, resulting in an anti-inflammatory shift and ultimately leading to reduced biological age and lower GlycanAge index [16–18]. As our study populations were smaller in number, without optimal gender ration, further research would be beneficial to verify our results. Additionally, longitudinal studies involving inactive, active, and professional groups would further expand the knowledge of the impact of training frequency and intensity on the inflammatory status of athletes. High amount of sedentary time, coupled with low amount of moderate to high intensity physical activity and low CFR are associated with poor metabolic health and higher metabolic syndrome and T2D risk [34]. Even the increase from low to moderate amount of moderate physical activity, while reducing sedentary time will increase CRF, resulting in reduced risks of cardiometabolic disorder occurrence, leading to a healthier life.

Conclusion

In summary, our results are reporting a decreased GlycanAge index in an active group of moderately exercising population, while both the inactive group and professional athletes group, when compared to the active group, demonstrate an increased GlycanAge index. Our findings support the notion that moderate physical exercise is a widely available, anti-inflammatory strategy with mild to no side effects for the general population. Physical activity has great potential to mitigate growing issues related to obesity and sedentary lifestyle, which are directly linked to an increased inflammatory status and the development of non-communicable diseases.

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Data Availability The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the Faculty of Kinesiology, University of Zagreb (31/2017) and Ethics Committee of School of Medicine, University of Zagreb (380-59-10106-18-111/110).

Conflict of interest G. Lauc is the founder and owner of Genos Ltd, a private research organization that specializes in high throughput glycomic analyses and has several patents in this field. N. Šimunić-Briški, M. Vinicki and A. Frkatović-Hodžić are employees of Genos Ltd. G. Lauc is also co-founder of GlycanAge Ltd and is listed as an inventor on patents EP3011335B1 and US9910046B2 that protect the use of GlycanAge® to predict biological age. All other authors declare no conflicts of interest.

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RESEARCH ARTICLE

MMP3 single-nucleotide polymorphisms are associated with noncontact ACL injuries in competing high-level athletes

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Abstract

Matrix metalloproteinases (MMPs) play an important role in matrix remodeling, as well as in ligament integrity. Anterior cruciate ligament (ACL) rupture is a severe and frequent knee injury in sports. The aim of this study was to investigate polymorphisms within the *MMP3* gene with the predisposition for noncontact ACL rupture in the Croatian professional athletes. One hundred eighty-seven (95 with ACL rupture occurring through a noncontact mechanism and 92 asymptomatic controls) unrelated Caucasians were recruited between 2016 and 2017. All participants were genotyped for three single-nucleotide polymorphisms (SNP) within the *MMP3* gene: rs591058 C/T, rs650108 A/G, and rs679620 G/A using the pyrosequencing method. For all three investigated SNPs, genotype frequencies have significantly differed between cases and controls. The MMP3 rs591058 TT ($p = 0.0012$, odds ratio [OR] = 38.541, 95% confidence interval [CI] = 1.7024–8.7254), rs650108 GG ($p = 0.0051$, OR = 23.338, 95% CI = 1.2899–4.2226) and rs679620 AA ($p = 0.0030$, OR = 34.750, 95% CI = 1.5266–7.9101) genotypes, as well as haplotype variant T-G-A ($p = 0.0104$, OR = 1.71, 95% CI = 1.13–2.59) were significantly overrepresented in cases compared to controls. These results support association between functional variants within the *MMP3* gene and the risk of ACL rupture. Still, further research is needed to corroborate these results in a larger population.

KEYWORDS

ACL Rupture, Genetic Association Study, Matrix Metalloproteinase 3, Single-nucleotide polymorphism, Sport Injury

1 | INTRODUCTION

Knee ligament injuries occur frequently during sport activities, primarily via noncontact mechanism, affecting both professional and recreational athletes. Skeletal ligaments, composed of collagenous fibers, form dense bands and are anchored to the bones at both ends, varying in size, location, and shape.[1] The anterior cruciate ligament (ACL) is one of four main knee ligaments, consisting of two major fiber bundles, anteromedial bundles (AM) and posterolateral bundles (PL). One of the most common sport injuries is the rupture of the ACL in the knee joint. In most cases, ACL injury occurs when an excessive force is applied and an excessive loading is applied to ACL.[2] Surgical reconstruction is currently most widely spread method for treatment of ACL ruptures in active patients,[3] as healing never occurs and it is the general practice for patients who develop knee instability, if not dealt in conservative manner. ACL rupture usually leads to devepoment of knee osteoarthritis.[4] ACL tears occur in 64% of athletic knee injuries and these injuries result in roughly 120,000–200,000 ACL reconstructions performed annually in United States of America, with estimated costs of 1.7 billion USD.[5] ACL

rupture risk depends on both extrinsic and intrinsic factors. Extrinsic factors include type and intensity of physical activity, type of footwear and playing surface, medication use, nutrition, and other physiological factors. Intrinsic factors include age, gender, anatomical factors, laxity and flexibility, phase of the menstrual cycle, familial predisposition, and genetic polymorphisms. Incidence of ACL rupture is four to six times higher in women than in men[6] and individual that has suffered ACL rupture will twice more likely have ACL rupture occurrence within a family.[7] Several studies have already suggested that genetic predisposition is a significant factor in its etiology,[8] one study showed that relatives of athletes with bilateral ACL injuries had increased incidence of ACL injuries compared to unrelated controls.[9] Matrix metalloproteinases (MMPs) are members of zinc-endopeptidases that regulate cell matrix composition and can degrade extracellular matrix.[10] MMPs are composed of 25 enzymes, divided into collagenases, gelatinases, stromelysins, matrilysins or membrane-type MMPs. MMP3, member of stromelysins, is responsible for extracellular matrix remodeling and degradation, cell proliferation, and angiogenesis.[11] Single-nucleotide polymorphisms (SNPs) account for 90% of the changes and are the most common variations, which occur every 100–300 base pairs throughout the genome. Several *MMP* genes, including *MMP1*, *MMP3*, *MMP10*, and *MMP12* are located on chromosome 11 (11q22.3).[12] Several sequence variants have been associated with different conditions and disorders, such as lumbar disc degeneration,[13] osteoarthritis,[14] and rheumatoid arthritis,[15,16] as well as chronic Achilles tendinopathy[17,18] and ACL rupture.[19–21] As the different ethnic populations can exhibit different heterogeneity,[22,23] it is important to carry out research with different populations. The aim of this study was to investigate whether the polymorphisms within the *MMP3* gene are associated with the susceptibility of ACL ruptures in Croatian subpopulation.

2 | METHODS

Our study is case–control study (Level 3).

2.1 | Participants

In this case–control genetic association study a total of 187 participants were recruited between 2016 and 2017. They were all either recreationally competing or professional athletes, self-reported Caucasians and unrelated. The ACL rupture group consisted of 95 participants (69 male and 26 female, average age 25.8 years), diagnosed with magnetic resonance and arthroscopically confirmed ACL rupture through the noncontact mechanism of the injury. All ACL participants were active competing athletes (soccer, handball, basketball, martial arts, skiing), having between three and seven coach supervised trainings per week, recruited at the time of the injury in the Orthopedic Clinic or by a team doctor specialized in orthopedic surgery. Control group consisted of 92 participants (72 male and 20 female, average age 39.0 years) that have finished their active athlete career and have reported not sustaining any ligament ruptures throughout their athlete career. Main selection criteria for the control group were recruited athletes who retired and have finished their professional career without sustaining ACL injury. Through their professional career control group was closely monitored by obligatory medical exams every year and their club doctors if they had any injury. In the time of the recruitment, they were all without any self-reported injuries. If someone from control group had any symptoms of knee injury at the time of recruitment orthopedic surgeon with over 30 years of clinical experience with sports and knee injury would do clinical exam and tests. Participants from control group were not subjected to MRI exam or knee arthroscopy for the purpose of this study. Control recruitment was monitored by two experienced observers to ensure background quality of the recruited controls. Orthopedic surgeon has over 30 years of clinical experience with sports and knee injury, working as chief officer at football club and national football team, his area of expertise being knee ligament reconstruction and retired professional athlete who is working as a professor at the Faculty of Kinesiology, specialized in biomechanics of movement and injury prevention exercise programs. This study was approved by the medical Ethics committee of the School of Medicine and by the Ethics committee of the Faculty of Kinesiology, University of Zagreb where the research was done. All participants have given written informed consent and have provided a biological sample for analysis.

2.2 | Genotyping

Genomic DNA was extracted from the oral epithelial cells using QIAamp DNA kit (Qiagen), followed by PCR amplification using MMP3 rs591058, rs650108, and rs679620 specific primer pairs (Metabion) and PyroMark PCR Kit (Qiagen). Amplified fragments were sequenced by pyrosequencing method (PyroMark Q24; Qiagen) according to the manufacturer's protocol and analyzed by Pyromark Q24 software.

2.3 | Statistical analysis

An odds ratio method was used for allelic, genotypic, and haplotype differences, respectively, using Statcalc program, AcaStat software. A statistically significant difference was defined when p was $p < 0.05$. Haplotype analysis was performed using Phase software. The Hardy–Weinberg equilibrium and linkage disequilibrium (LD) analyses were done by the Arlequin software version 3.5 (Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland). In this study, we used groups similar in size to previously reported studies[20,22,24,25] investigating genotype effects on various soft-tissue injuries, as those group sizes proved to be large enough to detect significant results, so power analysis was not performed. The Bonferroni correction was not used in this study, as it is considered

too conservative.[26] *P* values were adjusted for false discovery rate (FDR) using Benjamini–Hochberg procedure for adjusted *p* value. It was applied for each genotypic, allelic, and haplotype separately.[27]

3 | RESULTS

3.1 | Participants characteristics

Participants were divided into two groups: self-reported noncontact mechanism of ACL rupture occurrence and control group that has not self-reportedly suffered from any ligament injuries during their active years in competing professional sport. The control group was significantly older and heavier, with the higher BMI index than ACL group, but that is explained by the different lifestyle of the active professional athletes and retired athletes. Due to the similar average heights, we have assumed weight and BMI were similar between ACL rupture group and controls, when controls were active competing athletes, but at the presented data are not matched for weight and BMI, only for height, ethnicity, and gender (Table 1).

Genotype frequencies were significantly different between groups, TT genotype of rs591058, GG genotype of rs650108, and AA genotype of rs679620 were significantly overrepresented in ACL rupture group and associated with predisposition for ACL injury. Similarly, allelic frequencies significantly differed between groups. C allele of rs591058, A allele of rs650108 and G allele of rs679620 were significantly overrepresented in controls compared to cases and therefore classified as protective, while T allele of rs591058, G allele of rs650108 and A allele of rs679620 were significantly overrepresented in cases comparing to controls thus being considered as a predisposition for injury (Table 2).

All polymorphisms conformed to the Hardy–Weinberg equilibrium in both the ACL rupture and the control group (Table 2). SNPs rs591058, rs650108, and rs679620 were also in the LD, $D' = 1.000$; $r^2 = 0.4014$ and $D' = 0.8893$; $r^2 = 0.3242$, respectively. Apart from Bonferroni correction being considered too conservative for these types of studies, there is no need for it, as all three SNPs are in LD, meaning they are inherited as blocks.

Haplotypes constructed from all possible combinations of polymorphisms were analyzed (only relevant data is shown in Table 3). As all three polymorphisms are in LD, it was expected for T-G-A haplotype to be significantly overrepresented in cases, being associated with predisposition for ACL rupture, while C-A-G haplotype was significantly overrepresented in controls, being associated with protection.

TABLE 1 General characteristics of the ACL rupture (ACLR) group and control group.

	ACLR (*n* = 95)	Controls (*n* = 92)	*p* Value
Age (years)	25.8 ± 8.3	39.0 ± 11.4	**0.0001**
Height (cm)	178.8 ± 7.3	179.8 ± 9.6	0.4349
Weight (kg)	77.5 ± 11.8	84.6 ± 15.4	**0.0005**
BMI (kg/m^2)	24.2 ± 2.9	26.0 ± 3.3	**0.0001**
Ethnicity (Caucasian)	100% (95)	100% (92)	1.0000
Gender (% male)	73% (95)	78% (92)	0.4270

Note: Age, height, and weight are self-reported values in time of the recruitment. Ethnicity and gender are represented as a percentage, the remaining variables are expressed as a mean ± standard deviation. Significant *p* values are in bold.

4 | DISCUSSION

The main finding of this study was the allelic and genotype association of the investigated SNPs, TT genotype of rs591058, GG genotype of rs650108, and AA genotype of rs679620 within *MMP3* gene for the ACL rupture, as well as their haplotypes, showing a significant association with the risk of ACL rupture in Croatian subpopulation.

The *MMP* gene family codes for over 20 different zinc-dependent endopeptidases. MMP3 is a proteoglycan-degrading enzyme that plays a significant role in degradation of collagens and other structural components, mainly proteoglycans, fibronectin, laminin, aggrecan, and gelatine.[28] Out of 23 MMPs in humans, 9 of them form a cluster on the long arm of chromosome 11, resulting in an almost perfect LD of rs591058 and rs679620, and both being in high LD with the third rs650108.[18]

The MMP3 SNP rs679620 was previously associated with ACL rupture as part of the inferred haplotype in a South African cohort, together with the with the MMP10 rs486055C/T, MMP1 rs1799750 1G/2G, and MMP12 rs2276109 A/G polymorphisms, but there was no independent association for MMP3 rs679620, though GG genotype had a trend toward overrepresentations in controls,[29] which is in accordance with the data we have presented in our study, having statistically significant overrepresentation of GG genotype in our controls. In an additional study of South African cohort, investigating same MMP3 SNPs included in the present study, there were no significant associations between MMP3 SNPs and ACL group where rupture originated from both contact and noncontact mechanism of injuries,[22] possibly due to a nonhomogeneous group.

The 5A/6A polymorphism in the promoter region of the *MMP3* gene affects the regulation of *MMP3* gene expression. Study of the Thailand cohort found a significant difference for rs3025058 (5A/6A, MMP3 promoter) in genotype 5A/5A between the ACL patients with contact sport and without contact sport, while in allelic association significant differences were 6A: 81.2% in contact sports versus 89.1% in noncontact sports and 5A: 18.8% in contact sports versus 10.9% in noncontact sports.[20] More recent study of the Polish cohort found the MMP3 rs679620 G allele and the MMP3 rs591058 C allele could predispose for ACL ruptures in physically active Caucasians,[19] which is contradictory to our findings.

Different risk associations have already been reported in populations with independent ancestry, and even though the reason

TABLE 2 Allele and genotype frequency for MMP3 SNP.

MMP3			Allele frequency					
SNP rs591058	**C>T**	**ACLR (*n* = 95)**	**CON (*n* = 92)**	***p* Value**	**FDR**	**OR**	**95% CI**	**Association**
1	C	48.9% (91)	62.5% (115)	**0.0047**		0.5515	0.3651–0.8331	**Protection**
2	T	52.1% (99)	37.5% (69)	**0.0047**	**0.0047**	1.8132	1.2004–2.7388	**Predisposition**
			Genotype frequency					
SNP rs591058	**C>T**	**ACLR (*n* = 95)**	**CON (*n* = 92)**	***p* Value**	**FDR**	**OR**	**95% CI**	**Association**
11	CC	25.3% (24)	34.8% (32)	0.1567		0.6338	0.3372–1.1913	None
12	CT	45.3% (43)	55.4% (51)	0.165		0.6648	0.3736–1.1831	None
22	TT	29.4% (28)	9.8% (9)	**0.0012**	**0.0024**	38.541	1.7024–8.7254	**Predisposition**
HWE		0.364	0.080					
MMP3			**Allele frequency**					
SNP rs650108	**A>G**	**ACLR (*n* = 95)**	**CON (*n* = 92)**	***p* Value**	**FDR**	**OR**	**95% CI**	**Association**
1	A	27.4% (52)	39.1% (72)	**0.0161**		0.5862	0.3793–0.9057	**Protection**
2	G	72.6% (138)	60.9% (112)	**0.0161**	**0.0153**	1.706	1.1041–2.6362	**Predisposition**
			Genotype frequency					
SNP rs650108	**A>G**	**ACLR (*n* = 95)**	**CON (*n* = 92)**	***p* Value**	**FDR**	**OR**	**95% CI**	**Association**
11	AA	9.5% (9)	12% (11)	0.5836		0.7706	0.3035–1.9566	None
12	AG	35.8% (34)	54.3% (50)	**0.0112**	**0.0168**	0.4682	0.2604–0.8419	**Protection**
22	GG	54.7% (52)	33.7% (31)	**0.0051**	**0.0161**	23.338	1.2899–4.2226	**Predisposition**
HWE		0.331	0.177					
MMP3			**Allele frequency**					
SNP rs679620	**G>A**	**ACLR (*n* = 95)**	**CON (*n* = 92)**	***p* Value**	**FDR**	**OR**	**95% CI**	**Association**
1	G	48.4% (92)	62.5% (115)	**0.0064**		0.5633	0.3729–0.8507	**protection**
2	A	51.6% (98)	37.5% (69)	**0.0064**	**0.009**	1.7754	1.1754–2.6815	**predisposition**
			Genotype frequency					
SNP rs679620	**G>A**	**ACLR (*n* = 95)**	**CON (*n* = 92)**	***p* Value**	**FDR**	**OR**	**95% CI**	**Association**
11	GG	24.2% (23)	34.8% (32)	0.1142		0.5990	0.3171–1.1313	None
12	GA	48.4% (46)	55.4% (51)	0.3376		0.7547	0.4246–1.3416	None
22	AA	27.4% (26)	9.8% (9)	**0.0030**	**0.0064**	34.750	1.5266–7.9101	**Predisposition**
HWE		0.765	0.080					

Note: Allele and genotype frequencies are expressed as percentage with the number of participants (*n*) in parentheses. Results that have been classified as significant by the *p* value ($p < 0.05$) in bold and have been additionally checked for false discovery rate (FDR), using Benjamini–Hochberg adjusted p value.

Abbreviations: CI, confidence interval; HWE, Hardy–Weinberg equilibrium; OR, odds ratio.

TABLE 3 Haplotype frequency.

Hap code	Haplotype	CON	ACLR	*p* Value	FDR	OR	95% CI	Association
1	C-G-G	43	40	0.51		0.85	0.52–1.39	None
2	C-A-G	71	50	**0.0074**	**0.0222**	0.55	0.35–0.85	**Protection**
3	C-A-A	0	3	–	–	–		
4	T-G-G	0	2	–	–	–		
5	T-G-A	68	97	**0.0104**	**0.0156**	1.71	1.13–2.59	**Predisposition**

Note: Haplotype Hap code 3 and 4 were not predicted by PHASE in our samples. Results that have been classified as significant by the *p* value ($p < 0.05$) in bold and have been additionally checked for false discovery rate (FDR), using Benjamini–Hochberg adjusted *p* value.

Abbreviations: CI, confidence interval; OR, odds ratio.

is not fully understood, it has been hypothesized that study participants indirectly implicate a region where biologically informative variants can be found.[23] Apart from independent ancestry, the main difference in our study is that our control group that was not matched for age and weight with the ACL rupture group. We wanted to make sure controls never suffered from ligament injuries through their active competing career in professional sport, to have a real control group. If control group were active competing athletes, we could not be sure they would not suffer from ACL injury in their active sports future. To eliminate that, we decided to recruit participants after their competing sport career ended, so they are older than cases and their body weight is somewhat higher, but that does not reflect their body composition when they were actively competing. It is fair to assume their weight and BMI would be matched to an active ACL rupture group.

The Polish cohort had a group of recreationally active controls that cannot match volume of training when compared to professional athletes, and controls were matched for age with the ACL rupture group, aging only ~25 years, so it cannot be known whether they will suffer from ACL rupture in the future, especially as they do not have same training load as the ACL rupture group.

Taking into the account the importance of investigating a noncontact mechanism of injury as they occur due to the athlete's own movements, without any external force acting, we have included only noncontact mechanism of injury in our ACL rupture group. In a noncontact injury, that has higher intrinsic component than a contact injury, mechanisms are classified as sudden deceleration before the change of direction or before landing after jump. Contact injuries involve contact with another person, usually via collision or accident or unknown source.[30] Cost of ACL rupture is from $17,000 to $25,000 per injury, with conservative estimates of surgery and rehabilitation.[31] ACL injuries are particularly significant as 80% of all knee ligament injuries result in surgery and in general population sport activities account for 65% of those injuries.[32] Additional loss for professional athletes is absence from trainings, games, and competitions. Sport clubs also suffer significant financial losses. In some cases, injured athlete needs to be temporarily replaced with uninjured one, and that causes additional financial issues in professional sport. The results presented in this study could be used in future clinical practice when interpreted with caution. Limitations of this study are the small sample size, so any interpretation of the results should be done with caution. Gender distribution between groups was matched, but both consisted of a larger proportion of male cases, even though it was previously reported that females are in greater risk.[33–35] To our knowledge, this is a first study that has investigated MMP3 SNPs related to a noncontact injury of ACL in competing athletes, while having a control group of retired competing athletes who have finished their sport careers without ACL injuries. Future studies should recruit larger number of participants in both groups but should also consider the significance of controls that have had all the possibilities to suffer an injury and they have still finished their active career without them. Though they are not matched for age and weight with injury group, their genetic makeup gives a better picture than control groups consisting of active recreational athletes whose future and injury prospective is unknown in the long run.

5 | PERSPECTIVE

Genetic studies are novel field in the sports medicine. Our research is small step forward in understanding intrinsic risk factors for ACL rupture. Many books and articles wrote about connection of genes and ACL ruptures, but scientific evidence was poor.[7–9] This study showed genetic association between genotypes (TT genotype of rs591058, GG genotype of rs650108, and AA genotype of rs679620) and ACL rupture in professional athletes. With this knowledge we can develop “easy to use” screening tests for ACL rupture (ligament injury) in young athletes who have increased risk for injury. In this way coach or club can introduce preventive exercises, correct wrong biomechanical patterns and introduce better recovery protocols for young competing athletes with positive screening test for ACL rupture.

6 | CONCLUSION

There is an increased amount of genetic case–control association studies trying to identify genetic sequence variants within genes coding for soft tissue building blocks and extracellular matrix to associate them with the risk of developing soft tissue injuries. This study investigated one small fraction of variants within the gene coding for proteoglycan-degrading enzyme and whether three functional variants of the MMP3 gene are associated with a noncontact manner of ACL rupture in competing athletes. A novel finding of this study are significant associations of investigated genotypes (TT genotype of rs591058, GG genotype of rs650108, and AA genotype of rs679620) that were significantly overrepresented in ACLR group and associated with predisposition for ACL injury.

AUTHOR CONTRIBUTIONS

Nina Simunic-Briski, Goran Vrgoc, Zlatko Dembic, and Gordan Lauc substantially contributed to research design, acquisition, analysis, and interpretation of data, drafting the paper and revising it critically, approval of the submitted and final versions. All authors have read and approved the final submitted manuscript. Sasa Jankovic and Damir Knjaz substantially contributed to acquisition, analysis, and interpretation of data, drafting the paper and revising it critically, approval of the submitted and final versions.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The study was approved by the Medical ethics committee of the School of Medicine, University of Zagreb and by the Ethics committee of the Faculty of Kinesiology, University of Zagreb.

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4. Association of the matrix metalloproteinase 3 (MMP3) single nucleotide polymorphisms with tendinopathies case-control study in high level athletes

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ORIGINAL PAPER

Association of the matrix metalloproteinase 3 (MMP3) single nucleotide polymorphisms with tendinopathies: case-control study in high-level athletes

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Abstract

Background Matrix metalloproteinases (MMPs) play an important role in matrix remodelling, as well as in tendon integrity. Due to overuse, athletes often develop chronic tendinopathies. If not treated, they lead to severe impairment, even complete tendon ruptures.

Aim The main purpose of this study was to investigate whether three functional polymorphisms within the MMP3 gene are associated with increased risk of developing tendinopathies in high-level Croatian athletes.

Methods We have recruited one hundred fifty-five (63 high-level athletes with diagnosed tendinopathies and 92 asymptomatic controls) unrelated Caucasians for this case-control genetic study. All participants were genotyped for three single nucleotide polymorphisms (SNP) within the MMP3 gene: rs591058 C/T, rs650108 A/G and rs679620 G/A using the pyrosequencing method.

Results The MMP3 rs650108 GG ($P = 0.0074$) and rs679620 AA ($P = 0.0119$) genotypes were significantly over-represented in cases compared with controls, while rs591058 TT ($P = 0.0759$), as well as haplotype variant T - G - A ($P = 0.06$), implicated that there is an indication of predisposition for tendinopathies.

Conclusion These results support association between functional variants within the MMP3 gene and the risk of tendinopathies in high-level athletes. Further research is needed to replicate these results in a larger population.

Keywords Matrix metalloproteinase 3 (MMP3) · Single nucleotide polymorphism · Genetic association study · Achilles tendinopathy · Sport injury

Introduction

Soft tissue injuries occur frequently during sport activities, affecting muscles, tendons and ligaments. They can be classified either as acute or chronic injuries, so-called overuse injuries. Overuse injuries mostly are result of the repetitive microtraumatic events occurring over longer periods of time. Overuse injuries may be defined as an imbalance caused by overly intensive training and inadequate recovery, which subsequently leads to a breakdown in tissue reparative mechanisms [1, 2].

In sport, chronic tendinopathies (tendinitis/tendinosis) are of more interest, as they impact athlete's abilities to compete, and if not treated, they may result in full tendon ruptures [3]. Achilles and patellar tendon are the most commonly affected in lower extremities, while rotator cuff and tennis elbow (extensor carpi radialis brevis) tendons are the ones affected in

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upper extremities [4, 5]. Achilles tendon rupture is an example of acute injury, while Achilles tendinopathy is an example of the chronic overuse and is a common cause of disability in recreational and professional athletes [6]. In the last few decades, tendon injury occurrence has increased, and it is reported to be between 30 and 50% of sport-related injuries and is mostly traced back to exercise-related overuse [7]. The investigations of Achilles tendon injuries in collegiate-level athletes in the USA identified an injury rate (IR) of 2.17 (per 100,000 AEs). These injuries occurred most often in women's gymnastics (IR = 16.73), men's basketball (IR = 4.26) and women's basketball (IR = 3.32), respectively [8].

Tendons have inelastic structure and therefore can resist very high forces. As most of the connective tissues, 70% of total tendon weight is made of water, while dry weight of tendons is predominantly composed of collagens (collagen I, III, V, XII, XIII), ranging from 60 to 85%, the rest being made of different proteoglycans and glycoproteins [9]. Tendinopathy is a condition varying from the asymptomatic condition visible with ultrasound (US) or magnetic resonance imaging (MRI) to severe chronic changes that cause discomfort, pain and functional impairment. Chronic tendinopathy is becoming more and more frequent in both professional and recreational athletes, where professionals mostly train over their limits in order to achieve better results, while recreational athletes without trainer supervision do not adjust intensity of periodic training to their age and physical condition. As the result, professionals will not be able to participate in training and competition for a longer period of time, while recreational athletes might completely give up on physical activity, which has a consequence on burdening public health system. Australian study estimated direct annual health care costs to be around $377 million per year credited to physical inactivity [10]. Flemish study showed that total medical costs due to the sport injuries were 0.08% (over 15 million €) of the total budget spent on health care and 3.4% (over 111 million €) of the costs arising from absence from work [11]. Tendinopathies are followed by many molecular changes, especially in the extracellular matrix (ECM) where balance between degradation and synthesis is usually affected. Matrix metalloproteinases (MMPs) are members of zinc endopeptidases that can degrade connective tissue and ECM [12]. The matrix metalloproteinase 3 (MMP3), also known as stromelysin-I, is a proteoglycanase of a broad spectre and plays a major role in remodelling and maintenance of a normal tendon [13]. MMP3 is found to be downregulated in tendinopathies and in complete tendon ruptures. A genetic contribution has been proposed for several tendon injuries. Single nucleotide polymorphisms (SNPs) are sites located throughout the genome at which more than one nucleotide is found in a population. MMP3 genes, including MMP3, are located on chromosome 11, 11q22.3. Several studies have found association between the variants within the MMP3 gene and tendon injuries [14–17], as well as with rotator cuff injury [18], arthritis [19] and ACL injuries [20]. To estimate a correlation between genotypes and injuries, it is important to include as many different populations as possible, due to the exhibition of different heterogeneities in different ethnic populations. The aim of this research was to study the polymorphisms within the MMP3 gene associated with the tendinopathies in Croatian athlete population.

Methods

Participants

In this case-control genetic association study, a total of 155 participants were recruited between 2016 and 2017. They were all physically active athletes, self-reported unrelated Caucasian. The Achilles tendinopathy group (TEN) consisted of 63 participants (47 male and 16 female, average age 32 years old), with a clinically diagnosed Achilles tendinopathy, that included one or more of the following criteria: progressive pain, sensitivity to palpation, history of swelling and changes in lesion thickening within the tendon. Most of the TEN participants were active competing athletes (soccer, handball, basketball, martial arts, skiing), having between 3 and 7 coach-supervised training sessions per week, recruited at the time of the injury in Orthopaedics Clinic or by a team doctor, who was an orthopaedic surgeon. Control group (CON) consisted of 92 participants (72 male and 20 female, average age 39.0 years) that have finished their active athlete career and have self-reported not having tendinopathies throughout their athlete career. This study was approved by the Ethics Committee of the School of Medicine, University of Zagreb, Croatia, and by the Ethics Committee of the Faculty of Kinesiology, University of Zagreb, Croatia. All participants have provided a biological sample and given written informed consent.

Genotyping

Genomic deoxyribonucleic acid (DNA) was extracted from the oral epithelial cells using a QIAamp DNA kit (Qiagen, Germantown, MD, USA), followed by polymerase chain reaction (PCR) amplification using MMP3 rs591058, rs650108 and rs679620 specific primer pairs (Metabion, Planegg/Steinkirchen, Germany) and PyroMark PCR Kit (Qiagen, Germantown, MD, USA). Amplified fragments were sequenced by pyrosequencing method (PyroMark Q24, Qiagen, Germantown, MD, USA) according to the manufacturer's protocol and analysed by PyroMark Q24 software (Qiagen, Germantown, MD, USA).

Statistical analysis

To analyse allelic, genotypic and haplotype differences, we used an odds ratio method, using a StatCalc program (AcaStat software, Orange County, FL, USA). A statistically significant difference was defined when P was $P < 0.05$. Phase software was used for haplotype analysis. The Hardy-Weinberg equilibrium and linkage disequilibrium analyses were performed using the Arlequin software version 3.5 (Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland). In this study, we used groups similar in size to previously reported studies [12, 17, 18] investigating genotype effects on tendinopathies, as those group sizes proved to be large enough to detect significant results. The Bonferroni correction is considered too conservative [21], so it was not applied in this study. P values were adjusted for false discovery rate (FDR) using Benjamini-Hochberg procedure for adjusted P value. It was applied for each genotype, allele and haplotype separately [22].

Results

Participants' characteristics

Participants were divided into two groups: tendinopathy group (TEN) with diagnosed tendinopathy occurrence and control group that has not self-reportedly suffered from any tendinopathies during their active competing years. The control group (CON) was significantly heavier and chronologically older, due to being retired athletes, with a higher BMI index than TEN group, but that is due to the different lifestyle of the retired and competing athletes. As the average heights were comparable, it is assumed that BMI and weight were similar in the same time point of the career between controls and TEN group, as it is known that controls were active competing athletes. Presented data are not matched for weight and BMI, only for height, ethnicity and gender (Table 1).

Table 1 General characteristics of the tendinopathy group and control group

	TEN ($n = 63$)	Controls ($n = 92$)	P value
Age (years)	32.1 ± 12.8	39.0 ± 11.4	**0.006**
Height (cm)	180.5 ± 8.8	179.8 ± 9.6	0.6454
Weight (kg)	79.4 ± 14.9	84.6 ± 15.4	**0.0381**
BMI (kg/m^2)	24.1 ± 3.6	26.0 ± 3.3	**0.009**
Ethnicity (Caucasian)	100% (63)	100% (92)	1.0000
Gender (% male)	75% (47)	78% (92)	0.6639

Ethnicity and gender are represented as a percentage, and the remaining variables are expressed as a mean ± standard deviation. Significant P values are in bold. Age, height and weight are self-reported values in time of the recruitment

Genotype frequencies were significantly different between groups: GG genotype of rs650108 and AA genotype of rs679620 were significantly over-represented in TEN group and associated with predisposition for tendinopathies. TT genotype of rs591058 is associated with the higher risk of tendinopathies. Similarly, allelic frequencies significantly differed between groups. A allele of rs650108 and G allele of rs679620 were significantly over-represented in controls compared with cases and therefore classified as protective, while G allele of rs650108 and A allele of rs679620 were significantly over-represented in cases comparing with controls, thus being considered as a predisposition for development of tendinopathies. C and T allele of rs591058 did not show associations (Table 2).

All polymorphisms conformed to the Hardy-Weinberg equilibrium (HWE) in both the tendinopathy and the control group (Table 2). SNPs rs591058, rs650108 and rs679620 were also in the linkage disequilibrium (LD). Apart from Bonferroni correction being considered too conservative for these types of studies, there is no need for it, as all three SNPs are in LD, meaning they are inherited as blocks.

Haplotypes constructed from all possible combinations of polymorphisms were analysed (only relevant data is shown in Table 3), as all three polymorphisms are in LD. After correction for FDR, T - G - A haplotype indicates predisposition for tendinopathies, while C-A-G haplotype is associated with protection for development of tendinopathies.

Discussion

Based on previous studies on different populations, current study further investigated variations within MMP3 gene and risk of developing tendinopathies. In the present study, we identified significant allele and genotype association between GG genotype of rs650108 and AA genotype of rs679620 and predisposition for developing tendinopathies in Croatian athletes. After adjusting for FDR, TT genotype of rs591058 shown indication of predisposition for developing tendinopathies. Haplotype association analysis was preformed to validate findings of SNP-based association analysis. After adjusting for FDR, T - G - A haplotype, constructed of rs591058, rs650108 and rs679620, was associated with higher risk, while C - A - G haplotype was associated with reduced risk of developing tendinopathies in Croatian athletes. To our knowledge, this is the first genetic association study investigating these three MMP3 variants in Croatian population.

All three MMP3 variants were previously associated with musculoskeletal soft tissue injuries [14, 23]. Investigated polymorphisms, rs591058 (T/C), rs650108 (G/A) and rs679620 (A/G), are transition mutations, meaning that there

Table 2 Allele and genotype frequency

MMP3			Allele frequency					
SNP	C > T	TEN	CON					
rs591058		$n = 48$	$n = 92$	p	FDR	OR	95% CI	Association
1	C	53.1% (51)	62.5% (115)	0.1304		0.68	0.4125–1.1209	None
2	T	46.9% (45)	37.5% (69)	0.1304		1.4706	0.8922–2.4240	None
Genotype frequency								
SNP	C > T	TEN	CON					
rs591058			$n = 92$	p	FDR	OR	95% CI	Association
11	CC	27.1% (13)	34.8% (32)	0.3556		0.6964	0.3232–1.5006	None
12	CT	52.1% (25)	55.4% (51)	0.7056		0.8738	0.4340–1.7593	None
22	TT	20.8% (10)	9.8% (9)	0.0759		24.269	0.9118–6.4597	Indication of predisposition
HWE			0.751					
MMP3			Allele frequency					
SNP	A > G	TEN	CON					
rs650108		$n = 63$	$n = 92$	p	FDR	OR	95% CI	Association
1	A	26.2% (33)	39.1% (72)	**0.0187**		0.552	0.3363–0.9058	**Protection**
2	G	73.8% (93)	60.9% (112)	**0.0187**	**0.0187**	1.8117	1.1040–2.9731	**Predisposition**
Genotype frequency								
SNP	A > G	TEN	CON					
rs650108			$n = 92$	p	FDR	OR	95% CI	Association
11	AA	8% (5)	12% (11)	0.4221		0.6348	0.2093–1.9254	None
12	AG	36.5% (23)	54.3% (50)	**0.0299**	0.04485	0.4830	0.2504–0.9315	**Protection**
22	GG	55.5% (35)	33.7% (31)	**0.0074**	0.0222	24.597	1.2730–4.7527	**Predisposition**
HWE			0.658					
MMP3			Allele frequency					
SNP	G > A	TEN	CON					
rs679620		$n = 63$	$n = 92$	p	FDR	OR	95% CI	Association
1	G	50.8% (64)	62.5% (115)	**0.0410**	0.082	0.6194	0.3912–0.9805	**Protection**
2	A	49.2% (62)	37.5% (69)	**0.0410**	**0.041**	1.6146	1.0199–2.5561	**Predisposition**
			Genotype frequency					
SNP	G > A	TEN	CON					
rs679620			$n = 92$	p	FDR	OR	95% CI	Association
11	GG	27.0% (17)	34.8% (32)	0.3061		0.6929	0.3432–1.3990	None
12	GA	47.6% (30)	55.4% (51)	0.3392		0.7308	0.3842–1.3902	**Protection**
22	AA	25.4% (16)	9.8% (9)	**0.0119**	**0.0357**	31.395	1.2872–7.6573	**Predisposition**
HWE			0.707					

Allele and genotype frequencies are expressed as percentage with the number of participants (n) in parentheses. Results that have been classified as significant by the P value ($P < 0.05$) are in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg-adjusted P value. *OR* odds ratio. *95% CI* 95% confidence interval

is an interchange of either two pyrimidines or two purine nucleotides within the DNA sequence. While rs591058 and rs650108 variants are part of the intron 4 and 8, respectively, rs679620 variant is a non-synonymous mutation, within exon 2, resulting in change from lysine to glutamic acid [16].

MMP3 gene, member of MMP gene family coding for over 20 different endopeptidases, codes for proteoglycan-degrading enzyme which role is degradation of collagens together with other structural components, such as proteoglycans, aggrecan, laminin and gelatine [13]. In humans, nine out of 23 MMPs are located on the long arm of chromosome 11. MMP3 has a major role in regulation of tissue remodelling and remodelling of extracellular matrix of tendons. It is hypothesised that increased expression of MMP3 is necessary for tissue remodelling and prevention of pathological changes in tendons [24]. Tendon matrix is constantly remodelled, and the higher the strain, the higher the turnover rates. Tendons showing tendinopathy have increased

Table 3 Haplotype frequency

Hap code	Haplotype	CON	TEN	p	FDR	OR	95% CI	Association
1	C - G - G	43	31	0.51		0.85	0.52–1.39	None
2	C - A - G	72	33	**0.018**	*0.053*	0,55	0,34-0,91	Indication of protection
3	T - G - A	69	62	**0.04**	*0.06*	1,61	1,02-2,59	Indication of predisposition

Results that have been classified as significant by the *P* value ($P < 0.05$) are in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg-adjusted *P* value. *OR* odds ratio. *95% CI* 95% confidence interval

rate of matrix remodelling, making tendons less stable and thus more susceptible to injuries.

One of the first studies conducted on MMP3 variants found a significant association of GG genotype of rs679620, CC genotype of rs591058 and AA genotype of rs650108 and risk of Achilles tendinopathy ($n = 114$) in South African Caucasian cohort [14]. Observed results were subsequently replicated in two other cohorts. Association between MMP3 rs679620 variant and Achilles tendinopathy was not found in British cohort ($n = 93$), only association that was found was between small cohort subgroup of patients with tendon rupture ($n = 25$) and GG genotype of rs679620 [11]. Same set of MMP3 variants were analysed in Australian cohort as well, but none showed statistically significant results ($n = 77$); moreover, they demonstrated that alternate 6A-G-C-G-inferred haplotype (rs3025058, rs679620, rs591058 and rs650108) was associated with reduced risk for Achilles tendinopathy [16]. Findings of Australian cohort somewhat match our findings, as in Croatian cohort, G allele of rs679620 and C allele of rs591058 were associated with reduced risk for tendinopathies. Most recent study of the Chinese Han cohort in general population that was recreationally active reported that individuals with G allele of rs679620 would have an increased risk of chronic Achilles tendinopathy. Authors stated that study was based on the general population and that results might be different from those based on cohort of athletes [17]. Another study on Chinese Han population had evidence of AA allele of rs650108 being associated with frozen shoulder condition, where synovial fibrosis occurs, leading to imbalance between collagen synthesis and degradation, resulting in excessive collagen deposition leading to joint pain and stiffness [25]. Other studies have linked MMP variants with various conditions such as anterior cruciate ligament (ACL) rupture [16, 26], lumber disc degeneration [27], osteoarthritis [19] and rheumatoid arthritis [28]. It is important to underline relatively high occurrence of alternate risk associations between different populations [14–16, 29] and the importance of studying same variants in different populations in order to assess implications of how same variants impact conditions differently. In physically active population, overuse injuries related to sports are most commonly tendinopathies, while acute injuries are related to ligament ruptures. High-level athletes put much more force and strain on their tendons and ligaments, as their training schedule and competition calendar rarely leave enough time to rest and recover. It is expected that there will be differences between general population suffering from tendinopathies and high-level athletes, and so as controls in this study, we used 'retired' high-level athletes, choosing rather having some difference in age and weight (it is common for older people to have gained some weight) than acquiring controls matched for age and weight but without any assurance that they might suffer tendinopathies any time through the rest of their active competing career. It is important to state that the main limitation of this study is a small sample size, due to that small niche of high-level athletes that will have the injury reported to their orthopaedic doctor, because it interferes with their performance enough that it starts making a difference. Presented data needs to be interpreted cautiously and, if possible, replicate it within the larger population of both high-level athletes and recreational athletes presenting the same diagnosis and general population.

Genetic risk factors modulate and contribute to overall risk of soft tissue injuries. With the knowledge of one's genotype, athletes that have risk predisposition can modify environmental factors and adjust to the impact of the extrinsic factors with more time for rest, recovery and preventive exercises, so that they do not stir to the other direction and possibly avoid initiating event of injury all together. Sports genetics and genetics of sport injuries is a novel field with major limitations of study size, and, as shown, difference between populations can account for alternate risk associations.

Compliance with ethical standards

This study was approved by the Ethics Committee of the School of Medicine, University of Zagreb, Croatia, and by the Ethics Committee of the Faculty of Kinesiology, University of Zagreb, Croatia. All participants have provided a biological sample and given written informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

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5. DISCUSSION

Functional and structural properties of the immunoglobulin G (IgG) are substantially affected by the processes of glycosylation, with direct effects on the immune system [254]. Previous studies have shown the positive effect of exercise interventions on the immune system and metabolic effects in older adults [47,48]. Similarly, two studies carried out on young adults have linked exercise/and diet interventions with the overall anti-inflammatory IgG profile after the recovery period, but interestingly, both have reported indications of a pro-inflammatory IgG profile and significantly increased inflammatory IgG profile at the end of the intervention itself [172,173].

Tijardović et al. [173] reported indications of elevated levels of pro-inflammatory IgG glycans at the end of high-intensity repeated sprint training (RST) in young male adults, while after six weeks of the recovery period, authors reported the anti-inflammatory effect of RST intervention, with an increase in digalactosylated and monosialylated glycans, and a decrease in agalactosylated glycans. Sarin et al. [172] demonstrated that at the end of four months of intervention, involving dietary restrictions coupled with high-intensity aerobic exercise in young female adults, IgG pro-inflammatory glycans were significantly increased. Authors reported decreased levels of digalactosylated and sialylated N-glycans without bisecting GlcNAc, while agalactosylated and monogalactosylated glycans and glycans with bisecting GlcNAc increased. After the weight regain period over the course of four months, all glycans have returned to the baseline.

Very few studies have been carried out on the older population, but two studies have reported the anti-inflammatory effects of exercise interventions in the aging population. Hayes et al. [48] reported that a short-term aerobic exercise intervention lasting 6 weeks has lowered IL-6 levels in lifelong sedentary older adults, while age-matched life-long exercisers have had even lower levels of circulating IL-6. Rest et al. [47] reported the metabolic effects of 13-week lifestyle intervention, where they introduced a 12.5% caloric reduction and a 12.5% increase in energy expenditure through physical activity in older adults, aged 63 years old on average. They reported that fasting insulin levels were much less decreased, with more heterogeneous responses, but blood pressure, thyroid function, glucose, and lipid metabolism were significantly improved, demonstrating overall improvement of metabolic health.

Several studies have linked lifestyle changes, focusing on dietary interventions, as the key factors in bringing down inflammation rates, shifting the pro-inflammatory IgG towards the

anti-inflammatory IgG profile. As expected, more drastic interventions yielded significantly better results. Two studies that involved both bariatric surgery and follow-up diet reported significant anti-inflammatory IgG glycan shift in the previously obese populations [165,168], while more discrete anti-inflammatory trends were observed in the study that involved only a low-calorie diet, followed by a maintenance diet [166]. But when the restrictive diet intervention was introduced to a population with normal BMI, coupled with aerobic exercise to evoke a more prominent caloric deficit, IgG glycans shifted from an anti-inflammatory towards a pro-inflammatory profile, returning back to baseline after the weight-regain period, during recovery [172]. Furthermore, many previous studies have reported significant changes in the IgG glycome composition associated with different diseases, inflammatory states and aging [87,88,94,96,180]. Though some changes are irreversible, low-grade inflammation occurring in the aging process has been of key interest when trying to find environmental factors that could help in reversing the biological clock of aging.

With the age gap in previously investigated populations, between young adults aged 18 to 27 years old [172,173], and the senior population aged over 60 years old [47,48], the adults group (30 – 65 years old) was recruited to study the impact of exercise intervention on IgG glycosylation. Effects of three months of moderate exercise on IgG glycosylation in previously inactive, sedentary, middle-aged, overweight and obese populations were observed [174]. The mild pro-inflammatory shift of IgG glycans after the exercise intervention was reported. The pro-inflammatory shift was indicated by a decrease in digalactosylated, monosialylated and disialylated glycan structures and an increase in agalactosylated, asialylated and core-fucosylated glycan structures, what was expected in a previously sedentary population with the elevated BMI levels [133,169]. It was also in accordance with trends observed at the same timepoint in young adult populations [172,173].

In parallel, as part of this thesis, the investigation of the investigation of the training frequency and intensity impact on IgG glycosylation was performed, recruiting the adult population between 25 and 65 years old [177]. For this study, 276 healthy participants were recruited and divided into four different subgroups based on the level of exercise frequency and intensity. They were categorized and placed into physically inactive group (inactive), previously sedentary, but recently active group (recreational), life-long exercisers group (active), and professionally competing athletes (professional). The study results indicated a significant change in the GlycanAge index between the active and inactive groups, as well as between the

active and professional groups. The active group was on average 7.4 years younger when compared to the inactive group. Differences were even more prominent when stratified for sex; active women were on average almost a decade younger than their counterparts in the inactive group. A similar trend was observed in men, but the effect was only nominally significant and smaller in the effect size. When the professional competing athletes' group was compared to the active group, they were on average 7.5 years older than their active counterparts, just as the inactive group was. It draws the conclusion that too much exercise is as stressful and harmful for the organism as is the lack of exercise. The same trends were observed between women in competing professionals versus women in the active group, only here the difference was even more pronounced. Women in the professional group were on average 20 years older than their female counterparts in the active group, though it is important to underline a small number of professionally competing women in this study. Nonetheless, these results are in accordance with the results from Sarin et al. [172] study, when comparing results in the middle time point, just after the intervention, where they have reported the pro-inflammatory IgG glycome pattern in their cohort. Men in the professional competing athletes group lacked statistical significance when compared to their active counterparts, even though trends were the same, only less in effect size, similar to Tijardović et al. [173] study, which also reported indication of the pro-inflammatory IgG glycome, lacking a statistical significance at the end of the high-intensity RST intervention. The most important takeaway is that a lifelong moderate physical activity, or at least one continuously lasting for several years, will bring distinct benefits. Those are visible both in lower GlycanAge index or as the prevailing anti-inflammatory IgG glycome, which is in accordance with some previous studies, that presented lower IL-6 concentrations in life-long exercisers, indicating the overall anti-inflammatory effect [48].

Every physical activity brings a certain risk of injuries, especially if one has been physically inactive for a longer period of life. Assessing the genetic components as intrinsic factors for injury risk could be beneficial. Numerous studies have been conducted in the last two decades, peaking in volume around a decade ago, doing studies in different populations [205,221,226–228,230,237,238,244,248,252,255–260]. Some of the gene variants were replicated across different populations, but different ancestry once again proved to be troublesome, when having to replicate results across multiple populations.

One of the most extensively investigated polymorphisms, rs1800012 G>T within the *COL1A1* gene was associated with many conditions including pelvic organ prolapse [261], intervertebral

disc degeneration [262,263], osteoporosis [208,209], osteogenesis imperfecta [210] and anterior crucial ligament (ACL) ruptures [264–266]. COL1A1 rs1800012 was associated with a reduced risk of sport-related ligament injuries. The rare TT genotype was considered to have a protective role in developing ACL injuries [267]. Interestingly, it wasn't replicated in the European population, even though authors reported that the TT genotype is underrepresented. Statistical significance between the control and ACL rupture group was not met [268], much like results reported in Appendices, Table 1.

Following the proposed importance of collagen in the etiology of soft-tissue injuries, analysis of several collagen gene variants was conducted, in two sub-groups of professionally competing athletes. They were divided into a full ACL rupture (ACLR) group, which occurred in a non-contact manner (ACLR group: N = 95) and a tendinopathy group, where Achilles tendinopathy (AT) was diagnosed by the medical doctor (orthopedic surgeon) (TEN group: N=63). To have a high-quality control group, 92 healthy retired professionally competing athletes, who have not suffered from any types of tendon or ligament injuries during their sports career and competing period, were selected for the control group. This control group was recruited to ensure that they were under at least similar training conditions and training stress, as opposed to having controls from the general population [250,253]. Results included several findings within the ACL and TEN groups, mainly in accordance with some of the previous studies.

While no statistical significance between ACL ruptures and gene variant within the *COL1A1*gene was observed, statistically significant results were reported in the gene variant within the *COL27A1* gene. The rs946053 GG genotype within the *COL27A1* gene was considered to be protective (p = 0.0329, OR = 0.4504, 95% CI = 0.2277 - 0.8909), while the TT genotype was considered as the predisposition for the occurrence of ACL rupture (p = 0.0329, OR = 22.915, 95% CI = 1.1792 – 4.426). The rs240736 GG genotype within the *COL12A1* gene had only an indication of predisposition for the developmen oft ACL rupture, as it has lost significance after correcting for FDR.

In the TEN group analysis was conducted in two collagen gene variants and one variant within the *Tenascin C* gene (Appendices, Table 2). Similar to the variant within the *COL1A1* gene, results couldn't be replicated, as reported in the previously published studies that associated the rs12722 C>T polymorphism with the risk of AT [220,269]. This study lacked statistical

significance, much like one in the British cohort [259], but statistically significant results were observed in the gene variant within *COL27A1*gene. The rs946053 GG genotype within the *COL27A1* gene was considered to be protective (p = 0.0354, OR = 0.3444, 95% CI = 0.1503 – 0.7895), as reported in a previous study in two populations [230]. The rs2104772 TT genotype within the *TNC* gene was considered as the predisposition for the occurrence of ATs (p = 0.0267, OR = 25.357, 95% CI = 1.2628 – 5.0918), as it was reported before [230]. Furthermore, in the aforementioned TEN group, rs946053-rs2104772-rs12722 haplotype was constructed and it was reported that G-A-C haplotype was considered to be protective (p = 0.0424, OR = 0. 4310, 95% CI = 0.1880 - 0.9900), while the carriers of T-T-T haplotype were under the greater risk of tendinopathies (p = 0.0424, OR = 2.453, 95% CI = 1.107 - 5.434).

When checking for polymorphisms within the *MMP3* gene, statistically significant results were reported for both genotypes and constructed haplotypes, as was previously reported in the literature [247–249,252]. Within both the ACLR and TEN groups, there were the same findings. The rs650108 GG genotype was considered as the predisposition for the occurrence of both ACLR [253] and ATs [250] (p = 0.0161, OR = 23.338, 95% CI = 1.2899 – 4.2226 and p = 0.0222, OR = 24.597, 95% CI = 1.2730 – 4.7527, for the latter), as well as the rs679620 AA genotype (p = 0.0064, OR = 34.750, 95% CI = 1.5266 – 7.9101 and p = 0.0357, OR = 31.395, 95% CI = 1.2872 – 7.6573, for the latter). Moreover, when constructing the rs591058-rs650108-rs679620 haplotype it was reported that C-A-G haplotype was considered to be protective (p = 0.0222, OR = 0.55, 95% CI = 0.35–0.85), while the carriers of T-G-A haplotype were under greater risk of ACL ruptures (p = 0.0156, OR = 1.71, 95% CI = 1.13 – 2.59).

Numerous studies have suggested that there is a genetic component in developing musculoskeletal soft tissue injuries, predominantly focused on ligaments and tendons, closely followed by muscle injuries. Sports injuries are usually caused by training load, insufficient rest, and physical interactions, but individual genetic makeup shouldn't be dismissed [270]. For many musculoskeletal injuries, there is a significant amount of genetic contribution, but they are predominantly polygenic, with numerous gene-to-gene and gene-to-environment interactions. Coming back to the ever-debated nature vs nurture, perhaps it is time to acknowledge it is both. As one can't escape from their genes, what happens throughout the lifetime and how our gene products are post-translationally altered via mechanisms of methylation and glycosylation, brings nurture, or better to say environment to the prime focus. Though one can't escape from their genetic legacy, altering the environmental factors to

increase the odds of thriving is possible. While the concern of using genetic tests for talent identification is justified [271], perhaps the niche of genetic predispositions for sport-related injuries shouldn't be eliminated as fast, even though we have just scratched the surface. Unlike talent identification or training alterations, injury predispositions could be useful tools to adapt one's conditioning training, if it's not being misused to dampen one's potential and possible future results. Ethics falls on all parties involved, to use and not misuse the knowledge, insights, and information such analysis could provide.

We are fighting against the silent epidemics of obesity and with the prevalence of physical inactivity, every additional incentive towards the increase of physical activity counts. Injuries affect both professional and recreational athletes, and if the risk of occurrence can be mitigated, fewer sports injuries would directly lead to more physical activity. In the predominance of inactivity, any type of biomarker that could help on the path towards a healthier and more active lifestyle should be considered helpful.

6. CONCLUSIONS

Regular and long-term physical activity of moderate level has a beneficial effect on overall health. It is demonstrated by the difference in the GlycanAge index of biological age, between groups engaging in different levels of physical activity. The inactive population is significantly older than the long-term moderately active population when comparing their GlycanAge index. The same trend is observed in the population of professionally competing athletes. They are significantly older than their moderately active counterparts.

Every physical activity carries a certain risk of injury occurrence. The genetic screening for soft tissue injury risk might be valuable to those who would like to adapt their conditioning training or everyday activities. Certain training adaptations have been related to the reduced risks of soft tissue injuries, as they target and strengthen specific muscle groups, that can help in joint stabilization, resulting in less strain put on connective soft tissues.

Genetic predisposition for soft tissue injuries, as well as the biological age index, are information that could benefit those who have been leading a sedentary lifestyle. Physical inactivity is often paired with increased body weight, creating a further negative spiral of inactivity, possibly paired with the fear of injuries, when engaged in any sort of physical activity. That inevitably leads to unhealthy life choices, that will likely result in lower quality of life, earlier onset of diseases and overall dissatisfaction.

The benefits of incorporating regular physical activity in everyday life are vast. By using a biomarker that can track changes occurring within our bodies, feedback on our everyday routine is always available, as are the solutions on how to improve it further if needed. The relentless rise of obesity has taken its toll on the healthcare systems worldwide, but the increase in physical activity could help by reducing the inflammatory levels in an inactive population, which are usually significantly higher when compared to the active population. Furthermore, the proven positive effects of regular physical activity would likely delay the onset of non-communicable diseases related to obesity and inactivity.

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8. CURRICULUM VITAE

Nina Šimunić Briški started working in Genos in 2014, working in several areas including project management, marketing and research. In the early stages of her PhD research, she was awarded the IRCRO research grant, focused on investigating genetic components of soft tissue injuries in the sports genetics field. Transitioning to the glycobiology field, she started investigating the impact of physical activity intensity and dietary interventions on Immunoglobulin G N-glycosylation in different populations, funded by Centre of Competence and Croatian Science Foundation grants.

She obtained an M.Eng. degree in the field of Molecular Biotechnology from the University of Zagreb, Faculty of Food Technology and Biotechnology in 2010. During her studies, she was awarded two scholarships for excellent students (National scholarship and Pliva Ltd. scholarship). During and shortly after graduation she worked on two research projects in Germany, in the area of cancer biology. From 2011-2014 she worked in the R&D department of the soft beverages FMCG industry, using that period to obtain a dual degree; M.Sc. in Strategic Management from Kelley School of Business, Indiana University, USA and an MBA degree in Marketing and Finance from the Institute of Economics in Zagreb, Croatia.

Currently, she is involved in several ongoing research projects while working on new collaborations. Her main interest areas are exercise and nutrition interventions, as well as obesity's impact on inflammation status and healthy aging.

9. PROŠIRENI SAŽETAK

Redovna, umjerena, višegodišnja tjelesna aktivnost blagotvorno utječe na cjelokupno zdravlje ljudskog organizma. Iako su pozitivni utjecaji redovne fizičke aktivnosti brojni, trenutno se još uvijek zna jako malo o utjecaju vježbanja na glikozilaciju imunoglobulina G (IgG). U ovom doktorskom radu cilj je bio istražiti utjecaj učestalosti treniranja na glikanske biomarkere biološke dobi, kao i povezanost trenutno poznatih varijanti gena s ozljedama mekog vezivnog tkiva kod sportaša. Rezultati su pokazali značajno niži GlycanAge index (-7.4) u grupi redovnih umjerenih vježbača u odnosu na neaktivnu populaciju, dok su profesionalni sportaši u odnosu na redovno, umjereno aktivnu populaciju, imali 7.5 godina više. Neaktivna popoulacija i profesionalni sportaši su imali vrlo slične glikanske profile. Osim toga, pokazali smo značajnu povezanost genskih varijanti unutar *COL12A1, COL27A1, Tenascin C* i *matriks-metaloproteinaza 3 (MMP3)* gena s povišenim ili smanjenim rizikom od nastanka rupture prednjeg križnog ligamenta (eng. anterior cruciate ligament [ACL]) i tendinopatija u Ahilovoj tetivi, čestim sportskim ozljedama, koje pogađaju i rekreativce i profesionalne sportaše. COL27A1 rs946053 genotip GG je bio značajno zastupljen u ispitivanoj skupini u odnosu na kontrole, indicirajući predispozicije za rupturu ACL-a, dok je TT genotip bio značajno zastupljen u kontrolnoj skupini, indicirajući zaštitnu ulogu. COL12A1 rs240736 genotip GG je nakon FDR korekcije izgubio statističku značajnost, pa je ostala samo indikacija predispozicije za razvoj rupture ACL-a. TNC rs2104772 genotip TT, kao i haplotip T-T-T halotip, konstruiran od rs12722-rs946053-rs2104772, su bili značajno zastupljeni u ispitivanoj skupini u odnosu na kontrole, indicirajući predispozicije za tendinopatije Ahilove tetive. Istovremeno su COL27A1 rs946053 genotip GG i haplotip G-A-C bili značajno zastupljeni u kontrolama, indicirajući zaštitnu ulogu. Nadalje, MMP3 rs591058 genotip TT, rs650108 genotip GG i rs679620 genotip AA, kao i haplotip T-G-A, konstruiran od rs591058- rs650108-rs679620, su bili značajno zastupljeni u ispitivanoj skupini u odnosu na kontrole, implicirajući povišeni rizik od nastanka rupture ACL-a. Haplotip C-A-G je bio značajno zastupljen u kontrolama u odnosu na ispitivanu skupinu, sugerirajući zaštitnu ulogu. MMP3 rs650108 genotip GG i rs679620 genotip AA su bili značajno zastupljeni u ispitivanoj skupini u odnosu na kontrole, indicirajući predispozicije za tendinopatije Ahilove tetive, dok su rs591058 geotip TT, kao i haplotip T - G – A, konstruiran od rs591058- rs650108- rs679620 implicirali indikaciju zaštitne uloge kod razvoja tendinopatija Ahilove tetive. Tjelesna aktivnost ima pozitivan učinak na cjelokupno zdravlje, a uz informacije koje pružaju genske predispozicije za nastanak ozljeda mekog vezivnog tkiva, moguće je dodatno modificirati trening kako bi se rizici sveli na minimum. Time se maksimalno iskorištava pozitivan utjecaj tjelovježbe, uz minimaliziranje rizika od ozljeda.

10. APPENDICES

Table 1. Allele and genotype frequency of the rs1800012, rs240736 and rs946053 in relation to the ACL rupture

COL1A1			Allele frequency					
SNP	G>T	ACL	CON					
rs1800012		n = 95	n = 92	p	FDR	OR	95% CI	Association
1	G	82.1% (156)	77.7% (143)	0.2901		1.3155	0.7915 – 2.1866	none
2	T	17.9% (34)	22.3% (41)	0.2901		0.7602	0.4573 – 1.2635	none
			Genotype frequency					
SNP	G>T	ACL	CON					
rs1800012		n = 95	n = 92	p	FDR	OR	95% CI	Association
11	GG	68.4% (65)	58.7% (54)	0.1697		15.247	0.8372 – 2.7768	none
12	GT	27.4% (26)	38.0% (35)	0.1208		0.6137	0.3311 – 1.1372	none
22	TT	4.2% (4)	3.3% (3)	0.7330		13.040	0.2837 – 5.9936	none
HWE			0.504					
COL12A1			Allele frequency					
SNP	A>G	ACL	CON					
rs240736		n = 95	n = 92	p	FDR	OR	95% CI	Association
1	A	69.5% (132)	75% (138)	0.2337		0.7586	0.4815 - 1.1953	none
2	G	30.5% (58)	25% (46)	0.2337		1.13182	0.8366 – 2.0769	none
			Genotype frequency					
SNP	A>G	ACL	CON					
rs240736		n = 95	n = 92	p	FDR	OR	95% CI	Association
11	AA	52.6% (50)	54.3% (50)	0.8140		0.9333	0.5252 – 1.6585	none
12	AG	33.7% (32)	41.3% (38)	0.2824		0.7218	0.3984 – 1.3078	none
22	GG	13.7% (13)	4.4% (4)	**0.0348**	*0.104*	34.787	1.0930 – 11.1298	*indication of predisposition*
HWE			0.045					
COL27A1			Allele frequency					
SNP	G>T	ACL	CON					
rs946053		n = 95	n = 92	p	FDR	OR	95% CI	Association
1	G	41.1% (78)	56.5% (104)	**0.0029**		0.557	0.3553 - 0.8076	**protection**
2	T	58.9% (112)	43.5% (80)	**0.0029**		1.8667	1.2382 – 2.8141	**predisposition**
			Genotype frequency					
SNP	G>T	ACL	CON					
rs946053		n = 95	n = 92	p	FDR	OR	95% CI	Association
11	GG	17.9% (17)	32.6% (30)	**0.0219**	**0.0329**	0.4504	0.2277 - 0.8909	**protection**
12	GT	463% (44)	47.8% (44)	0.8361		0.9414	0.5299 – 1.6717	none
22	TT	35.8% (34)	19.6% (18)	**0.0144**	**0.0329**	22.915	1.1792 – 4.4526	**predisposition**
HWE			0.675					

Allele and genotype frequencies are expressed as percentage with the number of participants (n) in parentheses. Results that have been classified as significant by the P-value ($P<0.05$) in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg Adjusted P value. OR- odds ratio. 95% CI – 95% confidence interval.

Table 2. Allele and genotype frequency of rs12722, rs12722, rs946053 and rs2104772 in relation to tendinopathies

COL3A1			Allele frequency					
SNP	A>G	TEN	CON					
rs12722		n = 48	n = 92	p	FDR	OR	95% CI	Association
1	A	40.6% (39)	27.2% (50)	**0.0225**	**0.025**	1.8337	1.0891 – 3.0873	**predisposition**
2	G	59.4% (57)	72.8% (134)	**0.0225**	**0.050**	0.5454	0.3239 – 0.9182	**protection**
			Genotype frequency					
SNP	A>G	TEN	CON					
rs12722		n = 48	n = 92	p	FDR	OR	95% CI	Association
11	AA	16.6% (8)	10.9% (10)	0.3340		16.400	0.6011 – 4.4741	none
12	AG	48.0% (23)	32.6% (30)	0.0780		19.013	0.9305 – 3.8853	none
22	GG	35.4% (17)	56.5% (52)	***0.0190***	*0.0569*	0.4218	0.2051 – 0.8675	*indication of protection*
HWE			0.963					
COL5A1			Allele frequency					
SNP	C>T	TEN	CON					
rs12722		n = 63	n = 92	p	FDR	OR	95% CI	Association
1	C	42.1% (53)	45.1% (83)	0.5957		0.8835	0.5590 - 1.3962	none
2	T	57.9% (73)	54.9% (101)	0.5957		1.1319	0.7162 - 1.7888	none
			Genotype frequency					
SNP	C>T	TEN	CON					
rs12722		n = 63	n = 92	p	FDR	OR	95% CI	Association
11	CC	14.3% (9)	21.7% (20)	0.2456		0.6000	0.2533 - 1.4210	none
12	CT	55.6% (35)	46.7% (43)	0.2816		14.244	0.7481 - 2.7121	none
22	TT	30.1% (19)	31.6% (29)	0.8570		0.9381	0.4682 - 1.8795	none
HWE			0.267					
COL27A1			Allele frequency					
SNP	G>T	TEN	CON					
rs946053		n = 63	n = 92	p	FDR	OR	95% CI	Association
1	G	43.7% (55)	56.5% (104)	**0.0264**	0.0264	0.5959	0.3773 - 0.9412	**protection**
2	T	56.3% (71)	43.5% (80)	**0.0264**	0.0264	1.6782	1.0625 - 2.6506	**predisposition**
			Genotype frequency					
SNP	G>T	TEN	CON					
rs946053		n = 63	n = 92	p	FDR	OR	95% CI	Association
11	GG	14.3% (9)	32.6% (30)	**0.0118**	0.0354	0.3444	0.1503 - 0.7895	**protection**
12	GT	58.7% (37)	47.8% (44)	0.1829		15.524	0.8127 - 2.9656	none
22	TT	27% (17)	19.6% (18)	0.279		15.193	0.7118 - 3.2428	none
HWE			0.124					
TNC			Allele frequency					
SNP	T>A	TEN	CON					
rs2104772		n = 63	n = 92	p	FDR	OR	95% CI	Association
1	T	61.1% (77)	48.4% (89)	**0.0276**	0.0276	1.6774	1.0586 - 2.6579	**predisposition**
2	A	38.9% (49)	51.6% (95)	**0.0276**	0.0276	0.5962	0.3762 - 0.9447	**protection**

			Genotype frequency					
SNP	T>A	TEN	CON					
rs2104772		n = 63	n = 92	p	FDR	OR	95% CI	Association
11	TT	42.9% (27)	22.8% (21)	**0.0089**	0.0267	25.357	1.2628 - 5.0918	**predisposition**
12	TA	36.5% (23)	51.1% (47)	0.0745		0.5505	0.2857 - 1.0608	none
22	AA	20.6% (13)	26.1% (24)	0.4351		0.7367	0.3402 - 1.5869	none
HWE			0.066					

Allele and genotype frequencies are expressed as percentage with the number of participants (n) in parentheses. Results that have been classified as significant by the P-value (P<0.05) in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg Adjusted P value. OR- odds ratio. 95% CI – 95% confidence interval.

Table 3. Haplotype frequency of rs12722 - rs946953 - rs2104772 in relation to tendinopathies

Hap code	Haplotype	CON	TEN	p	FDR	OR	95% CI	Association
1	G - A - T	16	5	0.1038		0.4339	0.1547 - 1.2166	none
2	**G - A - C**	25	8	**0.0424**	**0.0424**	0.4310	0.1880 - 0.9900	**protection**
3	G - T - T	32	21	0.8678		0.9500	0.5193 - 1.7380	none
4	G - T - C	31	21	0.9666		0.9871	0.5379 - 1.8114	none
5	T - A - T	42	30	0.7729		1.0565	0.6186 - 1.8046	none
6	T - A - C	11	6	0.6441		0.7864	0.2831 - 2.1843	none
7	**T - T - T**	11	17	**0.0234**	**0.0424**	2.453	1.107 - 5.434	**predisposition**
8	T - T - C	16	18	0.1219		1.7500	0.8556 - 3.5792	none

Haplotype containing rs12722 - rs946953 - rs2104772. Results that have been classified as significant by the P-value (P<0.05) in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg Adjusted P value. OR- odds ratio. 95% CI – 95% confidence interval.