CORRELATION OF ENDOGENOUS SECRETORY RECEPTOR FOR ADVANCED GLYCATION END PRODUCT (esRAGE) WITH METABOLIC HEALTH RELATED BIOMARKERS OF SKELETAL MUSCLE

Jesse Pajari

Liikuntalääketieteen pro gradu -tutkielma

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ABSTRACT

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Many health benefits of regular physical activity are mediated via improved exercise-associated oxidative capacity that is strongly correlated to skeletal muscle metabolism, longevity and overall health. Endogenous secretory receptor for advanced glycation end product (esRAGE) is a major soluble form of RAGE and a potential biomarker for chronic diseases. Studies have shown that AGE-sRAGE-RAGE pathway is associated with the disruption of metabolic health. The main purpose of this study was to investigate the association of serum esRAGE concentration with metabolic health related biomarkers of skeletal muscle.

The selection of physical activity discordant twin pairs for this study is based on the TWIN-ACTIVE study. Physical activity was assessed based on the questionnaire data of leisure-time physical activity in 1975, 1981; retrospective follow-up physical activity interviews (1980, 1985, 1990, 1995, 2000 and 2005); and the laboratory visit in 2007 which included physical activity questionnaires and interviews. Ten twin pairs (3 MZ pairs, 7 DZ pairs) with 32-year-long discordance in physical activity habits and who participated to muscle biopsy group were identified. Data were analyzed using IBM SPSS Statistics 22. Pairwise analysis was used to study the intrapair differences between physically inactive vs. physically active co-twins. The normally distributed variables was studied by Student's paired t-test and Wilcoxon matched-pair signed-rank test was used for non-normally distributed variables. The partial correlation coefficient was utilized for the correlation of serum esRAGE concentration with biomarkers, adjusted by sex and age.

Serum esRAGE concentration was found to be significantly associated with many biomarkers related to skeletal muscle metabolism. The main finding was that esRAGE was inversely associated with energy metabolism related gene centroids; fatty acid metabolism ($p<0.05$), ubiquinone synthesis ($p<0.01$), oxidative phosphorylation ($p<0.05$), valine, leusine and isoleucine degradiation ($p<0.05$) and butanoate metabolism ($p<0.01$). Inverse correlation of esRAGE was also found with number of mitochondrial DNA copies ($p<0.01$) and IL-15 ($p=0.01$). Positive correlation was found with adiponectin ($p<0.01$) and resistin ($p<0.05$). The major differences observed between physically active and inactive co-twins were body fat percent (25.5±5.6% vs. 19.9±5.9%, $p<0.05$), intramuscular fat of thigh (11.3±5.4cm^2 vs. 7.5±4.2cm^2, $p<0.05$), estimated VO_{2peak} value (28.3±3.6 ml/kg/min vs. 33.0±5.0 ml/kg/min, $p<0.05$) and the amount of visceral fat (158.4±38.8cm^2 vs. 90.4±22.1cm^2, $p<0.05$).

In conclusion, our results showed that serum esRAGE concentration is correlated for biomarkers related to energy metabolism pathways. These findings provide a novel insight for the possible role of serum esRAGE level as biomarker of skeletal muscle metabolism. This support a hypothesis of esRAGE as a potential biomarker and gives a need for further research.

Key words: esRAGE, skeletal muscle, physical activity, metabolism

TIIVISTELMÄ

Pajari, J. 2016. Sisäeritteisen AGE-reseptorin (esRAGE) yhteys luurankolihaksen metaboliseen terveyteen liittyviin biologisiin merkkitekijöihin. Terveystieteiden laitos, Jyväskylän yliopisto, Liikuntalääketieteen pro gradu –tutkielma, 63 sivua.

Monet säännöllisen fyysisen aktiivisuuden terveyshyödyt välittyvät parantuneen liikuntaan liittyvään oksidatiivisen kapasiteetin kautta, jolla on vahva yhteys luurankolihaksen aineenvaihduntaan, pitkäikäisyyteen ja yleiseen terveyteen. Sisäeritteinen AGE-reseptori (esRAGE) on merkittävä liukoisen AGE-reseptorin (sRAGE) muoto, joka on mahdollinen kroonisiin sairauksiin liittyvä biologinen merkkitekijä. Tutkimukset ovat osoittaneet AGE-sRAGE-RAGE reitin olevan yhteydessä metabolisiin sairauksiin. Tutkimuksen päätarkoituksena on selvittää seerumin esRAGE konsentraation yhteys luurankolihaksen metabolista terveyttä kuvaaviin biologisiin merkkitekijöihin.

Tutkimukseen valikoituneiden fyysiseltä aktiivisuudelta poikkeavien kaksosparien valinta perustuu TWINACTIVE –tutkimukseen. Kaksosparien fyysistä aktiivisuutta on arvioitu kyselyaineiston perusteella, joka on kohdennettu vapaa-ajan fyysiseen aktiivisuuteen vuosina 1975, 1981; fyysisen aktiivisuuden seurantahaastattelut suoritettiin viiden vuoden välein (1980, 1985, 1990, 1995, 2000 ja 2005); lisäksi laboratoriokäynti vuonna 2007 sisälsi fyysiseen aktiivisuuteen liittyviä kyselyjä ja haastatteluja. Lopulta tutkimukseen valikoitui kymmenen kaksoparia (3 mozygoottia paria, 7 dizygoottia paria), jotka poikkesivat fyysisen aktiivisuuden osalta toisistaan vähintään 32 vuoden ajalta, ja jotka kuuluivat lihasbiopsia ryhmään. Tulosten analysoinnissa käytettiin IBM SPSS Statistics 22 –tilasto-ohjelmaa. Tutkimuksessa käytettiin parittaista analyysiä, kun selvitettiin kaksosparin välisiä eroja fyysisesti aktiivisen ja inaktiivisen parin välillä. Normaalisti jakautuneita muuttujia tutkittiin Studentin parittaisella t-testillä ja ei-normaalisti jakautuneita muuttujia Wilcoxonin sijalukujen merkkitestillä. Osittaiskorrelaatiota käytettiin tutkittaessa seerumin esRAGE konsentraation yhteyttä biologisiin merkkitekijöihin, kun sukupuoli ja ikä vakioitiin.

Seerumin esRAGE konsentraatio oli yhteydessä useisiin luurankolihaksen metaboliaa kuvaaviin biologisiin merkkitekijöihin. Päälöydöksenä havaittiin, että esRAGElla oli käänteinen yhteys lihaksen energia-aineenvaihduntaan liittyviin geeniryhmiin; rasvahappojen metabolia ($p<0.05$), ubikinoni synteesi ($p<0.01$), oksidatiivinen fosforylaatio ($p<0.05$), valiini, leusiini ja isoleusiini hajoaminen ($p<0.05$) ja butanoaatti metabolia ($p<0.01$). Käänteinen yhteys havaittiin myös esRAGEn ja mitokondrion DNA kopioiden ($p<0.01$) ja IL-15 ($p=0.01$) välillä. Seeruminen esRAGE konsentraatio oli positiivisesti yhteydessä adiponektiiniin ($p<0.01$) ja resistiiniin ($p<0.05$). Merkittävimmät erot fyysisesti aktiivisten ja inaktiivisten kaksosten välillä oli kehon rasvaprosentissa (25.5±5.6 vs. 19.9±5.9, $p<0.05$), reisilihaksen sisäisen rasvan määrässä (11.3±5.4cm2 vs. 7.5±4.2cm2, $p<0.05$), viskeraalirasvan määrässä (158.4±38.8cm2 vs. 90.4±22.1cm2, $p<0.05$) ja arvioidussa hapenottokyvyn huippuarvossa (VO2peak) (28.3±3.6 ml/kg/min vs. 33.0±5.0 ml/kg/min, $p<0.05$).

Yhteenvetona, tutkimuksen tulokset osoittavat seerumin esRAGE konsentraation olevan yhteydessä energia-aineenvaihduntaa kuvaaviin biologisiin merkkitekijöihin. Löydökset antavat viitteitä, että seerumin esRAGE taso saattaa olla mahdollinen terveyttä kuvaava biologinen merkkitekijä, joka vaatii lisätutkimusta.

Avainsanat: esRAGE, luurankolihas, fyysinen aktiivisuus, metabolia

ABBREVIATIONS

AGE	advanced glycation end product
CRF	cardiorespiratory fitness
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
DZ	dizygotic
esRAGE	endogenous secretory receptor for advanced glycation end product
HDL	high density lipoprotein
IL-6	interleukin-6
IMT	intima-media thickness
kDa	kilodalton
MET	metabolic equivalent of task
mtDNA	mitochondrial deoxyribonucleic acid
mtmDNA	mitochondrial messenger deoxyribonucleic acid
mtrDNA	mitochondrial transfer deoxyribonucleic acid
MZ	monozygotic
RAGE	receptor for advanced glycation end product
RNA	ribonucleic acid
ROS	reactive oxygen species
sRAGE	soluble receptor for advanced glycation end product

CONTENTS

INTRODUCTION

There have been observations of the health benefits of physical activity since the time of Hippocrates and Galen. The scientific evidence to support the benefits has started to gather relatively recently. In early 1950s Jeremy Morris and his colleagues were the first who recognized that physical inactivity was a risk factor for health in an occupational environment. They found that physically active conductors were protected from coronary heart-disease compared to more sedentary drivers of London's double-decker buses. Later these findings were reproduced with other occupations (Morris & Crawford 1958; Paffenberger et al. 2001). Today, it is clear that physical inactivity is a major risk factor for non-communicable diseases with significant economic impact globally (Lee et al. 2012; Ding et al. 2016). Evidence shows that high levels of physical activity possess many distinctive health benefits. It is associated with increased longevity and can be used as a therapy for many chronic diseases (Janssen et al. 2013; Hawley et al. 2014; Pedersen & Saltin 2015). Although habitual physical activity is associated with lower mortality, risk reduction is even larger with high cardiorespiratory fitness (CRF) (Lee et al. 2011b). Genetic factors are known to have effects for levels of physical activity, CRF and responsiveness to exercise (Bouchard et al. 1999; Bouchard & Rankinen 2001; Stubbe et al. 2006; Schutte et al. 2015).

The Finnish Twin Cohort has been used to select 32-year leisure time physical activity discordance twin pairs to fit TWINACTIVE study. Eventually, 16 middle-aged and older twin pairs with discordance in their physical activity habits were identified of which ten twin pairs (3 MZ, monozygotic pairs, 7 DZ, dizygotic pairs) took a part to muscle biopsy group (Leskinen et al. 2009). The fundament principle behind twin study is that MZ twins come from a single fertilized egg and share identical genetic material, so it can be postulated that any discordance between the co-twins are resulted from environmental factors. DZ twins comes from two separately fertilized eggs sharing 50% of same genes. Comparison of the genetic influence for a trait offers the estimate of the extent to which genetic variation determines phenotypic variation of that trait (Boomsma et al. 2002). The main aim of this master thesis is to study the association of serum esRAGE level, which is associated with many chronic health conditions, with energy metabolism related gene centroids. We hypothezise that serum esRAGE levels is associated with biological markers related to metabolic health of skeletal muscle. Therefore, serum esRAGE level may be considered as a potential biological marker for the health of skeletal muscle.

Finally, I want to declare my acknowledgements especially for professor Urho Kujala for the data and Tuija Leskinen. All the data used in this master thesis is based on the TWINACTIVE study. Tuija Leskinen with her co-workers have done all laboratory analyses and measurements.

2 LITERATURE REVIEW

2.1 Physical activity vs. physical inactivity

In 1985 Caspersen et al. (1985) defined physical activity,"as any bodily movement produced by skeletal muscles that requires energy expenditure." This has been the most popular and widely cited definition of physical activity (Strath et al. 2013, WHO 2014). Physical activity results an increase in energy expenditure above resting levels accounting for 15-30% of total daily energy expenditure under typical circumstances which has the most profound effect on human energy expenditure. The rate of energy expenditure is directly linked to the intensity of physical activity (McArdle et al. 2015, 196). Leisure-time physical activity (LTPA) is a broader description of the activities one participates in during free time based on personal interests and needs. The intensity and duration can vary considerably in these activities (Howley 2001). According to the most recent physical activity guidelines to promote and maintain health, all healthy adults need moderate-intensity aerobic physical activity for a minimun of 30 minutes on 5 days each week or vigorous-intensity aerobic physical activity for a minimun of 20 minutes on 3 days each week. Combinations of moderate- and vigorous-intensity activity can be performed, and also to perform multiple short bouts of physical activity throughout the day to meet these recommendations (Physical Activity Guidelines Advisory Committee 2008). Half of the U.S. adult population do not meet these guidelines (Bauer et al. 2014).

Once major adaptations of human survival were consonant with habitual physical activity. Most of the current human genome has probably evolved in the physically active hunter-gatherer environment and remains unchanged to this day. Estimates of physical activity in the late paleolithic period is much greater than today. This has lead to a dissonance between genes and current environment including sedentary lifestyle (Booth et al. 2002). At the same time with the technological revolution daily physical activity levels have declined over the last decades. Almost a third of adult population around the globe are not attained to recommended physical activity guidelines, and thus defined as physically inactive (Hallal et al. 2012; WHO 2014). According to the recent estimation over half million deaths may be averted each year worldwide, if physical inactivity is decreased by 10% (Lee et al. 2012). In addition, physical inactivity has been identified as the fourth leading risk factor for global mortality (WHO 2014). Several factors are known to affect for physical activity behavior including individual-level factors such as age, sex, health status, self-efficacy, and previous physical activity, as well as physical and social

environments, and also genetics (Bauman et al. 2012). Sedentary behavior is one aspect of physical inactivity that is defined as any waking behaviour characterized by an energy expenditure ≤1.5 METs while in a sitting or reclining posture has received a lot of attention over the past years. It has recently received a lot of attention, especially in western countries (Edwardson et al. 2012; Tremblay 2012). Prolonged sedentary time has an independent association with various disease states and all-cause mortality. Though, it seems that deleterious health effects will decrease substantially among persons who participate in higher levels of physical activity compared to lower levels (Ekblom-Bak et al. 2014; Biswas et al. 2015; Ekelund et al. 2016). Increased inactivity has contributed to the worldwide epidemic of non-communicable diseases including type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver disease, and colon cancer (Eckardt et al. 2011; Boyle et al. 2012; Oni et al. 2015; Wilson et al. 2015). Etiology of many chronic diseases points towards metabolic risk factors including increased fat mass, glucose intolerance and low oxidative capacity.

Physical activity induced energy expenditure reduce fat mass, if there is a meaningful long-term net energy deficit. Reduced fat mass points towards a reduction in adipocyte size rather than a change in number while the greatest relative loss takes place in visceral adipose tissue (Thompson et al. 2012). Physical activity has also effect to adipocyte metabolism and it prevents fat infiltration to the skeletal muscle even in older age (Wroblewski et al. 2011; Rönn et al. 2013). Even a single bout of exercise is known to improve whole-body insulin sensitivity for up to 48 hr after cessation. Exercise increases skeletal muscle glucose uptake through an insulin-independent pathway indicating that muscle contraction directly impacts glucose homeostasis (Egan & Zierath 2013). When daily physical activity is decreased significantly for two weeks, peripheral insulin sensitivity and lean mass is reduced (Krogh-Madsen et al. 2010). Importantly, it seems that physical inactivity but not age is a determinant factor of muscle oxidative capacity, mitochondrial function and insulin sensitivity (Rimbert et al. 2004). Only 7 days of total physical inactivity is enough to lower metabolic capacity and affects metabolic regulation in a human skeletal muscle (Ringholm et al. 2011). For all its positive health effects, regular exercise comes with relatively low risk of side effects. Finally, although evidence strongly highlights that higher physical activity levels and cardiorespiratory fitness are related to lower cardiovascular and all-cause mortality, physical activity remains underused to reduce detrimental health effects (Tucker et al. 2011; Myers et al. 2015; Celis-Morales et al. 2016). There is still a global need to promote physical activity and to reduce overall sedentary time during the day (Healy et al. 2015).

2.2 Exercise and metabolic health

Exercise is a mode of physical activity, defined as structured, planned, repetitive, and purposive. The objective of exercise is to improve one or more components of physical fitness. That is a set of attributes that people have or achieve, further defined as a set of components that influences exercise ability and performance in sports (Caspersen et al. 1985; Schutte et al. 2015). Health benefits of exercise extend from skeletal muscle and exercise performance to a metabolic health of whole-body (TABLE 1). Regular exercise improves biomarkers of lipid and lipoprotein metabolism, and affects positively to glucose intolerance and insulin resistance, systemic inflammation, and hemostasis (Mann et al. 2014; Lin et al. 2015). Regular exercise is also associated with lower blood pressure, higher HDL cholesterol levels, skeletal muscle structure, function and metabolism, higher physical fitness and lower body fat. The benefits of exercise are mediated via many disease-specific mechanisms (Kujala 2009).

Exercise has been suggested to have benefits similar to hormesis, a biological concept which states that exposure to a low dose of a noxious or toxic agent can bring about results deemed beneficial to the long-term welfare of the organisms (Ji et al. 2016). Exercise challenges whole-body homeostasis, and is followed by acute and adaptive responses at the cellular and systemic levels. Responses are meant to minimize extensive disruptions. Exercise also provokes widespread changes in numerous cells, tissues, and organs that are caused by or are a response to the metabolic activity of contracting skeletal muscle (Egan & Zierath 2013). Exercise-associated adaptations include an array of physiological responses related to metabolic health which are based on: (1) muscle contraction per se, (2) the elevated energy expenditure, and (3) the increased substrate turnover during exercise. Effects are reflected by changes in contractile protein and function, mitochondrial function, metabolic regulation, intracellular signaling, and transcriptional responses. Adaptations are the result of coordinated alterations in gene expression of muscle cells that are induced during each bout of contractile activity. Repeated, transient bursts of gene expression and thus mRNA levels are essential for cellular adaptations to exercise (Ost et al. 2016). Timing and responsiveness of individual mRNA species to different types of contractile activity is variable. Peak values for genes generally occur 4-8 hour after an exercise bout, and after that mRNA levels return to pre-exercise state within 24-hour. Exercise also regulates mRNA and protein abundance by altering DNA methylation status, histone modifications, and micro-RNA expression (Hawley 2014).

TABLE 1. Adaptations and health benefits of aerobic exercise to selected skeletal muscle and metabolic health characteristics (modified from Egan & Zierath 2013).

Aerobic (endurance)	
Skeletal muscle morphology and exercise performance	
Muscle hypertrophy	↔
Muscle fiber size	↔↑
Myofibrillar protein synthesis	↔↑
Mitochondrial protein synthesis	↑↑
Mitochodrial density and oxidative function	↑↑↑
Endurance capacity	↑↑↑
Anaerobic capacity	↑
Whole body and metabolic health	
Percent body fat	↓↓
Lean body mass	↔
Insulin sensitivity	↑↑
Inflammatory markers	↓↓
Cardiovascular risk profile	↓↓↓

↑, values increase; ↓, values decrese; ↔, values remain unchanged; ↑ or ↓ small effect; ↑↑ or ↓↓, medium effect; ↑↑↑, large effect; ↔↑, no change or light change.

2.2.1 Cardiorespiratory fitness

Many health benefits of regular exercise are mediated via improved maximal exercise-associated oxidative capacity, called as cardiorespiratory fitness (Lin et al. 2015). Cardiorespiratory fitness is usually reported as mlO_2*kg*min or METs (1 MET= 3.5 mL O_2 uptake*kg/body mass/min) which is highly correlated with maximal oxygen uptake (VO_{2max}) (DeFina et al. 2015; Shuval et al. 2015). It has estimated that each 1-MET increase is associated with 10%-25% improvements in survival. This is a relatively small increment achievable by most individuals (Kaminsky et al. 2013). VO_{2max} represents the maximal capacity of cardiovascular and respiratory systems to transport O_2 to vital organs and skeletal muscle and is determined by the combined capacities of the central nervous system to recruit motor units, the pulmonary and cardiovascular systems to deliver O_2 to contracting skeletal muscles, and the ability of those muscles to consume O_2 in the oxidative, metabolic pathways. After its description in 1925, VO_{2max} is now recognized as a better predictor of mortality than any other established risk factor

or biomarker for cardiovascular disease (Hawley 2014: Korpelainen et al. 2016). The gains in VO_{2max} in subjects exposed to several months of endurance exercise are on average 15-25%, but the heterogenity in response is considerable ranging from zero or very low gains up to >50% (Bouchard et al. 2011). On the contrary, a total physical inactivity for 3 weeks has shown to decrease VO_{2max} by 26% (DeFina et al. 2015). Age, sex, and ethnic origin are not major determinants of human responses to regular exercise, whereas previous training history has a considerable impact in some cases (Bouchard & Rankinen 2001).

Strong relationship is found between measured and self-reported cardiorespiratory fitness and health (Holtemann et al. 2015). Evidence highlights that cardiorespiratory fitness is a predictor of cardiovascular disease and all-cause mortality in men and women, it has a more profound effect for all-cause mortality than body mass index (BMI) or high physical activity levels (Wei et al. 1999; Kodama et al. 2009; Lee et al. 2011b; Barry et al. 2014). The addition of cardiorespiratory fitness to traditional risk factors significantly improves reclassification of the risk of CVD mortality across short-term and long-term follow-up (Gupta et al. 2011). There is a gradient of mortality risk even in men with low cardiorespiratory fitness (Farrell et al. 2015). High cardiorespiratory fitness has some protective effect against prolonged sedentary time by lowering mortality risk (Shuval et al. 2015). Regardless of individual's current health status, higher levels of physical activity and cardiorespiratory fitness improve the overall CVD risk profile (Myers et al. 2015). Evidence has also demostrated independent, but overlapping, effects of cardiorespiratory fitness and high physical activity levels for cardiovascular health (DeFina et al. 2014). The direct association between the amount of regular physical activity and level of cardiorespiratory fitness is well documented. Thus, increasing the volume of weekly physical activity or the intensity of physical activity improves cardiorespiratory fitness (Kaminsky et al. 2013).

2.2.2 Skeletal muscle as a secretory organ

Skeletal muscle accounts for 30% of the resting metabolic rate in adult humans and comprises 40% of total body mass in mammals (Egan & Zierath 2013). It is composed of different, specialized fibers and shows an enormous malleability in functional adaptation and remodeling in response to contractile activity (Ost et al. 2016). Skeletal muscle has myriad beneficial effects for health via metabolism and muscle function. Muscle mass and function described as strength

and power have their vital independent role in human health. Loss of muscle mass with loss of muscle function such as decreased grip strength and gait speed is defined as sarcopenia which plays a key role in the development of the frailty syndrome (Mitchell et al. 2012; Clegg et al. 2013; Cederholm & Morley 2015).

Genomic and proteomic approaches have identified several hundred so called myokines which are defined as factors produced and secreted by muscle cells including a protein factors such as cytokines (Karstoft & Pedersen 2016). Since the introduction of myokines these proteins have suggest to mediate anti-inflammatory and metabolic effects that may contribute to the preventive effects of exercise againts chronic diseases (Pedersen 2009; Pedersen & Febbraio 2012) (FIGURE 1). Among others, myokines such as IL-6, IL-15, irisin and decorin have their distinctive roles in human metabolism and health (Karstoft & Pedersen 2016). Skeletal muscle gene expression is affected very differently by an acute exercise bout and regular exercise, which is also reflected by myokine gene expression. There is only a minor overlap between myokines released by acute exercise and in response to regular exercise training. An acute bout of strenuous exercise increases pro-inflammatory myokines and regular exercise training is considered as a mean to control and counteract chronic low-grade inflammation (Ost et al 2016).

Almost all identified myokines are also produced by other tissues and cell types including macrophages and adipose tissue. Exercise-induced myokines can have autocrine or paracrine effects and are not necessarily released into plasma (Ost et al. 2016). Importantly, in insulin resistant conditions myokine production and secretion is regulated differently. This underlines a profound role of skeletal muscle in metabolic disease states (Karstoft & Pedersen 2016).

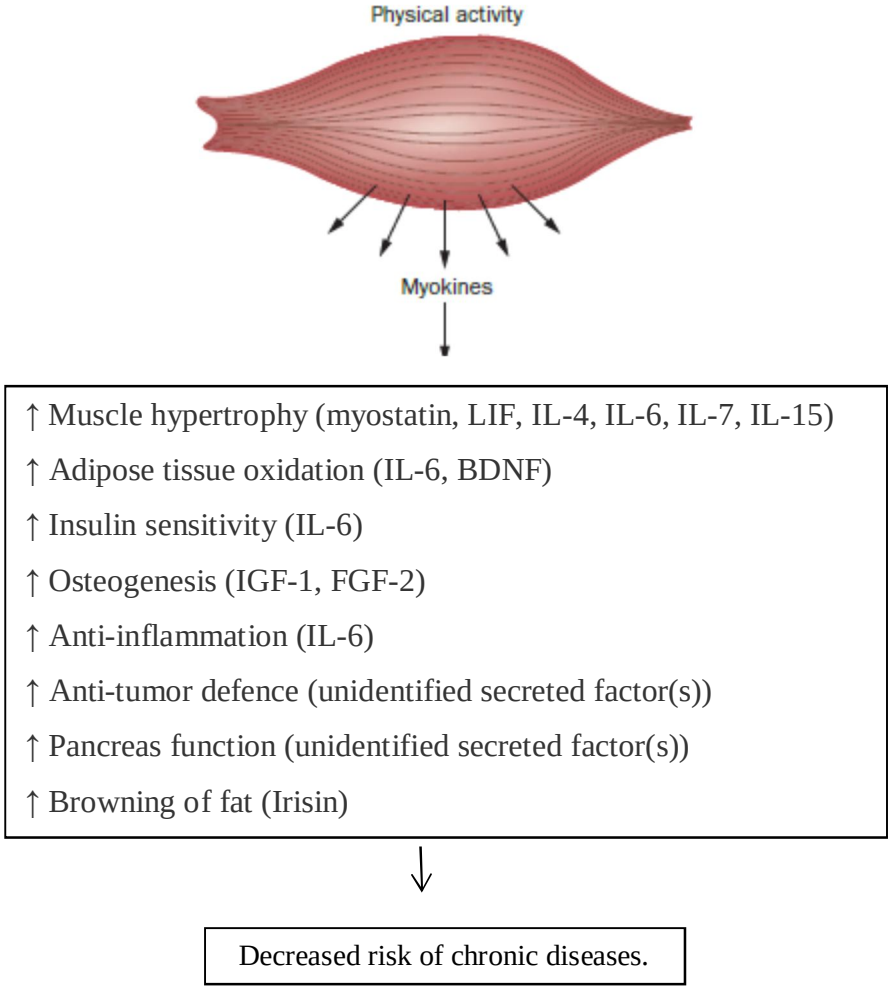

FIGURE 1. Skeletal muscle produces and releases myokines which provides conceptual basis for understanding some of the molecular mechanisms that link physical activity to protection against chronic diseases (modified from Pedersen & Febbraio 2012). LIF, leukemia inhibitory factor; IL-4, interleukin-4; IL-6, interleukin-6; IL-7, interleukin-7; IL-15, interleukin-15; BDNF, brain-derived neutrophic factor; IGF-1, insulin-like growth factor-1; FGF-2, fibroblast growth factor-2.

2.2.3 Mitochondria

Normal control of skeletal muscle metabolism is essential for health. Essence for the regulation of skeletal muscle metabolism is the transcriptional control of metabolic enzyme expression and epigenetic mechanisms such as DNA methylation and histone acetylation (Howlett & McGee 2016). Maximal exercise-associated oxidative capacity is strongly correlated to muscle

metabolism, longevity and overall health. That is reflected by correlation of high cardiorespiratory fitness with higher levels of VO_{2max}, skeletal muscle oxidative enzyme capacity, and proteins such as peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α). Therefore, impaired regulation of oxidative pathways in mitochondria may be the common factor linking reduced cardiorespiratory fitness to cardiovascular and metabolic risk. Cardiorespiratory fitness may be a reflection of overall physiological health and function thereby poor cardiorespiratory fitness may be a marker of early physiological manifestation of these conditions (Kaminsky et al. 2013). This highlights the central role of mitochondria in cellular metabolism and further underlines mitochondria as potential loci of disease with extensive clinical relevance (Hood 2001; Overmyer et al. 2015).

Mitochondria (Ø from 0.5 to 1.0μm) as a membrane-enclosed organelles are found in most eukaryotic cells and are composed of five compartments: the outer mitochondral membrane, the inter-membrane space, the inner mitochondrial membrane, the cristae (formed by infoldings of the inner membrane), and the matrix (space within the inner membrane) (Bishop et al. 2014). In skeletal muscle, mitochondria are highly organized in a tubule reticulum and form, by fusion and fission, a dynamic reticular network which allows them to share mtDNA (Barbieri et al. 2015). The main role of mitochondria is the production of adenosine triphospate (ATP). The production of ATP occurs during the reactions tricarboxylic acid cycle, located within the matrix of mitochondria, and via the electron transport system, located along the inner membrane. The electron transport chain (FIGURE 2) consists of 5 multi-polypeptide complexes (I-V) located in the inner membrane of mitochondria that receive electrons from the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2), produced in the tricarboxylic cycle. Electrons are transfered along complexes of the electron transport chain, with O_2 serving as the final acceptor at complex IV. Along the process, protons are pumped out of the matrix into the inter-membrane space producing an electrochemical gradient that represents the driving force enabling complex V to produce ATP by phosphorylation of adenosine diphosphate (ADP). These last two processes are jointly described as oxidative phosphorylation (OXPHOS) (Bishop et al. 2014).

Mitochondrial biogenesis implies the cellular processes involved in the synthesis and degradation of the organelle, and more accurately the generation of new mitochondrial components (Bishop et al. 2014; Barbieri et al. 2015). Mitochondrial biogenesis requires the coordination

of multiple cellular events, including transcription of two genomes, synthesis of lipids and proteins, and the stoichiometric assembly of multisubunit protein complexes into a functional respiratory chain (Hawley 2014). Exercise is a potent stimulus for the mitochondrial biogenesis, especially aerobic training or endurance training increases cardiorespiratory capacity and mitochondrial content of skeletal muscle (Ji et al. 2016).

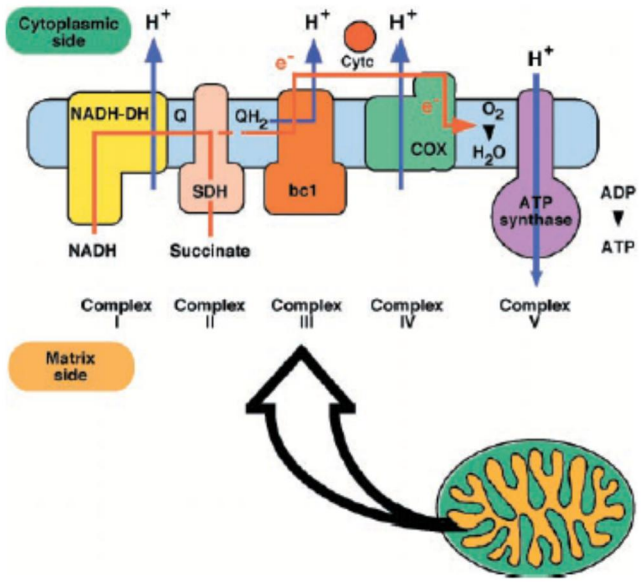

FIGURE 2. The simplified illustration of the electron transport system. NADH-DH, nicotinamide adenine dinucleotide dehydrogenase; SDH, succinate dehydrogenase; bc1, cytochrome bc_1; COX, cytochrome oxidase (modified from Saraste 1999).

Repeated contractile activity-induced mitochondrial adaptations in skeletal muscle are the result of cumulative effects of continuous acute bouts of activity. The initiation of mitochondrial biogenesis in muscle during an exercise begins with the putative signals by muscle contractions within seconds of the onset of contractile activity (FIGURE 3) (Camera et al. 2016). These swift responses to exercise are related to the type, intensity and duration of contractile activity, as well as the muscle fiber type. Changes in mitochondrial biogenesis initiated by exercise are a result of sequential molecular events of which primary pathways include (1) initiation of biogenesis by activation of signaling kinases, (2) the induction of coactivator proteins such as PGC-1α and nuclear respiratory factor (NRF) transcription factor proteins, and their transactivation of target genes, (3) the import of these precursor proteins into mitochondria, and (4) the coordinated incorporation of both mitochondrial and nuclear gene products into an expanding organelle reticulum (Ljubicic et al. 2010).

Exercise is capable to increase mitochondrial content also in aged subjects. The extent of the change is dependent on the age of the subjects, their health, absence or presence of different co-morbidities, duration, and the intensity and frequency of the exercise dose. Mitochondrial function can be restored at least partially with exercise in older subjects. In some cases, the extent of mitochondrial adaptations can potentially be similar between young and old subjects (Carter et al. 2015).

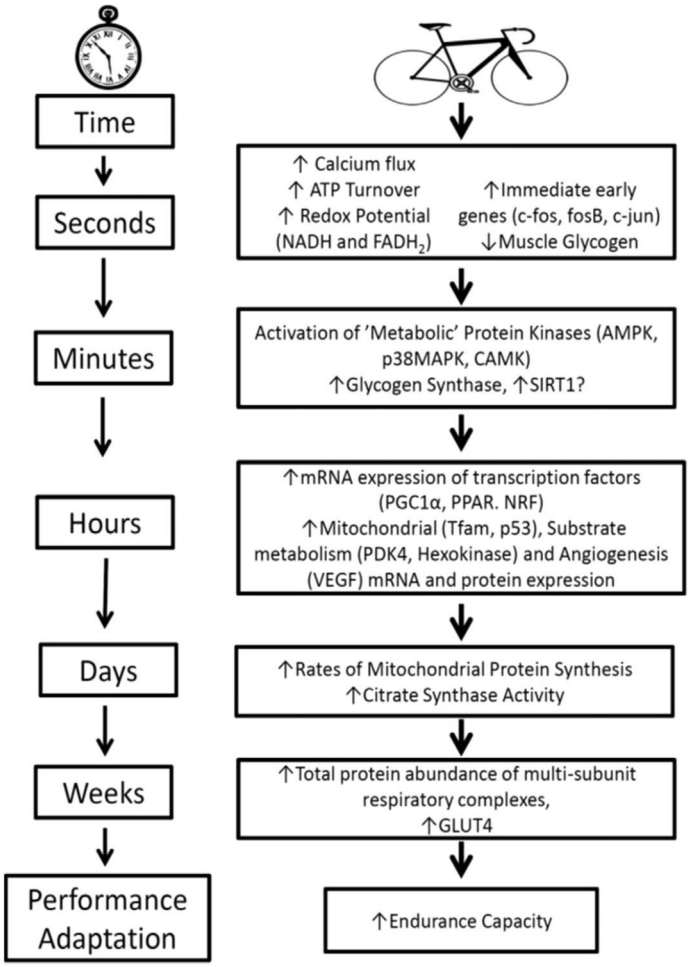

FIGURE 3. Time-dependent changes in molecular responses to endurance exercise in skeletal muscle. Following endurance exercise immediate fluctuations in intracellular calcium, ATP and redox potential activate metabolic kinases, followed by an upregulation in the levels of transcription factors and mitochondrial, substrate and angiogenesis related mRNAs. These signalling events culminate in mitochondrial biogenesis which results enhanced substrate metabolism and improved endurance performance (adapted from Camera et al. 2016).

2.3 AGE, RAGE and health

A limited formation of advanced glycation end products (AGEs) is a part of normal metabolism, but excessive accumulation has detrimental effects to the health of nearly every organ system in disease states such as diabetes mellitus, neurodegenerative diseases, chronic obstructive pulmonary disease, renal failure, rheumatoid arthritis, inflammatory myopathies and cardiovascular diseases (Heidland et al. 2001; Haslbeck et al. 2005; Goh & Cooper 2008; De Groot et al. 2011; Hegab et al. 2012; Hoonhorst et al. 2014; Li et al. 2012; Mallipattu & Uribarri 2014). Many of the adverse health effects of AGE overload are related to hyperglycaemia which is a significant driving force for AGE formation (Vlassara & Uribarri 2014). AGE transmits its effects via the plasma membrane receptor for AGEs (RAGE) which is highly expressed in immune cells, neurons, skeletal cells, muscle, lung, and heart. This interaction activates a number of downstream signalling pathways which relate to increased oxidative stress and/or acute or chronic inflammation (Nedic et al. 2013).

AGEs also interact with circulating soluble receptors for AGE, named endogenous secretory RAGE (esRAGE) and soluble RAGE (sRAGE). Soluble RAGE is proteolytically cleaved from cell surface receptor and esRAGE is a splice variant of RAGE and is secreted from the cells. These soluble RAGE variants acts as a decoy for RAGE ligands and thus have a cytoprotective effect against AGEs-RAGE interaction. The RAGE and its soluble variants forms in a different pathological states a complex and highly regulated biological system (Mahajan & Dhawan 2013). It has proposed that esRAGE alone or AGE/sRAGE or AGE/esRAGE may be a biomarker for AGE-RAGE-related pathologies (Kalea et al. 2011; Prasad 2014).

2.3.1 AGEs and disease

A process which leads to AGE formation was first observed in 1912. It was noted that amino acids heated in the presence of reducing sugars develop a yellow-brown colour. Since then, the process has been called the Maillard, or browning reaction (John & Lamb 1993). In the mid-1970s it was realised that the reaction also occurs slowly in biological systems in vivo and may cause some of the tissue modifications that takes place in various pathologies and during aging (Ulrich & Cerami 2001). Today, glycation is recognized as only one of many nonenzymatic modifications of proteins (Thorpe & Baynes 2003). The accumulation of AGEs on tissue proteins has been implicated in the ageing of tissues and the progression of chronic, age-related

degenerative diseases such as atherosclerosis, chronic renal failure, Alzheimer's disease and diabetes (Hu et al. 2015).

Body's AGE pool is determined mostly by diet, smoking, endogenous AGE formation, renal metabolism and tissue catabolism (Uribarri et al. 2005; Prasad et al. 2015). Advanced glycation occurs over a period of weeks (Singh et al. 2001). The most crucial factors to the formation of endogenous AGEs include the degree of hyperglycaemia, the rate of turnover of proteins for glycoxidation, and the extent of oxidant stress in the environment. AGEs are produced when reducing sugars such as glucose, react non-enzymatically with amino groups in proteins, lipids and nucleic acids (Goldin et al. 2006). AGEs could be formed in all tissue when protein meets glucose (Hu et al. 2015). The process begins when glucose reacts with a free amino group to form adduct referred to Schiff-base. During the process reversibly formed Schiff-base adducts converts to covalently bound Amadori rearrangement which undergo further rearrangement, chemical reactions that result in the formation of irreversibly bound AGEs (Nedic et al. 2013). There are three general mechanisms how these intracellular AGE precursors damages target cells. Firstly, AGEs cause modification of intracellular proteins, which alters their function. Secondly, AGE precursors modify extracellular matrix components of which impairs interaction with other matrix components and with the receptors for matrix proteins on cells. Thirdly, plasma proteins modified by AGE precursors bind to AGE receptors on cells inducing receptor-mediated production of reactive oxygen species (Brownlee 2001).

AGE interaction with its receptor RAGE has an important role in development and progression of vascular complications, especially in diabetes (FIGURE 4) (Basta 2008). The circulating AGE level is positively associated with inflammatory biomarkers, endothelial dysfunction, coronary artery disease and cardiovascular death in both diabetic and nondiabetic subjects (Kilhovd et al. 1999; Ueda et al. 2012). In type 1 diabetic patients, higher baseline plasma levels of AGEs were associated with incident fatal and non-fatal cardiovascular disease and all-cause mortality during the 12-year follow-up (Nin et al. 2011). Even in young and healthy subjects, serum level of AGEs could be a biomarker having an ability to predict cardiovascular health (Ueda et al. 2012). Impaired AGE metabolism exacerbates insulin resistance and play an important role in the etiology of cardiovascular pathology (Vasdev et al. 2007; Cassesse 2008). It has also proposed that the correlation of high serum levels of AGE with markers of inflammation and impaired metabolism may link healthy obesity to at-risk obesity (Uribarri et al. 2015).

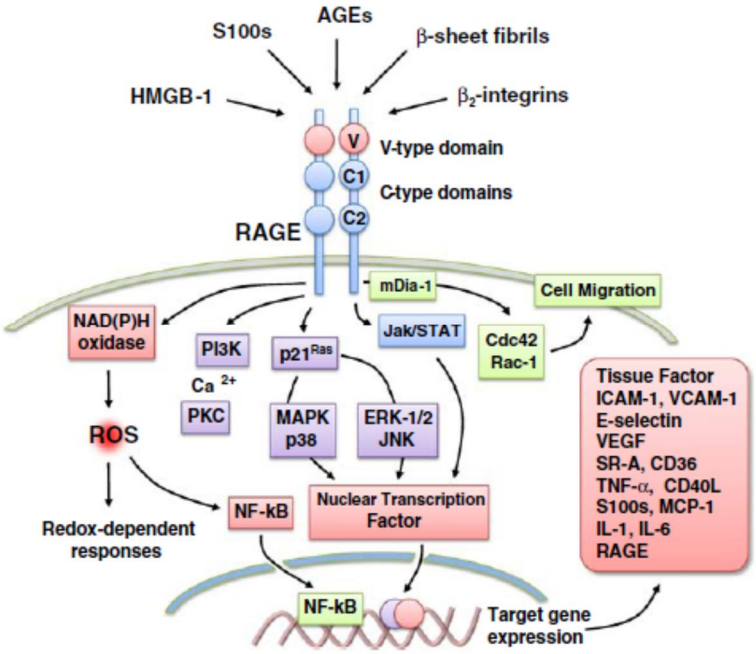

FIGURE 4. AGE-RAGE interaction. The activation of RAGE initiates the activation of a diverse array of signaling cascades. RAGE activation enhances the generation of reactive oxygen species (ROS) by activating NAD(P)H oxidase and other intracellular sources of ROS, such as mitochondrial electron transport chain. AGE-RAGE interaction also activates nuclear transcription factors, including nuclear factor (NF)-κB, and consequent target gene transcription (adapted from by Vazzana et al. 2009).

Besides its harmful effects for cardiovascular health and formation of collagen crosslinks in to the heart with age, AGEs accumulate also to other long lived proteins such as a human cartilage collagen and muscle proteins (Verzijl et al. 2000; Willemsen et al. 2011; Ramamurthy & Larsson 2013). AGEs are known to affect a human skeletal muscle during aging. In rats, AGEs accumulate progressively in the intracellular as well as the sarcolemmal regions of the muscle fiber, and also myosin is glycated irrespective of fiber type during aging (Ramamurthy & Larsson 2013). In a mouse study, accumulation of AGEs in the diabetic skeletal muscles was associated with myscle atrophy and muscle dysfunction through the RAGE-mediated AMPK-down-regulated Akt signalling pathway (Chiu et al. 2016). AGE cross-linking is significantly increased in a muscle in healthy, sedentary elderly people, although aging has no effect on the intramuscular collagen concentration (Haus et al. 2007). AGE accumulation is associated with reduced muscle strength and power, not only in older people, but also in younger adults. Moreover, slower walking speed have observed in older men and women with higher plasma level

of AGEs (Semba et al. 2010). Thus, it is hypothesized that AGEs may have a role in mechanisms, which may eventually lead to sarcopenia in older age (Momma et al. 2011).

Regarding the negative health effects of impaired AGE metabolism, lifestyle interventions such as exercise and nutrition are important determinants to decrease AGE levels. Short-term lifestyle modification has the ability to decrease the level of serum AGEs (Yoshikawa et al. 2009; De Courten et al. 2016). Together, aerobic exercise with low AGE diet is effective strategy to decrease circulating levels of AGEs (Macías-Cervantes et al. 2015).

2.3.2 Receptor for AGE

Neeper et al. (1992) were the first who isolated the receptor for advanced glycation end products (RAGE) from a bovine lung and characterized it to be a cell surface receptor for AGEs. Later RAGE was specified to be a 47-55 kDa protein and described as a pattern recognition receptor. It is a member of the immunoglobulin superfamily and due to its multiligand nature a receptor for certain S100 proteins, amyloid fibrils, high-mobility group box-1 (HMGB1), DNA and RNA. RAGE structure contains an extracellular region made of V-, C1- and C2-type domains, a short membrane-spanning domain, and a relatively short cytoplasmic and transducing domain (Sorci et al. 2013; Ott et al. 2014; Muth et al. 2015). RAGE is expressed in multiple tissues and cell types, but its physiological function is largely unresolved (Ott et al. 2014).

The common feature of RAGE biology is ability of RAGE ligands to activate signal transduction cascades and induce cellular migration. RAGE ligands transduce their effects on gene expression and cellular properties through the cytoplasmic domain of RAGE (Yan et al. 2010). This interaction activates multiple downstream signalling pathways (Xie et al. 2013). Functional consequences are strongly dependent on the cell type, the context, the identity and local concentration of its ligands, and also intervening intracellular events such as the amount of produced reactive oxygen species (ROS), the redox status, energy metabolism, cell-specific transcription and activity of definite genes. Thus, the activation of RAGE impacts both the organization of the cytoskeleton and transcription (Sorci et al. 2013). Truncation of the cytoplasmic domain in vitro or in vivo prevents RAGE ligands from activating signaling cascades and modulating expression and activity of central transcription factors (Yan et al. 2010).

RAGE is a prominent factor in the fine regulation of the balance between injury and repair.

Short-term RAGE signalling triggered by low concentrations of its ligands may support tissue homeostasis and concur to tissue repair by promoting cell proliferation and differentiation. On the other hand, long-term RAGE signalling triggered by high concentrations of its ligands may be deleterious by amplifying and perpetuating the inflammatory response, excessive stimulating cell survival, proliferation and migration or causing a lethal accumulation of ROS. RAGE ligands induced stress further potentiates the formation and accumulation of AGEs and subsequent RAGE overexpression (Sorci et al. 2013).

In endothelial cells, activation of RAGEs by circulating AGEs transduces multiple signals resulting in activation and translocation of nuclear transcription factors, including NF-kB, which transcribes its target genes. These genes consist among others vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, vascular endothelial growth factor (VEGF), and also proinflammatory cytokines, including IL-1a, IL-6, and tumor necrosis factor-a, and RAGE itself (Golding et al. 2006). In addition, AGE binding to RAGE results in the depletion of cellular antioxidant defense mechanisms and the generation of reactive oxygen species (ROS) (Stoner et al. 2013). RAGE also interacts with other endogenous nonglycated peptide ligands. Many of them have a significant regulatory role in inflammation (Prakash et al. 2015). Blockade of ligands/RAGE interaction supresses markers of inflammation, such as cytokine production and activation of NF-kB (Ramasamy et al. 2009). Therefore, it has proposed that RAGE plays a considerable role in regulation of inflammation and oxidative stress, and it is involved in microvascular and macrovascular complication in diabetes (Prakash et al. 2015).

2.3.3 Soluble receptors for AGE

Soluble RAGE (sRAGE) isoforms contains the spliced and several cleaved forms of sRAGE (Kalea et al. 2013). Each form of the sRAGEs is expressed at a lower level compared to full length RAGE. Total sRAGE/RAGE ratio is tissue specific and varies between 0.59 and 1.79 (Xie et al. 2013). sRAGE may act by binding up ligands and preventing their engagement and activation of the cell-surface receptor, when administrated as a ligand decoy (FIGURE 5) (Ramasamy et al. 2009). In several animal models sRAGE administration is successfully prevented or reversed RAGE effects in disorders such as diabetic atherosclerosis, altered wound healing,

and tumor growth and invasion (Falcone et al. 2013). Moreover, sRAGE also affect to the activity of other RAGE-ligand receptors such as toll-like receptors and scavenger receptors (Vazzana et al. 2009). A single measurement of circulating sRAGE could be a useful measure as concentrations of sRAGE within an individual remains relatively stable over three-year period (Bower et al. 2014). Genetic (29%) and family environmental (22%) components have a notable effect for the total sRAGE variation. Variation may also be affected by gender, age, race-ethnicity, concomitant diseases, renal function and medication. Serum sRAGE levels decrease with age and women tend to have significantly higher levels than men (Yan et al. 2010; Hudson et al. 2014; Prakash et al. 2015).

Circulating sRAGE is associated with different chronic disease states such as myasthenia gravis, kidney disease, acute coronary syndrome, sepsis and asthma or chronic obstructive pulmonary disease with neutrophilic airway inflammation (Moser et al. 2012; Sukkar et al. 2012; Prasad 2014; Jensen et al. 2015; Matsumoto et al. 2015; Rebholzl et al. 2015). There is some controversy about the role of sRAGE in diabetes and its effects to development and progression of cardiovascular diseases (Kajikawa et al. 2015). In a study by Biswas et al. (2015) sRAGE levels were not altered between prediabetic and normoglycaemic subjects, but there are also conflicting results (Huang et al. 2015). Elevated sRAGE level is an independent risk factor for increased risk of cardiovascular disease event in type 2 diabetics (Fujisawa et al. 2013). Also in young type 1 diabetics, sRAGE was positively correlated with carotid atherosclerosis, but in multi-ethnic population the association was not found (Heier et al. 2015; Hudson et al. 2015). In other study sRAGE levels were inversely associated with atherosclerosis and levels were lower in patients with coronary artery disease with peripheral artery disease than patients with coronary artery disease alone and control subjects (Falcone et al. 2013).

Circulating sRAGE and markers of obesity-related comorbidity risk factors including BMI are negatively correlated in adult and pediatric population (Accacha et al. 2013; Brickey et al. 2014; He et al. 2014; Hudson et al. 2014; Prakash et al. 2015). As sRAGE is associated with fat free mass and adiposity, it may be involved with the body muscular mass (Davis et al. 2014; Prakash et al. 2015). In summary, circulating sRAGE level could be an early biomarker of cardiometabolic disease risk even in youth (Brickey et al. 2014).

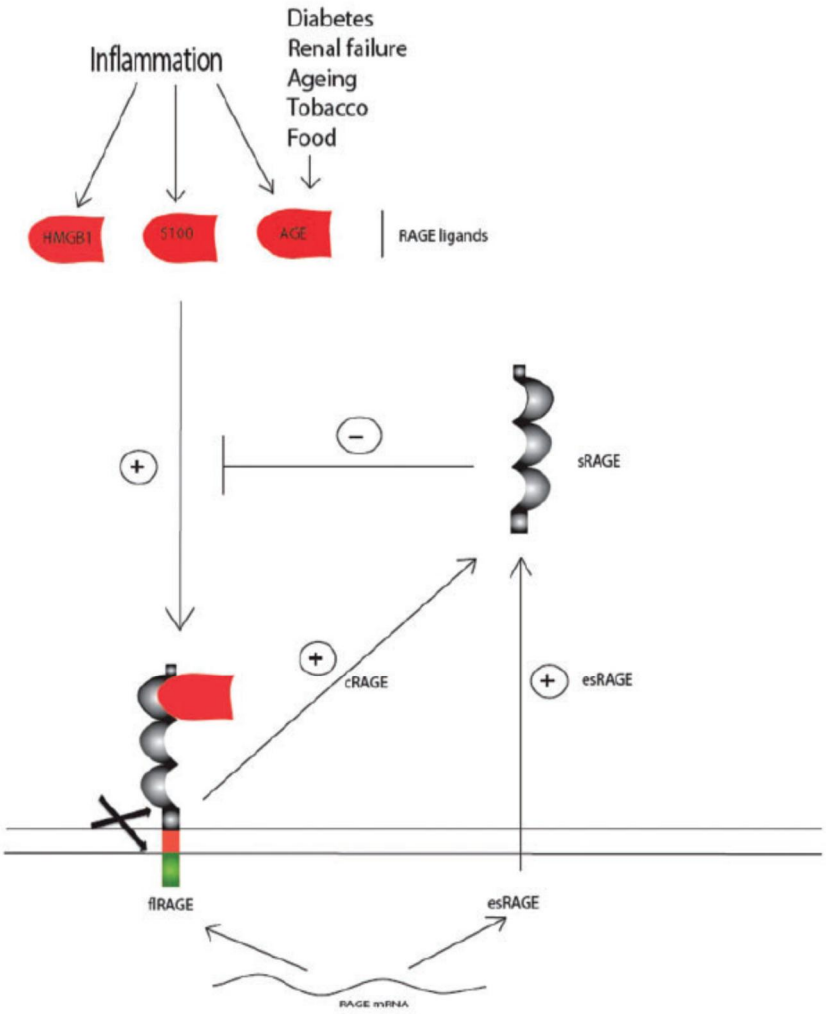

FIGURE 5. Feedback mechanism in AGE-RAGE-sRAGE system. Inflammation and other pathophysiological states enhance the expression of RAGE and AGE, resulting in increased formation of sRAGE as a negative feedback on RAGE with its ligands (adapted from Maillard-Lefebvre et al. 2009).

2.4 esRAGE - a biological marker for disease

EsRAGE/RAGEv1 is secreted and migrates as ≈48 kDa protein. This protein has been recognized to be a primary secreted splice variant of RAGE gene (Hudson et al. 2008). Alternative splicing of mRNA is an evolutionarily old process, which occurs through the utilization of different exons, introns, promoters and polyadenolation sites. The central function of alternative splicing is to increase the diversity of mRNA expressed from the genes (Kelemen et al. 2013). EsRAGE is produced due to the alternative splicing of intron 9 and the removal of exon 10. This altered exon/intron organization changes the reading frame of the transcript, and subsequently removes the transmembrane and cytoplasmic domain of RAGE (Kalea et al. 2011).

EsRAGE is determined to be the second most prevalent RAGE isoform after full-length RAGE (Hudson et al. 2008). Plasma levels of esRAGE are approximately five times lower than total sRAGE in healthy subjects, but relative proportion of esRAGE concentration may be higher in younger population (Koyama et al. 2005; Heier et al. 2015; Schmidt 2015). Evidence over the last decade has suggested that esRAGE may be a biological marker for disease. Based on the findings that serum esRAGE levels are decreased in many disease states. It is proposed that the decoy function of esRAGE may exhibit a feedback mechanism by which esRAGE prevents the activation of RAGE signalling (Koyama et al. 2007; Kalea et al. 2011; Prasad 2014).

Yonekura et al. (2003) were the first who proposed that esRAGE is present in the circulating blood and extracellular fluids in the vascular walls of our bodies. They discovered that esRAGE binds to an AGE ligand, and this activity neutralizes AGE action, and therefore have a protective effect against vascular injury (Yonekura et al. 2003). Plasma/serum esRAGE levels are inversely associated with many metabolic and inflammatory conditions (Schmidt 2015). Circulating esRAGE, but not sRAGE, is associated with metabolic syndrome, and inversely associated with quantitatively determined atherosclerosis in the carotid and femoral arteries (Koyama et al. 2005; Momma et al. 2014). EsRAGE levels are already reduced in pre-diabetic adults and this is correlated with oxidative stress (Huang et al. 2015). Hence, it is proposed that esRAGE may be a component of body's antioxidant defense system (Huang et al. 2015; Piarulli et al. 2013). Lower levels are related to pro-inflammatory state, which adresses that esRAGE may has a regulatory role in inflammation (Choi et al. 2009; Crasto et al. 2011; Santilli et al. 2015).

Serum esRAGE levels are mainly reduced in type 1 and 2 diabetes, although some conflicting results has reported in diabetes-associated complications such as kidney diseases and cardiovascular diseases (Koyama et al. 2007; Nishizawa & Koyama 2008; Colhoun et al. 2011; Chen et al. 2012; Prasad 2014). Serum esRAGE levels are decreased and inversely correlated to carotid intima-media thickness (IMT) in young type 1 diabetics and older type 2 diabetics (Katakami et al. 2005; Katakami et al. 2007). In type 1 diabetics, esRAGE, but not sRAGE, is independently associated with early carotid atherosclerosis. Further, both are risk factors for the progression of this condition, but only low sRAGE level is associated with plaque calcification (Katakami et al. 2008; Katakami et al. 2009; Moriya et al. 2014). Serum esRAGE levels are also decreased in diabetics with concomitant hyperlipidemia compared to diabetics without hyperlipidemia (Turk et al. 2014). EsRAGE, but not sRAGE, is inversely associated with hypertension, especially systolic blood pressure in patients with obstructive sleep apnea (Cai et

al. 2015). In contrast, according a study by Colhoun et al. (2011) both serum sRAGE and esRAGE levels are elevated in type 2 diabetics with coronary artery disease. In older women, high total serum sRAGE and esRAGE has been shown to predict cardiovascular mortality (Semba et al. 2009). These partly conflicting results may reflect the complexity of AGE-esRAGE and AGE-sRAGE axis and their distinctive roles of regulating AGE-RAGE pathway (Heier et al. 2015).

2.5 Twin study design

The classical twin study design has been used for a long time to estimate the importance of genetic and environmental influences on variation of complex traits. Study design compares phenotypic similarity of MZ and DZ twins. MZ twins derive from a single fertilized egg and share nearly 100% of their segregating genes, whereas DZ twins are derived from two distinct zygotes and share on average 50% of their segregating genes (van Dongen et al. 2012). The first estimation of the extent to which genetic variation determines phenotypic variation related to certain trait or disease can be made, when the resemblance of MZ twins for that same trait or disease is compared to resemblance of DZ twins (Boomsma et al. 2002). Thus data from MZ and DZ twins allow for the examination of causal relations in the comorbidity of traits (van Dongen et al. 2012).

Results of the family, twin and adoption studies have shown variance of human traits including physical, medical and biochemical characteristics that are influenced by genetic variation. Together with a fact of obvious environmental contribution to health behaviours, it can be said that diseases or traits have multifactorial nature with complex genetics. It is important to distuingish between environmental and genetic influences affecting the incidence of variability of a trait or disease for different reasons. First, the research resources can be target to the most important topics. Second, predisposition of genetics can be evaluated concerning diseases or traits. This has implications for primary or secondary prevention proposals (Kaprio & Koskenvuo 2002; van Dongen et al. 2012). As it said, ultimate objective of twin studies is to increase understanding of the role of the genetic and the environmental factors contributing to complex diseases and health-related factors, and to define their causal role in these conditions (Kaprio & Koskenvuo 2002).

Co-twin control studies are perfectly matched for genes and family background (Boomsma

2002). Along with a near complete match for age, gender, genes, intrauterine and childhood environment, and stadardization of both familial and genetic factors, co-twin control study is unique study design with which to research the effects of physical activity on health. These studies have highlighted the associations between long-term physical activity and health measures and studies have also pointed out the causal relationship of regular physical activity for health outcomes (Leskinen & Kujala 2015).

3 AIMS OF THE STUDY

High physical activity levels and/or aerobic fitness as a phenotype are associated with increased longevity and lower cardiovascular disease risk (Kujala et al. 1998; Myers et al. 2002; Kodama et al. 2009; Lee et al. 2011a; Gebel et al. 2015). The health benefits of regular physical activity seem to be explained mechanistically through the complex networks of signalling pathways and regulatory molecules that coordinate adaptive responses to physical activity. These adaptations are related to changes in fitness, body composition and metabolic health profile (Egan & Zierath 2013).

In this study 10 middle-aged and older twin pairs (7 DZ pairs, 3MZ pairs) with long-term discordance in their physical activity habits were identified from the Finnish Twin Cohort. Discordance was first defined in 1975 and after 32-year-long follow-up the same co-twin remained substantially more physically active. The design of the study considers the genetic background and thus is an appropriate model to study the associations between physical activity vs. inactivity and health outcomes.

The main aim of this master thesis is to study the correlation of serum esRAGE level, which is associated with different disease states, with biomarkers related to metabolic health of skeletal muscle among the habitually physically active vs. inactive twins. We hypothesize that serum esRAGE level is associated with biological markers of skeletal muscle metabolism and thus may be a potential marker of metabolic health.

The main research question:

Is serum esRAGE level associated with biomarkers related to metabolic health of skeletal muscle among study subjects?

4 METHODS

4.1. Twin pairs

The selection of physical activity discordant twin pairs for this study is based on the TWINACTIVE study (FIGURE 4) (Leskinen 2013). The selection was carried out in two waves, and same three assessments were conducted in both waves; baseline identification based on the questionnaire data of leisure-time physical activity in 1975, 1981; retrospective follow-up physical activity interviews (1980, 1985, 1990, 1995, 2000 and 2005); and the laboratory visit in 2007 which included physical activity questionnaires and interviews. The first wave of the study (Finnish Twin Cohort) includes all same-sex twin pairs born in Finland before 1958, and with both co-twins alive in 1967 compiled from the Central Population Registry of Finland. In a first follow-up in 1981, the cohort compromised of 5663 healthy same-sex twin pairs (1772 MZ and 3551 DZ, age 24 to 60 years). From these twin pairs 146 were found to be discordant for leisure-time physical activity both in terms of volume of the activity and in the participation in vigorous activity according to the questionnaires done in 1975 and 1981. Metabolic equivalent task (MET) index (intensity (MET) * duration (h) * frequency (per day)) was used as a quantifiable measure of the leisure-time physical activity volume. This was expressed as the total score of MET hours/ day. Strenuous activity was assessed based on the question: is your physical activity during leisure-time about as strenuous on average as: (1) walking, (2) alternately walking and jogging, (3) jogging, (4) running? Co-twins who answers 2, 3 or 4 were classified as engaging strenuous activity (Leskinen et al. 2009). Twin pairs in which the other twin did strenuous activity equal or higher to a volume of 2 MET hours/ day and his/ her co-twin engaged in activity of less than 2 MET hours/ day in both assessments were selected to the study (Leskinen 2013).

A retrospective assessment of physical activity was carried out in 2005. A telephone interview included the group of 146 twin pairs. Finally, the interview was conducted in 111 twin pairs, as only those pairs were included in which both twins were still alive, both were living in Finland, and both spoke Finnish as their mother tongue. The telephone interview included two sets of questions on current and past leisure-time physical activity. Past physical activity was assessed at five-year intervals from 1980 to 2005. The same physical activity questions were used than in questionnaires in 1975 and 1981 (TABLE 1). The overall leisure time MET index was total

score of leisure-time activity and commuting activity. These were calculated as follows: (((frequency*duration*intensity)/60min)/30 days) and ((frequency as five times per week*duration* intensity of 4 METs)/60min/7 days) (Leskinen 2013).

TABLE 1. A retrospective assessment of leisure-time and commuting activities (modified from Leskinen 2013)

How long does the physical activity last at one session on average?
a) less than 15 minutes (coded as 7min)
b) 15 min to less than 30 min (22min)
c) 30 min to less than 1 hour (45min)
d) 1 hour to less than 2 hours (90min)
e) over two hours (150 min)
Presently how many times per month do you engage in physical activity during your leisure time?
a) less than once a month (coded as 0,5 times per month)
b) 1 to 2 times per month (1,5)
c) 3 to 5 times per month (4)
d) 6 to 10 times per month (8)
e) 11 to 19 times per month (15)
f) more than 20 times per month (25)
Which of the alternatives would describe the best of the intensity of your average leisure time activity?
a) walking (coded as 4 MET/h)
b) alternatively walking and jogging (6 MET/h)
c) jogging (10 MET/h)
d) running (13 MET/h)
How much of your daily journey to work is spent in walking, cycling, running and/ or cross-country skiing?
a) less than 15 min (coded as 7 min)
b) 15 min to less than an half an hour (22 min)
c) half an hour to less than an hour (45 min)
d) hour or more (75 min)
e) I am presently not at work (0 min)

A second set of questions asked in the retrospective follow-up interview was a definite assessment of the volume of leisure-time, daily and commuting activity over the previous 12-months (Leskinen 2013). The questionnaire was a modified and revised version of the Kuopio Ischemic

Heart Disease Risk Factor Study Questionnaire (Lakka & Salonen 1997). A monthly frequency and duration of each physical activity session was reported together with self-rated physical activity intensity. Intensities were transferred into MET and the average duration per session of each activity was reported to calculate overall dose of activity (MET*average duration*frequency. MET h/ day). The past 12-month dose of overall physical activity was further calculated by summing all the different forms of physical activity; daily, leisure-time and commuting activities. A total of 89 twin pairs completed all the physical activity questions in the follow-up interviews. Of these pairs, 42 pairs (5 MZ, 37 DZ pairs) had been consistenly discordant for physical activity. Eventually 14 DZ and 4 MZ pairs were invited to the study measurements. Among the 47 pairs, who were not consistently discordant for thirty years, 3 MZ pairs, who were discordant for physical activity at least in four out of the six time points, and with whom discordance for physical activity was seen over the previous 12 months were selected. After all, 7 MZ and 14 DZ twin pairs discordant for physical activity were invited to the TWINACTIVE study measurements (Leskinen 2013).

The second wave of the study was intended to increase the number of physical activity discordant MZ twin pairs. An extra 151 MZ twin pairs who were discordant for the volume of leisure-time physical activity by 2 MET h/ day or more both in 1975 and 1981 were selected from the original Finnish Twin Cohort. Among the pairs, 19 pairs have activity discordance between co-twins at least 3 MET h/ day in both 1975 and 1981, and the participation in strenuous activity was the same or greater in active co-twin. After exclusions five extra MZ twin pairs from this latter sub-group selection were invited to participate in the TWINACTIVE study (Leskinen 2013). A total 12 MZ and 14 DZ pairs having 30-year-long discordance in physical activity habits were invited to the TWINACTIVE study measurements (Leskinen 2013). After health examination and comprehensive intrapair assessment of the leisure-time MET index from 1975 to 2007 7 MZ (5 male pairs, 2 female pairs) and 9 DZ pairs (6 male and 3 female pairs) fulfilled all discordance criterias. From this group, ten twin pairs (3MZ pairs, 7DZ pairs) took part to muscle biopsy group. Other pairs were not participated due to type 2 diabetes (2 pairs), muscle artrophy (1 pair) and denial (3 pairs) (Leskinen et al. 2009a).

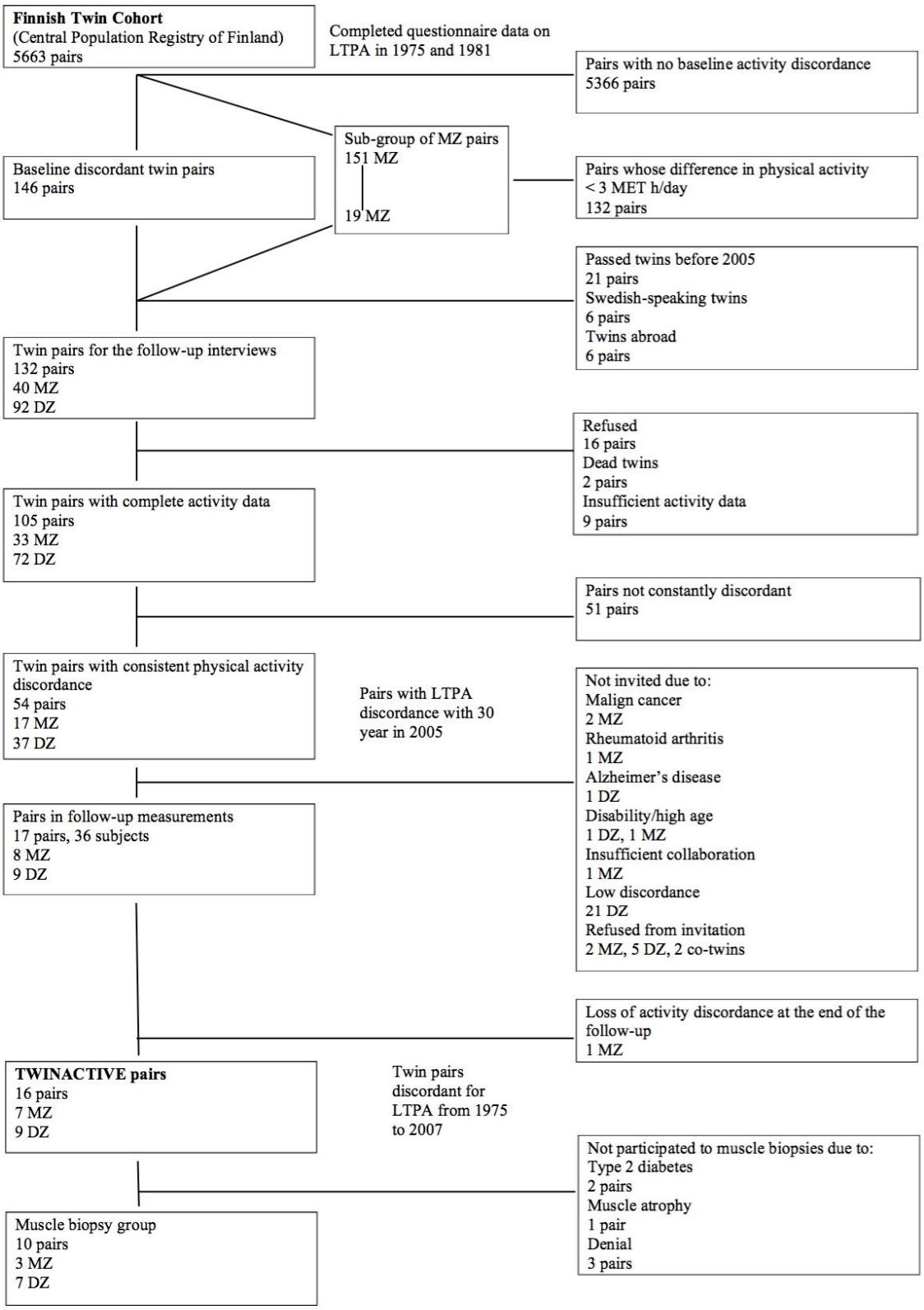

FIGURE 4. Summary of the selection of twin pairs discordant for leisure time physical activity (modified from Leskinen 2013).

4.2 Measurements

A detailed description of the timetable of the study and instructions to laboratory visits are presented in a work by Leskinen (2013).

4.2.1 Assessment of physical activity and fitness

The volume of physical activity (MET index) and participation in strenuous activity were assessed with the identical questions as used at the baseline and in follow-up assessments (TABLE 1). Detailed assessment of the volume of total physical activity including leisure-time, daily and commuting activity over the previous 12-month (12-month MET index) was also carried out. Lifestyle habits such as smoking, alcohol consumption were collected. The assessment of cardiorespiratory fitness using a slightly modified WHO protocol was performed by symptom-limited maximal clinical exercise test with cycle ergometer. The testing protocol was started by learning stage (20W) and warm-up stage (25W) 2-minute per stage. Thereafter the increment of workload was 25W per stage. Recovery stage lasted at least five minutes and was performed 25W. At the end of each stage, electrocardiogram was used to record heart rate, blood pressure was measured and subject was requested to rate their perceived exertion according to Borg's scale 6-20. Measurements were re-collected at the end of 1, 3 and 5 minutes during the recovery stage. Cardiorespiratory fitness was defined by total exercise time, peak load, and estimated oxygen uptake (Leskinen et al. 2009a).

4.2.2 Anthropometrics

Waist circumference (midway between the spina iliaca superior and the lowest rib) and hip circumference (at the level of the greater trochanters) was both measured to the nearest half centimeter. Body composition was measured using an InBody (720) (Biospace, Seoul, Korea) eight-point tactile electrode multifrequency impedance plethysmograph body composition analyzer after an overnigt fast (Leskinen et al. 2009b).

4.2.3 MRI measurements

Magnetic resonance imaging from the thigh was performed with a matrix of 384 x 256, field of view of 40 x 28 cm, and T1-weighted fast spin echo sequence with a repetition time/echo time

of 540/15.18 ms with 90° flip angle (FSE-XL PulsSeg.). A single axial image of the thigh was carried out in a position where the midpoint of the femur was lengthwise, the greater trochanter and head of the tibia was used as anatomical landmarks. After the imaging, OsiriX software (OsiriX Foundation, Geneve, Switzerland) was used to segment the areas of muscle, subcutaneous and intramuscular fat (Leskinen et al. 2009b).

The T1-weighted magnetic resonance scans of the abdomen was carried out using a 1.5-T scanner (GE Signa Excite HD CVI (General Electric Healthcare, Milwaukee, WI, USA)) with a torso phase-array coil using a matrix of 256 x 192, field of view of 40 x 30 cm and gradient echo sequence with a repetition time/echo time of 150/2.16 ms with a flip angle of 90° in the out-phase and 150/4.97 ms with a flip angle 90° in the in-phase images (FSPGR PulsSeg.). A single slice image 5 cm above the L4-L5 intervertebral disc using the open-source image analysis software OsiriX (OsiriX Foundation, Geneve, Switzerland) was used to segment manually abdominal subcutaneous and visceral fat (Leskinen et al. 2009b).

A single slice image as a difference in mean signal intensity between in-phase (water +, fat +) and out-phase (water +, fat -) images were used to calculate the liver fat score. Six regions of interest (ROIs) (1cm^2) was drawn and placed in the liver parenchyma to the following segments using OsiriX software: (1) segment, lobus caudatus; (2) superior subsegment of lateral segment left lobe; (4) left medial segment of the lobe; (8) superior subsegment of anterior segment of right lobe; (7) superior subsegment of posterior segment of right lobe. The spleen was used as a place for reference ROI. In in- and out-phase images were used the same ROI locations. Location was minimally changed only if there were vessels passing through the ROI. The liver fat score was formed by the fat fraction derived from the difference between the average pixel signal intensity of all the ROIs in in- and out-phase images (Leskinen et al. 2009b).

4.2.4 Muscle and fat tissue biopsies

Tissue samples were taken after an overnight fast (between 8 am and 10 am) under local anaesthesia after cooling of the skin and disinfection. The muscle biopsy was taken from the midpart of m. vastus lateralis, the midpoint between the lateral joint line of the knee and the greater trochanter using Bergström's needle (Æ 5mm) biopsy technique with suction, and a needle biopsy (12 G needle, Æ 2mm) of subcutaneous abdominal adipose tissue was taken at the level

of the umbilicus. All the samples were cleaned of any visible muscle and connective tissue. One part of the biopsies was frozen in liquid nitrogen after withdrawing from the needle and stored at -80°C until used for mRNA analysis. The second part of the muscle biopsy used for succinate dehydrogenase analysis was mounted transversely on a cork with Optimal Cutting Temperature compound (Tissue Tekä, Miles, Elkhart, In, USA; Sakura, Cat. # 4583). Then, frozen rapidly during 10-15 seconds in 2-Methylbutane (isopentane) (Fluka, Cat. # 59080) precooled to -160°C in liquid nitrogen and stored at -80°C (Leskinen et al. 2010).

4.2.5 Gene-expression array

Trizol-reagent (Invitrogen, Carlsbad, CA) was utilized to isolate total RNA from muscle biopsy samples of m. vastus lateralis homogenized on FastPrep FP120 apparatus (MP Biomedicals, Illkirch, France). Total RNA was isolated from adipose tissue following needle suspension with Ambion's RNAqueous –Micro Kit (AM 1931, Applied Biosystems) along with the manufacturer's instructions. To inspect the RNA quality and concentration Experion (Bio-Rad Laboratories, Hercules, CA) was used for this purpose and only high- quality RNA was used in the subsequent analysis (260/280 ratio >1.8). According to the manufacturer's intructions, an Illumina RNA amplification kit (Ambion, Austin, TX) was utilized to obtain biotinlabeled cRNA from 500 ng of total RNA, and after amplification Experion was used to carry out the quality control. The Finnish DNA Microarray Center at Turku Center for Biotechnology carried out hybridizations (one array per tissue) to Illumina HumanWG-6 v3.0 Expression BeadChips (Illumina Inc., San Diego, CA, USA) entailing probes for 48803 transcripts according to the Illumina BeadStation 500x manual (Revision C). The twin and his/ her co-twin were hybridized always on the same chip, and a total 6 samples were hybridized on the same chip. Cyanin-3-streptavidin (1mg/ ml, Amersham Biosciences, GE Healthcare, Uppsala, Sweden) using Illumina BeadArray Reader (Illumina Inc.) and BeadStudio v3 sofware (Illumina Inc.) was used to detect hybridized probes (Leskinen et al. 2010).

R software environment for statistical computing (http://www.R-project.org), including Bioconductor development software (http://www.bioconductor.org) was used to run initial data analysis. Affy package of Bioconductor was applied to quantile-normalize the raw data of each chip. To assess the data quality Pearson correlations were calculated and clustering was performed. Normalized data was exported to Excel and SPSS statistical package for the pairwise

analysis. To calculate the fold change (FC) between twin pairs the normalized expression value of each gene of the active twin with the corresponding value of the inactive co-twin was divided. For this data statistical analysis was done using one-sample t-test (FC vs. 1). Lists of genes at different significance levels ($p<0.05$, $p<0.01$ and $p<0.001$) were made in both analy sis. The raw data sets and the gene expression data are available in the GEO database, accession number GSE20536 for adipose tissue and GSE20319 for skeletal muscle data. During array data generation, preprocessing, and analysis, MIAME guidelines were followed (Leskinen et al. 2010).

4.2.6 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (version 2.0; GSEA, http://www.broad.mit.edu/gsea/) was used to cluster differentially expressed genes into functional groups and to determine the significance of their distributions between groups. GSEA is described as, "a computational method that determines whether an a priori defined set of genes shows statiscally significant, concordant differences between two biological states (e.g. phenotypes)". All transcripts on the chip were ranked according to the intrapair expression ratio. These were utilized in the GSEA analysis with 1000 "gene set" permutations. The most notable gene clusters provided from the GSEA analysis were correlated to physiological, anthropometric and biochemical data. For this account, the leading-edge genes (genes producing the GSEA enrichment score) were determined as they contribute to the enrichment score in each pathway. The centroid for each gene expression pathway was calculated by normalizing the sum of expression levels of leading-edge genes to a mean of zero (Leskinen et al. 2010).

4.2.7 Blood studies

Plasma samples were collected after ten-hour fasting by venipuncture after 10 minute of supine rest. Testee was advised not to exercise strenuosly during the 2 days before their visit to laboratory (Leskinen et al. 2012).

4.2.8 Measurement of esRAGE

Serum esRAGE concentrations were measured using the esRAGE Human ELISA Kit (B-Bridge International, Inc). The kit specifically detects the endogenous secreted form of RAGE including esRAGE in human plasma and serum samples. The measurement was carried out

according to the manufacturers' protocol. Primary anti-esRAGE antibody was used to precoat the microplate. For detection, the secondary esRAGE antibody conjugated to HRP was added. After incubation, hydrogen peroxide and 3,3",5,5"-tetramethylbenzidine (TMB) were added as a colorimetric substrate for color development. Color was measured at 450 nm. The detection range of measurement is between 0.05- 3.2 ng/ml and limit of detection 0.025 ng/ml (B-Bridge International, Inc 2016).

4.2.9 Zygosity

The Paternity Testing Laboratory (National Public Health Institute, Helsinki, Finland) verified the zygosity of the co-twins. DNA was extracted from a venous blood sample with a battery of ten highly polymorphic gene markers (Leskinen 2013).

4.2.10 Ethics

The TWINACTIVE study was conducted according to the guidelines for good clinical and scientific practice founded by the Declaration of Helsinki. The study plan was approved by the ethics committee of the Central Finland Health Care District, on August 15, 2006. Informed consent was written by participants before the study measurements (Leskinen 2013).

4.3. Statistical methods

Data were analyzed using IBM SPSS Statistics 22. Pairwise analysis was used to study the intrapair differences between physically inactive vs. physically active co-twins. Shapiro-Wilk test was used to assess the normality of mean variables. The normally distributed variables was studied by student's paired t-test and Wilcoxon matched-pair signed-rank test was used for non-normally distributed variables. Confidence intervals of ninety-five percent (95% CI) were calculated for the absolute mean differences between the physically inactive vs. physically active co-twins. The partial correlation coefficient was utilized for the correlation of serum esRAG level with gene centroids, adjusted by sex and age.

5 RESULTS

5.1 Characterics of physically active discordance twin pairs

Following comprehensive selection of twin pairs from the original Finnish Twin Cohort, 16 twin pairs with 32-year discordance in physical activity habits were selected for TWINACTIVE study. Ten twin pairs (3MZ pairs, 7DZ pairs) were further selected for this study as these pairs took part to muscle biopsy group. Eventually, study group included 6 male twin pairs and 4 female twin pairs with mean age 60 years (ranging 50 to 76).

Characteristics of physically active vs. inactive co-twin pairs are presented below (TABLE 2). Pairwise analysis was used for study intrapair differences and analyze statistical differences between co-twins. No statistically significant differences were observed between co-twins in most anthropometric characteristics, including weight ($p=0.14$), height ($p=0.28$), BMI ($p=0.20$) and waist circumference ($p=0.11$) excluding whole body fat percent (measured by InBody (720) body composition analyzer) ($p<0.05$) which differed significantly between co-twins.

Moreover, significant differences between active vs. inactive twin pairs were found in several metabolic health related characteristics. Visceral fat ($p<0.05$) and mid-thigh intramuscular fat ($p<0.05$) areas were lesser in physically active co-twins. Also liver fat score ($p=0.07$) differed between co-twins, but the difference was not statistically significant. Estimated VO_{2peak} mean value, based on symptom-limited maximal exercise test, was nearly 4.7 ml/kg/min higher ($p<0.05$) in physically active vs. inactive co-twins. Skeletal muscle mitochondrial DNA copy number differed, but not statistically ($p=0.10$) between co-twins. Serum esRAGE levels ($p=0.37$) did not differed between co-twins.

TABLE 2. Characteristics of co-twin pairs (n=20) with 32-year discordance in physical activity habits.

Characteristics	Inactive n=10	Active n=10	Mean difference (95% CI)	p-value
Sex (n, male: female)	6:4			
Zygosity (pairs)	3MZ : 7DZ			
Age (years, range)	60 (50-67)			
Body weight (kg)	78.4±23.0	69.1±11.7	9.3 (-4.9 to 23.6)	0.14
Body height (cm)	170.7±9.8	168.6±8.9	2.1 (-2.0 to 6.3)	0.28
BMI (kg/m^2)	26.5±4.3	24.2±2.8	2.3 (-1.4 to 6.0)	0.20
Waist circumference (cm)	95.3±15.7	87.8±9.8	7.6 (-3.2 to 18.3)	0.11
Body fat percent (%)[a]	25.5±5.6	19.9±5.9	5.6 (1.2 to 10.0)	0.02*
Visceral fat area (cm^2)[b]	158.4±38.8	90.4±22.1	33.5 (-3.4 to 70.4)	0.04*
Liver fat score	22.1±26.8	8.7±8.6	13.3 (-1.1 to 27.8)	0.07
Thigh IMAT area (cm^2)[c]	11.3±5.4	7.5±4.2	3.9 (-0.7 to 8.6)	0.04*
esRAGE (ng/ml)	0.25±0.16	0.22±0.12	0.03 (-0.05 to 0.11)	0.37
Skeletal muscle mtDNA	312±105	392±153	-79.6 (-178 to 19)	0.10
Estimated VO_{2peak} (ml/kg/min)[d]	28.3±3.6	33.0±5.0	-4.7 (-8.6 to 0.81)	0.02*
MET follow-up 1980-2007	2.0±1.9	11.4±3.0	-9.4 (-11.2 to -7.6)	0.01*

*$p<0.05$ statistically significant difference between groups. BMI, body mass index; IMAT, intramuscular (extra myocellular) fat; HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a]measured by InBody (720) body composition analyzer (Biospace Co. Seoul, Korea), [b]measured by MRI, [c]measured by MRI, [d]calculated from symptom-limited maximal exercise test.

5.2 Correlation of esRAGE with metabolic health associated biomarkers of muscle

In our study serum esRAGE level was negatively correlated with energy metabolism related gene centroids (TABLE 3). Centroids for fatty acid metabolism ($p<0.05$), ubiquinone synthesis ($p<0.01$), oxidative phosphorylation ($p<0.05$), valine, leusine and isoleucine degradiation ($p<0.05$) and butanoate metabolism ($p<0.01$) were significantly associated with serum esRAGE level. As centroid is defined as a mean expression of the coregulated genes within a subset and based on earlier studies which have shown correlation of centroids with distinctive metabolic and physiological parameters, centroids may be used to study for gene expression patterns that are related to metabolic disease states (Kivelä et al. 2010).

Serum esRAGE level was negatively correlated with IL-15, IL-6, HbA1C and skeletal muscle mtDNA number, of which IL-15 ($p=0.01$) and mtDNA number ($p<0.01$) were correlated significantly. Serum esRAGE level was positively correlated with resistin ($p<0.05$) and adiponectin ($p<0.01$).

TABLE 3. Partial correlations of serum esRAGE level with energy metabolism related gene centroids and metabolic health related biomarkers (n=20).

Biomarker	r	p-value
	esRAGE ng/ml	
Fatty acid metabolism	-0.49	0.038*
Ubiquinone biosynthesis	-0.59	0.009*
Oxidative phosphorylation	-0.59	0.010*
Valine, leusin and isoleucine degradiation	-0.59	0.010*
Butanoate metabolism	-0.61	0.007*
Propanoate metabolism	-0.60	0.008*
IL-15 (pg/ml)	-0.59	0.010*
IL-6 (pg/ml)	-0.39	0.107
Resistin (ng/ml)	0.54	0.022*
Adiponectin	0.63	0.005*
HbA1C	-0.47	0.051
Skeletal muscle mtDNA copy number	-0.62	0.007

*$p<0.05$ significant correlation between variables. Partial correlations are adjusted by age and sex.

6 DISCUSSIONS

Main finding of our study identified the correlation of serum esRAGE concentration with number of biological markers related to energy metabolism of skeletal muscle. The results demonstrated inverse association between energy metabolism related gene centroids, number of mitochondrial DNA copies and IL-15 with serum esRAGE concentration, whereas adiponectin and resistin showed positive correlation. The major differences observed between physically active and inactive co-twins were body fat percent, intramuscular fat of thigh, estimated VO_{2peak} value and the amount of visceral fat. There is no earlier research that has studied the association of esRAGE to these biomarkers. Thus, our findings provide a novel insight how serum esRAGE concentration is related to biomarkers of skeletal muscle. This gives further evidence to consider the significance of esRAGE as a potential biomarker for metabolic disease states.

6.1 Physical activity and AGE-RAGE-sRAGE axis

Physical activity has shown to reduce AGEs, but maybe not as much as AGE restricted diet (Macías-Cervantes et al. 2015). Mechanistically, the reduction of circulating AGEs can inactivate the expression of RAGE and lead to the reduction of sRAGE (Yamagishi et al. 2006). Kotani et al. (2011) suggest this may be an adaptive response to increased physical activity in healthy subjects. The improvement of the sedentary status can attenuate inflammatory state leading to the reduction of sRAGE. There may also be an increase of the clearance of sRAGE from the circulationz as a result of hemodynamic changes induced by the increase in physical activity (Kotani et al. 2011). Few studies have straightforwardly investigated the effects of physical activity to AGE-RAGE-sRAGE system. In a study by Boor et al. (2009) a 10-week moderate exercise program decreased advanced glycation and showed renoprotective effects in a rat model of type 2 diabetes. Interestingly, there were no differences in inflammatory markers, oxidative stress or insulin sensitivity between sedentary and physically active obese Zucker rats. The profile of urinary metabolites indicated higher energy metabolism in exercising rats (Boor et al. 2009). Another study showed that exercise training could inhibit the activation of RAGE in aged aortas of rats which may indicate exercise-induced inactivation of AGE-RAGE axis, and that may contribute to the effects of exercise training on oxidative stress and inflammation (Gu et al. 2014). In human studies, aerobic exercise training has shown to increase esRAGE levels by 61% during 8-week training period in 22 sedentary subjects along the improvement with platelet activation (Santilli et al. 2013). Conversely, a 6-month physical activity

program reduced serum sRAGE level significantly in elderly. Concurrently, no apparent changes were identified in general cardiometabolic risk factors (Kotani et al. 2011). A study by Choi et al. (2012) demonstrated significant increase in serum levels of sRAGE along the improvement in cardiorespiratory fitness and other cardiometabolic risk factors during 12-week aerobic training program in type 2 diabetic patients. Master athletes with life-long endurance training has shown to have lower AGE cross-link density and 11% lower skin AGE levels than in control subjects (Couppé et al. 2014). In osteoarthritic patients 12-week resistance training increased RAGE expression in skeletal muscle (Mattiello-Sverzut et al. 2013).

These findings have demonstrated that exercise may have a potential ability to increase serum sRAGE and/or esRAGE level and the increase may be accompanied by improvement in other cardiometabolic markers. However, it seems that this is not always the case which may be explained at least partly by the intensity and duration of exercise program as suggested by Kotani et al. (2011). Finally, according to the current evidence we can not draw definite conclusion, does regular physical activity increase serum esRAGE and/or sRAGE level in subjects with chronic disease or in subjects with cardiometabolic risk factors. The possible age-specific differences should be considered. The question, is potential increase in serum esRAGE levels associated with better overall health outcomes, is also debatable. Complex nature of the regulation of AGE-sRAGE-RAGE axis may help to explain the results of our study as we did not identify any difference in serum esRAGE level in physical activity discordant twin pairs. Other lifestyle factors should also take account. Food intake and its AGE-content and smoking can increase circulating AGE level, and therefore may have an effect on our results. However, recent findings suggest that AGE-content of food and cigarette smoking may not have effect on serum esRAGE level (Prasad et al. 2015; Di Pino et al. 2016, in press). It is also worth to consider that genetic factors may potentially affect to persistently elevated sRAGE level which may have implications to longevity, as it has seen when compared healthy centenarians to healthy young subjects (Reynaert et al. 2016, in press).

6.2 esRAGE - a biomarker for health of skeletal muscle

It is proposed that circulating esRAGE works in concert with a number of proinflammatory abnormalities that may suggest that the activation of the AGE-RAGE pathway may contribute to metabolic disease and the disruption of cardiovascular health (Santilli et al. 2015). This statement is also questioned based on conflicting results of studies regarding the association of

sRAGE/esRAGE with chronic disease states and the observation that plasma sRAGE concentrations are approximately 1000 times lower than that of AGEs (Reynaert et al. 2016, in press).

RAGE is upregulated in acute and chronic inflammatory diseases. Enhanced expression along with its ability to bind AGEs leads to aggravagation of downstream inflammatory responses and the activation of different signaling pathways including nuclear factor kappa (NF-κB), which results in the production of inflammatory mediators. AGEs can cause a prolonged and sustained NF-κB activation that overcomes the endogenous auto-regulatory feedback loops by synthesis of the p65 subunit of NF-κB (Reynaert et al. 2016, in press). Chronic activation of NF-κB is associated pathological states, such as insulin resistance and muscle wasting. Therefore, excess AGE-RAGE signaling may induce pro-inflammatory state in skeletal muscle. Chronic NF-κB activation also alters the production of genes, including those encoding proinflammatory cytokines (Kramer & Goodyear 2007). AGER gene that regulates RAGE expression is also NF-κB regulated gene. In addition, RAGE activation initiates other signaling pathways including the mitogen activated protein kinase members (MAPKs) that are interconnected to regulate cellular responses to various stimuli and stress conditions. Excess AGE accumulation also impair DNA repair mechanisms and reduce antioxidant defense mechanisms by increasing production of reactive oxygen species (ROS) via enhanced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity (Reynaert et al. 2016, in press). It was recently shown that the accumulation of AGEs was associated with muscle atrophy and muscle dysfunction through a putative mechanism of the RAGE-mediated AMPK-down-regulated Akt signaling pathway in the diabetic skeletal muscles (Chiu et al. 2016). These findings help to explain how excess AGE accumulation may drive chronic proinflammatory state. We can propose that inhibition of AGE-RAGE signaling pathway may deteriorate its harmful effects to health and this can be obtained by a regulatory role of sRAGE, esRAGE as its main variant. Thus, it is essential to determine how esRAGE is associated with other markers related skeletal muscle health and further determine the potential implications of that.

In our study we found that some myokines and adipokines were significantly associated with esRAGE. For instance, higher IL-15 level was associated with lower esRAGE level. As expressed in human skeletal muscle, IL-15 is an important regulator of oxidative metabolism and muscle metabolic phenotype, but it also plays a part in lipid metabolism and is involved in muscle-adipose tissue crosstalk (Pedersen & Febbraio 2012; Quinn et al. 2013). IL-15 protein content in human skeletal muscle is increased by endurance training as it relates to adaptive

responses following endurance training (Rinnov et al. 2014; Ye 2015). We also found that adiponectin, classified as adipokine, was positively correlated to esRAGE. Adiponectin, secreted and synthesized mainly by adipose tissue, has a beneficial effect on metabolism via improvement in insulin sensitivity, glucose tolerance and lipid profile. Exercise with weight loss has shown to increase adiponectin levels in different populations. Exercise is also related to adiponectin receptor expression changes which activates the AMPK and PPAR-a pathways. These changes may lead to improvement of insulin sensitivity in skeletal muscle. Adiponectin has also shown to stimulate fatty acid oxidation in skeletal muscle (Passos & Gonçalves 2014). As adiponectin, resistin showed positive correlation for esRAGE. Resistin is also called as adipokine, when it was first identified to be released from adipocytes impairing glucose metabolism in skeletal muscle of mice (Pravenec et al. 2003). Resistin is mainly produced by macrophages and monocytes in humans. Other pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α induce transcription of the resistin gene (Ouchi et al. 2011). Our results help to define the metabolic profile of skeletal muscle in relation to serum esRAGE concentration which can have useful impact for future studies.

Our main finding demonstrated inverse association between energy metabolism related gene centroids and number of mitochondrial DNA copies with serum esRAGE concentration. Mitochondrial DNA includes a limited number of genes, which consist of those encoding 13 polypeptides that are needed for energy production via oxidative phosphorylation, and 22 transfer ribonucleic acid (tRNA) and 2 ribosomal ribonucleic acid (rRNA) genes. Although the mtDNA can replicate independently of nuclear DNA (nDNA), the nDNA controls the expression of all other myofibrillar and mitochondrial proteins in muscle cells. The cooperation of both the nuclear and mitochondrial genomes are needed to mitochondrial synthesis. The concentrations of mtDNA, mitochondrial messenger RNA (mtmRNA) and mitochondrial transfer RNA (mtrRNA) all vary in direct proportion to changes in oxidative capacity. Mitochondrial density is also proportional to the oxidative capacity. These characteristics indicate that the expression of mitochondrial genes in skeletal muscle is proportional to their copy number. Both myonuclear and mitochondrial density are important for maintaining synthesis of proteins associated with oxidative metabolism. High oxidative skeletal muscle fibers have higher capacity for mitochondrial protein synthesis than low oxidative fibers as they express higher densities of myonuclei and mitochondria, and because mitochondrial biogenesis requires both mitochondrial and nuclear DNA (van Wessel et al. 2010). We found association between lower serum

esRAGE concentration with higher number of mtDNA copies and higher expression of oxidative energy metabolism related gene centroids; fatty acid metabolism, ubiquinone biosynthesis, oxidative phosphorylation, valine, leusine and isoleucine degradiation, butanoate metabolism and propanoate metabolism. These gene centroids are previously identified to be up-regulated in skeletal muscle among active compared to inactive co-twins (Leskinen et al. 2010). There was also difference in number of mtDNA copies between co-twins (p=0.10). This non-significant difference may be explained due to small sample size. Our results suggest that lower serum esRAGE concentration is potential marker to describe higher oxidative energy metabolism status of a subject, when adjusted by sex and age which may implicate better metabolic health.

Physical inactivity induces collective down-regulation of genes associated with muscle oxidative capacity. Low mitochondrial oxidative capacity is known to be related detrimental health effects as we have earlier demonstrated. Based on these findings, impaired regulation of oxidative pathways in mitochondria may link reduced total-body aerobic capacity to cardiometabolic disease states (Wisloff et al. 2005; Gram et al. 2014). This supports the need to study more the role of esRAGE as a biomarker of metabolic health.

6.3 Strengths and limitations

Strength of our study was that we used a co-twin control design using twin pairs with 32-year discordance for physical activity habits. This is a unique study design when both genetic and familial factors are standardized with the complete or close match for genes, age, gender, and birth and living environment in childhood (Kaprio & Koskenvuo 2002). Our study has some limitations. Number of twin pairs was rather low due to difficulty to identify substantial amount of twin pairs with long-term discordance in physical activity. Thus, a small sample size limits to generalise these results to a general population. Moreover, measurement of total sRAGE would have helped to differentiate the role of esRAGE from total sRAGE and give more specific insight to their contributing role regarding the findings of the study. Dietary and other lifestyle factors may also influence to the results of our study. AGE intake from food, smoking and alcohol consumption are known to have a possible influence on AGE-sRAGE-RAGE-axis (Uribarri et al. 2005; Yamagishi et al. 2006; Prasad et al. 2015). The significance, role and function of esRAGE in a systemic level is somehow unclear, so we need to be cautious how we interpret our results locally in skeletal muscle. There are also considerations regarding the measurement of esRAGE and storage of samples that may have influence to measured serum

esRAGE concentrations in our study.

7 CONCLUSIONS

In conclusion, our findings provide a novel insight for the role esRAGE as a biomarker for the metabolic health of skeletal muscle. We found that serum esRAGE concentration was correlated for biomarkers related to energy metabolism pathways. In addition to our findings, there is a need to understand the role of esRAGE and its function in a systemic level more closely, so we need to be careful how we interpret our results.

Our findings provide the foundation for future studies. Firstly, larger study sample is needed to confirm our results. Secondly, it is important to take account the total sRAGE concentration in future studies. Only then, we can even present a more profound question, what is the value of esRAGE to be independent biomarker for health of skeletal muscle. Thirdly, the mechanism of how serum esRAGE concentration affects the metabolism of skeletal muscle should be studied more closely. Finally, it would be interesting to study of which magnitude physical activity has an effect on serum esRAGE concentrations in different populations. After further research, we can make more precise decisions about the role of esRAGE as a potential biomarker for metabolic health.

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