Novel biomolecules of ageing, sex differences and potential underlying mechanisms of telomere shortening in coronary artery disease

Trine B. Opstad^{1,2,3}, Are A. Kalstad^{1,2,3}, Alf Åge Pettersen^{1,2,4}, Harald Arnesen^{1,2,3},
Ingebjørg Seljeflot^{1,2,3}

¹ Center for Clinical Heart Research, Department of Cardiology, Oslo University Hospital,
Ullevål, Norway

² Center for Heart Failure Research, Oslo University Hospital, Norway

³ Faculty of Medicine, University of Oslo, Norway

⁴ Ringerike Hospital, Vestre Viken HF, Norway

Corresponding author:

Trine Baur Opstad

Center for Clinical Heart Research

Department of Cardiology

Oslo University Hospital, Ullevål

Pb 4950 Nydalen

N-0240 Oslo

Norway

E-mail: trineoa@medisin.uio.no

Abbreviations

BMI: body mass index

CAD: coronary artery disease

CVD: cardiovascular disease

DNA: deoxy ribonucleic acid

GDF11: growth differentiate factor 11

HT: hypertension

IFN γ : interferon gamma

IGF1: insulin growth factor 1

IL: interleukin

LTL: leukocyte telomere length

MI: myocardial infarction

mTor: mechanistic target of rapamycin

NAD $^{+}$: nicotinamide adenine dinucleotide

PCR : polymerase chain recation

RNA : ribonucleic acid

ROS: reactive oxygen species

RQ : relative quantification

SIRT1: sirtuin1

TNF α : tumor necrosis factor alpha

Abstract

Telomere length (TL), growth differentiate factor (GDF)11, insulin growth factor (IGF)1, sirtuin (SIRT)1 and inflammatory processes have been related to ageing and age-related diseases, like coronary artery disease (CAD). We aimed to investigate the associations between leukocyte TLs (LTLs), chronological age, sex and comorbidities in CAD patients. Any covariations between LTL, GDF11, IGF1, SIRT-1 and pro-inflammatory cytokines were further assessed.

Methods

In 300 patients with stable CAD (age 36-81 years, 20 % females), DNA and RNA were isolated from whole blood for PCR analysis and relative quantification of LTLs and gene-expression of GDF11, IGF1, SIRT1, IL-12, IL-18 and IFN γ , respectively. Serum was prepared for the analyses of circulating IL-18, IL-12, IL-6 and TNF α .

Results

Patients with previous myocardial infarction (MI) presented with 20 % shorter LTLs vs. patients without ($p = 0.019$) indicating LTLs to be of importance for CAD severity. The observation however, was only observed in men ($p = 0.009$, $n = 115$), in which the upper LTL quartile associated with 64 % lower frequency of previous MI compared to quartile 1-3 ($p = 0.005$, adjusted). LTLs were not differently distributed according to sex or comorbidities such as hypertension, diabetes type 2 and metabolic syndrome. LTLs and GDF11 were inversely correlated to age ($r = -0.17$; $p = 0.007$ and $r = -0.16$; $p = 0.010$, respectively), however, separated in gender, LTL only in women ($r = -0.37$) and GDF11 only in men ($r = -0.19$) ($p = 0.006$, both). GDF11 and SIRT1 were strongly inter-correlated ($r = 0.56$, $p \leq 0.001$), suggesting common upstream regulators. LTLs were moderately correlated to GDF11 and SIRT1 in overweight women ($BMI \geq 25\text{kg/m}^2$) ($r = 0.41$; $p = 0.027$ and 0.43 ; $p = 0.020$, respectively), which may reflect common life-style influences on LTLs and these markers.

In all women, we observed further that the highest LTL quartile associated with higher GDF11 and SIRT expression and lower circulating levels of IL-12, IL-18 and TNF α , as compared to quartile 1, which may indicate lifestyle influences on female LTLs. In men, the highest LTL quartile associated with lower IFN γ expression and lower circulating TNF α . Overall, the results indicate an association between chronic low-grade inflammation and LTLs.

Conclusions

Shorter LTLs in CAD patients with previously suffered MI may indicate telomere attrition as part of its pathophysiology in men. The inverse association between LTLs and age exclusively in women underpins the previously reported decline in attrition rate in men with increasing age. As elevated GDF11 and SIRT1 along with attenuated pro-inflammatory cytokines seem to positively affect LTL in women, we hypothesize a potential sex-dimorphism in LTL regulation, which may implicate sex- adjusted health-preventive therapies.

Keywords: Telomere lengths; GDF11; IGF1; SIRT1; inflammatory cytokines; coronary artery disease

1.1. Introduction

Ageing is now widely hypothesized as a consequence of telomere shortening [1]. Telomeres consist of repetitive nucleotide structures (TTAGG) at the end of chromosomes that protect DNA from deterioration [2]. Telomere lengths decline with increasing chronologic age due to loss of the repetitive DNA sequences during cell-division. Consequently, and when the shortened telomeres are reduced below a critical length, cells are triggered into replicative senescence [3]. Little knowledge also exists on sex differences in telomere length regulation, other than reported dissimilar telomere shortening curves throughout life [4, 5]. Inflammation, oxidative stress, life-style and environmental factors are all thought to influence the rate of the senescence process and shortened telomeres have been associated with the onset of age-related diseases like cardiovascular disease (CVD) states [6-8]. However, the molecular mechanisms behind these effects are uncertain.

In recent research, several potential regulators of life-span and telomere lengths have been suggested. Growth differentiation factor 11 (GDF11), a member of the TGF β superfamily with homology to myostatin, retards the aging process via yet unknown mechanisms. In parabiosis experiments, it was demonstrated that older mice sharing blood supply with younger developed a younger phenotype with improved olfaction, regression of myocardial injury and better memory, and it was suggested that GDF11 was the rejuvenating factor causal for these alterations [9, 10]. However, due to questions concerning methodology [11] and reported increased circulating levels of GDF11 with increasing age both in mice and humans [12], its influence on lifespan is unclear.

Ageing research has lately also paid attention to the intracellular mechanistic target of rapamycin (mTor) signaling pathway, mediated by the growth hormone and insulin growth

factor1 (IGF1) [13], sensitive to insulin and caloric restriction. IGF1 is commonly shown to be higher in subjects affected by age-related diseases or obesity than in healthy and lean subjects, however, low circulating IGF1 levels have also been associated with increased all-cause mortality in several population studies [14].

Sirtuins (SIRT 1-7), localized in different cell compartments, are another group of mediators that are associated with promoted longevity. By deacetylating histones or non-histone substrates in a nicotinamide adenine dinucleotide (NAD⁺) dependent manner, sirtuins and especially SIRT1, located in the nucleus, can regulate gene-expression. Because NAD⁺ levels are sensitive to diet and exercise, sirtuins are involved in metabolism and sense life-style changes, thus impacting health status [15]. Being capable to translocate into cytoplasm, SIRT1 also seems to affect the production of reactive oxygen species (ROS) and to influence apoptosis. However, the role of SIRT1 in human aging is still unclear [16].

Chronic low-grade inflammation with elevation of pro-inflammatory cytokines increases with age and has also been associated with reduced length of telomeres [6], although the link between inflammation and telomere lengths is not well understood.

Due to accessibility, leukocyte telomere length (LTL) is now widely used as a measure of telomere lengths, which seems highly correlated to telomere lengths in other cells and tissue, although debated [17-20].

Ageing is one of the strongest non-modifiable disease-risk factors, especially for CVD [21], thus more research is needed to explore molecular mechanism associated with ageing in these patients. We therefore aimed to investigate associations between LTLs and 1) chronological age, sex and comorbidities in patients with coronary artery disease (CAD) 2) leukocyte gene expression of the potential telomere length regulators GDF11, IGF1 and SIRT1

and 3) levels of selected pro-inflammatory cytokines, partly as genetically expressed and circulating proteins.

1.2. Material and Methods

1.2.1. Study Population

The present investigation is a sub-study of the Norwegian ASCET trial (ASpirin non-responsiveness and Clopidogrel Endpoint Trial), which included angiographically verified stable CAD patients [22]. This cross-sectional analysis was performed on baseline data from the 300 first included patients in the main trial (age range 39-81 years, 20 % women, 97 % of Western European descent). Relatedness in the population was < 1%. The ASCET study was approved by The Regional Committee of Medical Research Ethics in South-Eastern Norway, and all subjects gave their written informed consent to participate. The ASCET study was performed according to the Declaration of Helsinki and is registered at clinicaltrials.gov; identification number NCT00222261.

The following comorbidities were recorded: Previous myocardial infarction (MI); Current smoking, defined as regular tobacco use or cessation ≤ 3 months prior to inclusion; Diabetes Type 2, defined as fasting glucose ≥ 7 mmol/L or previously diagnosed; Hypertension (HT), defined as treated HT or known diagnosis; overweight, defined as body mass index (BMI) > 25 kg/m², and Metabolic syndrome (MetS), as fulfilling $\geq 3/5$ criteria from a modified National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III definition [23].

1.2.2. Blood sampling and preparation

In fasting conditions and prior to morning medication (8.00-10:30 a.m.), blood samples were collected by venipuncture at entrance into the study. Routine analyses were performed by conventional laboratory methods. Serum was prepared by centrifugation within 1 hour at 2.500xg in 10 min for the analysis of circulating cytokines. EDTA blood and PAXGene Blood RNA tubes (Pre-Analytix, Qiagen GmbH, Germany) were collected for DNA extraction and RNA isolation, respectively. Circulating leukocytes in peripheral blood were hence the source for DNA and RNA. All materials were kept frozen at -80 °C until further preparation and analysis.

DNA was automatically isolated with the MagNA Pure LC DNA Isolation kit on the MagnaPure Instrument LC (Roche Diagnostics, GmbH, Mannheim, Germany). Total RNA was extracted using PAXGene Blood RNA kit (Pre-Analytix), with an extra cleaning step (RNeasy MinElute Cleanup kit, Qiagen). DNA and RNA purity and quantity was tested on the NanoDrop, ND-1000 (Saveen Werner, Sweden).

1.2.3. Leukocyte Telomere Length (LTL) determination

Equal amount of DNA per experiment was used to measure relative LTL by quantitative real-time PCR [24]. PCR amplification was performed on the VIIa7 instrument (Applied Biosystems by Life Technologies, Foster City, CA, USA), using telomere-specific primers (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) (Supplementary Table 1) and GoTaq®qPCR Master Mix (Promega, Madison, WI, USA). LTL was relatively quantified (RQ) to the single-copy-gene (SCG) SB34 (Invitrogen by Thermo Fisher Scientific) (Supplementary Table 1). PCR conditions for both targets were as follows; an initial step at 95°C for 10 min

followed by 40 cycles of 95°C for 15s and 60°C for 1 min. All samples were run in triplicates and LTLs were successfully analyzed in 264 samples.

1.2.4. Gene-expression analysis

Equal amount of total RNA per experiment (100 ng) was reversely transcribed into complementary DNA (cDNA) by use of qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, USA). Leukocyte expression of GDF11 (Hs00195156_m1), IGF1 (Hs01547656_m1) SIRT1 (Hs01009006-m1) and the selected pro-inflammatory genes interleukin (IL) -18 (Hs00155517_m1), IL-12p40 (Hs01011218_m1), and interferon (IFN) γ (Hs00989291_m1) were normalized to β -2-microglobulin (Hs99999907_m1), previously tested as a valid house-keeping gene in this population [25]. The gene expression analyses were measured on the VIIa7 instrument using TaqMan Universal PCR Master Mix , No AmpErase UNG, and the TaqMan assays as noted above (Applied Biosystems), as relative quantification (RQ) ($2^{-\Delta\Delta C_t}$ method) [26]. Number of successfully analyzed samples was for GDF11 (n = 274), IGF1 (n = 206), SIRT1 (n = 273), IL-12 (n = 235), IL-18 (n = 240) and for IFN γ (n = 234). Discrepancies in number of available results relate to limited sample material.

1.2.5. Determination of circulating cytokines

The following ELISA kits were used to measure serum levels of the selected pro-inflammatory cytokines: IL -18 (Medical Biological Laboratories, Naka-ku Nagoya, Japan), IL-12 (IL-12p40/p70, Invitrogen Corp. CA 93012, USA), IL-6 and tumor necrosis factor (TNF) α (R&D Systems Europe, Abingdon, Oxon UK, both). The following inter-assay coefficients of variation for the assays were 8.1%, 4.1 %, 6.0 % and 8.5 %, respectively.

1.2.6 Statistics

Data are presented as mean ($\pm$ SD), median (25th, 75th percentile), or proportions as appropriate. Student *t*-test and Mann Whitney test were used to compare continuous data with normal or skewed distributions, respectively. Kruskal Wallis test was used to compare skewed data across categorized groups. Proportional data were analyzed using the Chi square test. Correlation analyses were performed with Spearman's rho. Related multiple comparisons were adjusted for by Bonferroni correction, as appropriate. Multivariate logistic regression was performed to explore association between the frequency of previous MI in men and quartiles of LTLs, adjusted for age and other variables differently distributed according to presence of previous MI or not (BMI, triglycerides, glucose and HT). Linear regression was performed to explore associations between quartiles of LTLs and the log-transformed continuous variables GDF11, SIRT1, IGF1 and the selected pro-inflammatory cytokines, adjusted for age, sex and previous MI, as appropriate. P-values ≤ 0.05 were considered statistically significant throughout. Statistical analyses were performed using IBM®SPSS® Statistics software, version 24.

1.3. Results

Demographic data for the cohort in total and as related to sex are presented in Table 1, showing normal distribution on most of the variables and statistically significantly higher age, total-, low density lipoprotein (LDL)- and high density lipoprotein (HDL)- cholesterol in women compared to men, and numerically higher number of previous MI in men versus women.

Table 1 Demographic and laboratory data in the total cohort and in men and women separately

Variable	Total	Men (n = 240)	Women (n = 60)	<i>p</i>
Age (range)	62 (38-81)	61 (39-81)	64 (40-80)	0.034
Men/Women n (%)	240/60 (80/20)	-	-	-
Previous MI n (%)	159 (53)	133 (55.4)	26 (43.3)	0.093
Current smoker n (%)	66 (22.1)	52 (21.7)	14 (23.7)	>0.732
Diabetes Type 2 n (%)	74 (24.7)	57 (23.8)	17 (28.3)	>0.461
MetS n (%)	104 (34.7)	83 (34.6)	21 (35.0)	>0.952
HT n (%)	156 (52)	121 (50.4)	35 (58.3)	>0.272
BMI (kg/m ²)	27.5 (24.8, 30.3)	27.5 (25.1, 30.2)	25.5 (24.1, 30.7)	> 0.850
Total Cholesterol mmol/L	4.7 (0.9)	4.6 (1.0)	5.0 (0.8)	<0.001
LDL Cholesterol mmol/L	2.6 (0.7)	2.6 (0.8)	2.8 (0.7)	0.052
HDL Cholesterol mmol/L	1.3 (0.4)	1.2 (0.3)	1.5 (0.5)	<0.001

Triglycerides mmol/L	1.44 (0.96, 1.90)	1.43 (0.98, 1.9)	1.45 (0.90, 1.91)	> 0.515
Fasting Glucose mmol/L	6.3 (2.24)	6.3 (2.2)	6.4 (2.2)	> 0.875
HbA _{1c} %	6.2 (1.0)	6.2 (1.1)	6.2 (0.8)	> 0.2

Values are number (percentages), mean (SD) or median (25, 75 percentiles) if not otherwise stated.

MI; myocardial infarction, MetS; metabolic syndrome, HT; hypertension, BMI; body mass index, LDL; low density lipoprotein, HDL; high density lipoprotein

After Bonferroni correction, p-values in bold remained significant (p=0.004 by 13 performed associations)

1.3.1. LTLs in relation to chronological age and CAD subgroups

LTLs were investigated as related to age as continuous variable and categorized into age quartiles. LTLs were weakly and inversely correlated to age ($r = -0.17$, $p = 0.007$) and were differently distributed through age quartiles (Supplementary Table 2) ($p = 0.003$). When analyzed separately by gender, LTLs showed stronger correlation to age in women (Figure 1), whereas no significant correlation to age was observed in men. When dichotomizing age at the 75 percentile, 14 % shorter LTLs (RQ values) were recorded in subjects above 69 years ($n = 70$) compared to age below (0.57 vs. 0.65, $p = 0.043$). This association was especially predominant in older women ($n = 23$) holding 30 % shorter LTLs compared to the younger (0.57 vs 0.80, $p = 0.013$). After Bonferroni correction, ($p = 0.010$, by 5 performed associations), the associations between LTLs and age (as continuous variables as well as quartiles and sex categorized variable) remained statistically significant, when analyzed in the total population.

Figure 1 Correlation between TL and age analyzed as related to gender

Spearman Rho: $r = -0.125$ $p = 0.07$ in men, $r = -0.368$ $p = 0.006$ in women

Spearman Rho correlation is performed. Red stars and line indicate the female gender, black open dots and line indicate the male gender

Patients with previous MI presented with 20 % shorter LTLs, as compared to patients without ($p = 0.019$) (Table 2), however, when analyzed as related to gender, exclusively in men ($p = 0.009$) (Figure 2A). To further explore this relationship in men, LTLs were divided into quartiles (Figure 2B). A significant trend of lower number of previous MI across quartiles of LTLs appeared ($p = 0.033$). When dichotomized at the distinct cut-off level between quartile 3 and 4, as indicated by the figure, 64 % lower frequency of previous MI in the upper quartile compared to quartile 1-3 was observed [OR = 0.36 (95 % confidence interval 0.17, 0.74)] ($p = 0.005$), adjusted for age and other potential covariates (BMI, triglycerides, glucose and HT). Notably, none of the investigated age-related markers and the pro-inflammatory cytokines was differently distributed according to previous MI (data not shown).

Other CAD risk factors as cigarette smoking, diabetes type 2, and overweight were not significantly associated with LTLs (Table 2). Total- and HDL cholesterol were not correlated to LTLs ($r < 0.1$, both, $p = 0.837$ and $p = 0.876$, respectively), as was also not BMI ($r = 0.087$, $p = 0.159$).

Table 2 **Levels of LTLs in different CAD subgroups**

	LTL RQ	<i>p</i>
Men (n = 240)	0.63 (0.46, 0.93)	0.753
Women (n = 60)	0.65 (0.48, 1.03)	
MI + (n = 159)	0.58 (0.44, 0.84)	0.019
- (n = 141)	0.70 (0.49, 1.05)	
Current smoker + (n = 66)	0.57 (0.45, 0.96)	0.578
- (n = 233)	0.63 (0.46, 0.95)	
MetS + (n = 104)	0.64 (0.45, 0.93)	0.794
- (n = 196)	0.61 (0.47, 0.95)	
Diabetes Type 2 + (n = 74)	0.64 (0.46, 0.92)	0.834
- (n = 226)	0.62 (0.46, 0.96)	
HT + (n = 156)	0.64 (0.48, 0.97)	0.523
- (n = 144)	0.58 (0.45, 0.93)	
BMI $\geq$ 25 (n = 219)	0.65 (0.47, 0.99)	0.109
< 25 (n = 80)	0.58 (0.43, 0.79)	

RQ: relative quantification of LTLs; median values (25, 75 percentiles)

p-values represent differences in LTLs between CAD subgroups: MI; myocardial infarction, MetS; metabolic syndrome, HT; hypertension, BMI; body mass index

Figure 2 LTLs as related to previous MI

A. LTL measured as relative quantification (RQ values) in relation to previous MI in both gender and in men and women separately.

Grey column presents without previous MI, dark grey column presents with previous MI

The p-values refer to difference in LTLs levels between the groups with and without previous MI, Mann Whitney test.

B. LTLs divided into quartiles (Qs) as related to numbers with previous MI in men.

Qs are presented as follows: Q1 light grey, Q2 grey, Q3 dark grey, Q4 black.

Relative quantification (RQ) values of LTLs in Q1: ≤ 0.459 , Q2: $0.459-0.629$, Q3: $0.629-0.945$, Q4: ≥ 0.945 .

The p-value refers to difference in number (%) with previous MI through quartiles of LTLs, Kruskal Wallis test

1.3.2. Inter-correlations between LTLs and gene-expression of GDF11, IGF1 and SIRT1

Correlations between these markers are presented in Table 3, showing no association between LTLs and the investigated age-related factors. Due to observed gender differences in the association between LTLs and age (Figure 1) and higher expressed GDF11 and IGF1 in women compared to men (Supplementary Table 3) and the fact that SIRT1 is lifestyle-impressionable, correlation analysis was performed accordingly in actual subgroups, showing a tendency between LTLs and GDF11 in females ($r = 0.27$, $p = 0.071$, $n = 60$), statistically significant in overweight women ($r = 0.41$, $p = 0.020$, $n = 29$), as was also the correlation between LTLs and SIRT1 ($r = 0.43$, $p = 0.027$). These associations were not observed in men separately.

Further analyses in the total cohort showed strong correlation between GDF11 and SIRT1 whereas SIRT1 and IGF1 were weakly inter-correlated (Table 3).

Table 3 **Correlations between LTLs and genetically expressed GDF11, IGF1, SIRT1 in the total population**

	GDF11 RQ	IGF1RQ	SIRT1RQ
LTLs RQ	$r < 0.1$ $p = 0.427$	$r < 0.1$ $p = 0.808$	$r < 0.1$ $p = 0.906$
GDF11RQ		$r = 0.13$ $p = 0.057$	$r = 0.56$ $p < 0.001$
IGF1RQ			$r = 0.16$ $p = 0.026$

Coefficient of correlation; Spearman Rho

RQ; relative quantification of gene-expression

After Bonferroni correction, p-values in bold remained statistically significant ($p = 0.008$ by 6 performed correlations)

1.3.3. GDF11, IGF1 and SIRT1 levels in relation to age and CAD subgroups

The gene expression of GDF11 associated inversely and significantly with age ($r = -.016$, $p = 0.010$) and age quartiles ($p = 0.027$), whereas IGF1 and SIRT were not correlated to age ($r < 0.1$, both, $p = 0.352$ and $p = 0.148$, respectively), although numerically declining with increasing age quartiles (Supplementary Table 2). GDF11, IGF1 and SIRT1 were all higher expressed in women compared to men (Supplementary Table 3), however, only IGF1 remained statistically different after Bonferroni correction. We observed furthermore that IGF1 was significantly higher expressed in current vs. non-smokers (0.62 vs. 0.45) ($p = 0.011$) and SIRT1 was lower expressed in MetS vs. non-MetS subjects (1.50 vs 1.55) ($p = 0.045$). GDF11, IGF1 and SIRT1 were not differently expressed according to previous MI or other comorbidities (data not shown). The investigated cytokines were not differently distributed between gender (Supplementary Table 3).

1.3.4. Distribution of GDF11, IGF1, SIRT1 and pro-inflammatory cytokines as related to LTLs quartiles in the total cohort and in men and women separately.

When LTLs were categorized into quartiles, the investigated genes were not differently distributed, except for altered IFN γ and SIRT1 expression (Figure 3A). Along with above mentioned sex differences, the association to IFN γ was significant only in men (Figure 3B) and SIRT1 exclusively in women (Figure 3C). Circulating levels of the cytokines IL-18, IL-6 and TNF α were also significantly differently distributed through quartiles of LTL in all, with the highest cytokine levels in first quartile compared to quartiles 2, 3 and 4 (Figure 3A). A similar pattern was observed when analyzed in women separately (Figure 3C). As the levels in quartile 1 of LTLs differed especially from quartile 4 in most of the investigated variables, these two quartiles were compared with regard to the examined markers. In the total cohort, when the

model was adjusted for age and sex, LTLs in the upper quartile associated with lower IFN γ expression ($p = 0.005$) and lower circulating TNF α , IL-18 and IL-6 as compared to quartile 1 ($p = 0.006$, $p = 0.002$ and $p = 0.016$, respectively). In the female patients, a clear tendency emerged: LTLs in the upper quartile associated significantly with higher expression of GDF11 ($p = 0.047$), SIRT1 ($p = 0.010$) and IL-18 ($p = 0.002$), as well as with lower circulating levels of IL-12 ($p = 0.016$), IL-18 ($p = 0.027$) and TNF α ($p = 0.004$), as compared to quartile 1. In men separately, only IFN γ expression and circulating TNF α associated to LTLs in the same manner ($p = 0.043$ and $p = 0.025$, respectively, adjusted for previous MI).

Figure 3 Levels of GDF11, IGF1, SIRT1 and pro-inflammatory cytokines through quartiles (Qs) of telomere lengths in the total population (A) and separately in men (B) and women (C).

Qs are presented as follows: Q1 light grey, Q2 grey, Q3 dark grey, Q4 black.

Relative quantification (RQ values) of LTL in Q1: ≤ 0.459 , Q2: $0.459-0.629$, Q3: $0.629-0.945$, Q4: ≥ 0.945 .

The p-values refer to differences in levels of the markers through quartiles of LTLs, Kruskal Wallis test

1.3.5. GDF11, IGF1, SIRT1 and their associations to pro-inflammatory cytokines.

Correlations between the age-related factors and the selected pro-inflammatory cytokines are presented in Supplementary Table 4. After Bonferroni corrections, expression of GDF11 and SIRT1 were both significantly associated with expression of IL-12 and IL-18 and inversely correlated to circulating levels of IL-6.

1.4. Discussion

In this exploratory study of patients with stable CAD, we observed a sex-specific influence in the association between 1) LTLs and age, with stronger correlation in women, 2) LTLs and CAD severity, with significantly lower frequency of previous MI in the upper quartile of LTLs in men, 3) LTL and its covariation with the investigated lifestyle and lifespan-regulating factors, GDF11 and SIRT1, which was only present in women and 4) LTLs, GDF11, SIRT1 and pro-inflammatory cytokines, which was stronger in women. Additionally, IGF was higher expressed in women than in men, as also reported for circulating levels by others [27], and GDF11 and SIRT1 expression were strongly inter-correlated, independently of whichever analyzed subgroup, not previously reported.

Although highly significant, some observed results needs to be carefully interpreted. First, the study was performed in CAD patients, in which the delicate balance between the investigated molecular markers and telomeres length already may be disturbed due to the pathogenesis of their cardiovascular disease. Additionally, any influence of medication on telomere lengths cannot be excluded. Secondly, despite that not even a tendency in the association between LTLs and previous MI was observed in women, the absence of any relationship may be due to statistical error type 2. Third, our observed results on sex-differences in the relationship between LTLs , GDF11, SIRT1 and the pro-inflammatory cytokines may be taken with caution, as the number of women is limited, i.e. the number in some subgroups is low. However, the study has an exploratory character and the results may nevertheless be hypothesis generating.

The fact that LTLs decline with age is indisputable. We observed, however, that LTLs are seemingly more influenced by age in the female gender, contrary to similar correlations

between gender observed by others [28]. Men and women seem to have equal telomere lengths at birth, but thereafter male telomeres tend to shorten faster [4]. However, the decline in telomere shortening rate at higher age has been shown to be stronger in men than women, indicating a steeper telomere shortening curve in older female subject [5] . As half of our female patients were between 70-80 years, this may partly explain our observation. Additionally, the percentage of current smokers and presence of HT and diabetes type 2 was numerically more frequent in women, reflecting higher disease burden, which may result in higher telomere shortening rate.

LTLs associated significantly with previous MI in males at inclusion, also observed by others [29], probably reflecting a more advanced stage of their disease. Other risk factors, like cigarette smoking, hypertension, diabetes and overweight, were not associated with shorter telomere lengths in our study, which may be due to their already established CAD, however contrarily reported by others (28). The observed covariation between quartiles of LTLs and GDF11 and SIRT1 expression in women has to our knowledge not previously been reported. The observed novel and strong inter-correlation between GDF11 and SIRT1 may indicate common upstream effectors and/or common intracellular pathways in regulating cell-senescence, which may hold sex-specific influences on LTLs. GDF11 was inversely associated with age in our population and the lowest GDF11 expression levels were observed in patients >69 years. This is consistent with previous research stating GDF11 as a youthful-promoting factor, which declines with age [30] and not as claimed by others, increases with age [31].

Lack of any significant association between IGF1 and age and the other investigated markers, may be due to its differentiated profile [32] . While only numerically declined expression of IGF1 was observed in our study, circulating IGF1 has been reported to decrease with age [33].

However, IGF1 levels have been shown to be U-shaped related to CVD [32]. This may indicate a delicate balance and narrow range of what is beneficial and harmful for this marker. High enough levels are maybe required up to a certain age, in light of its growth-stimulating effect, whereas extensively elevated levels, due to presence of age-related risk factors, may be harmful. Consistently, overexpression of IGF1 is thought to abolish its anti-apoptotic properties, therefore persistent high levels may lead to cell-senescence [34].

Elevated circulating levels of the pro-inflammatory cytokines TNF α , IL-6, IL-12 and IL-18 associated with the shortest telomeres in our patients, especially in women. Association of telomere lengths with biomarkers of inflammation in the elderly has also been reported by others [35]. With age, the production of mitochondrial ROS increases, as also the production of pro-inflammatory cytokines, which raises the question of LTLs in women being potentially more susceptible to progression of these changes, often mediated by an unhealthy lifestyle. In opposite, women may be more receptive to health promoting life-style adjustments, whereas the same profile in men may be harder to modify, or that men evolutionarily are less susceptible or more resistant to environmental or lifestyle fluctuations influencing LTLs. A greater control over mitochondrial function and turnover in females has correspondingly been suggested, allowing better response to diet and environment [36] and the sex-specific biological environment was shown to have considerable influence over telomere dynamics [37]. Additionally, non-heritable factors might have a greater impact on telomere length dynamics in women during life. Antioxidants, calorie restriction, and exercise have been shown to prevent both cardiac telomere attrition and progression of heart disease [20]. Whether this positive effect holds gender differences in humans, remains to be elucidated. The observed association between LTLs and expression of the pro-inflammatory IFN γ gene, predominantly produced by leukocytes,

substantiate inflammation as drivers of telomere attrition. The telomerase enzyme, elongating telomeres, is thought to be involved in modulating the activity of the transcriptional factor NF- κ B, which mediate expression of inflammatory cytokines, thus a potential link is suggested [6]. GDF11 and SIRT 1 were also significantly and inversely associated with circulating IL-6, which correspondingly may indicate inhibition of these strongly inter-correlated youthful-promoting factors at a progressive inflammatory state, or a SIRT1- mediated suppression of NF- κ B expression, as indicated [38]. The strong correlation between GDF11, SIRT1 and genetically expressed IL-12 and IL-18 may indicate leukocytes not being the main source of these mediators, and/or a compensatory upregulating of IL-12 and IL-18 in leukocytes as a result of lower circulating IL-12 and IL-18 levels.

Due to multiple comparisons, Bonferroni correction has been performed, and mainly associations with p-values below 0.01 or even below 0.001 were considered. However, as this study has an explorative character, it is worth to notice that tendencies of an unhealthy lifestyle, manifested with high BMI, presence of MetS and cigarette smoking, influenced primarily the relationship between LTLs and both GDF11 and SIRT expression, which was observed strengthened in overweight women. Secondly, SIRT-1 expression was observed attenuated in MetS, also reported by others [39], and IGF1 expression was observed elevated in smokers, the latter contradictory reported [40].

1.4.1. Limitations

Other factors, such as loss of telomeric proteins that decrease telomere lengths i.e. lack of telomere-lengthening telomerase activity, have not been taken into considerations in this study. Different gender frequency of genetic polymorphisms in the telomerase enzyme needs to be further elucidated. Even though previous studies states LTLs to be indicative for telomere

lengths in other tissue, measuring cardiac telomere lengths would probably better indicate any association between telomere lengths and cardiac disease. Finally, additionally measuring circulating IFN γ and genetically expressed IL-6 and TNF α would have improved the results. Notably, expression of genes was investigated in the same cell type as telomere length measurements in the present study, which strengthen the results. However, any direct molecular impact cannot be confirmed.

1.4.2. Conclusion

In this cohort of stable CAD patients, LTLs were more related to age in women, indicating faster telomere attrition rate in elderly females compared to men. The observed association between pro-inflammatory cytokines and LTLs accompanied by LTLs covariance with the life-span regulatory molecules GDF11 and SIRT1, apparently in the female gender, may indicate sex-dimorphism in LTL and in life-span dynamics and regulation. This may implicate sex-adjusted health-preventive therapies. Although the study cannot confirm any causality, the observed significantly shorter LTLs in male subjects with previous MI, may indicate telomere attrition as part of MIs' pathophysiology, which may request extended preventive therapy in subjects at higher risk of MI.

1.4.3. Acknowledgement

MSc Sissel Åkra and MSc Vibeke Bratseth are acknowledged for laboratory assistance, including cytokine measurements, in the ASCET trial.

1.4.4. Funding

The work was funded by the Norwegian Council for Cardiovascular Diseases and Stein Erik Hagen Foundation for Clinical Heart Research, Oslo Norway. These funding sources were not involved in the research and preparation of the article.

1.4.4. Conflict of Interest: None

Reference list

1. Mikhelson VM, Gamaley IA: **Telomere shortening is a sole mechanism of aging in mammals.** *Current aging science* 2012, **5**(3):203-208.
2. Blackburn EH: **Structure and function of telomeres.** *Nature* 1991, **350**(6319):569-573.
3. Takubo K, Aida J, Izumiyama-Shimomura N, Ishikawa N, Sawabe M, Kurabayashi R, Shiraishi H, Arai T, Nakamura K: **Changes of telomere length with aging.** *Geriatrics & gerontology international* 2010, **10** Suppl 1:S197-206.
4. Barrett EL, Richardson DS: **Sex differences in telomeres and lifespan.** *Aging cell* 2011, **10**(6):913-921.
5. Needham BL, Diez Roux AV, Bird CE, Bradley R, Fitzpatrick AL, Jacobs DR, Ouyang P, Seeman TE, Thurston RC, Vaidya D *et al*: **A test of biological and behavioral explanations for gender differences in telomere length: the multi-ethnic study of atherosclerosis.** *Biodemography and social biology* 2014, **60**(2):156-173.
6. Zhang J, Rane G, Dai X, Shanmugam MK, Arfuso F, Samy RP, Lai MK, Kappei D, Kumar AP, Sethi G: **Ageing and the telomere connection: An intimate relationship with inflammation.** *Ageing research reviews* 2016, **25**:55-69.
7. Samani NJ, van der Harst P: **Biological ageing and cardiovascular disease.** *Heart* 2008, **94**(5):537-539.
8. von Zglinicki T: **Oxidative stress shortens telomeres.** *Trends in biochemical sciences* 2002, **27**(7):339-344.
9. Loffredo FS, Steinhauser ML, Jay SM, Gannon J, Pancoast JR, Yalamanchi P, Sinha M, Dall'Osso C, Khong D, Shadrach JL *et al*: **Growth differentiation factor 11 is a circulating factor that reverses age-related cardiac hypertrophy.** *Cell* 2013, **153**(4):828-839.
10. Katsimpardi L, Litterman NK, Schein PA, Miller CM, Loffredo FS, Wojtkiewicz GR, Chen JW, Lee RT, Wagers AJ, Rubin LL: **Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors.** *Science* 2014, **344**(6184):630-634.
11. Rodgers BD: **The Immateriality of Circulating GDF11.** *Circulation research* 2016, **118**(10):1472-1474.
12. Egerman MA, Cadena SM, Gilbert JA, Meyer A, Nelson HN, Swalley SE, Mallozzi C, Jacobi C, Jennings LL, Clay I *et al*: **GDF11 Increases with Age and Inhibits Skeletal Muscle Regeneration.** *Cell metabolism* 2015, **22**(1):164-174.
13. Junnila RK, List EO, Berryman DE, Murrey JW, Kopchick JJ: **The GH/IGF-1 axis in ageing and longevity.** *Nature reviews Endocrinology* 2013, **9**(6):366-376.
14. Laughlin GA, Barrett-Connor E, Criqui MH, Kritz-Silverstein D: **The prospective association of serum insulin-like growth factor I (IGF-I) and IGF-binding protein-1 levels with all cause and cardiovascular disease mortality in older adults: the Rancho Bernardo Study.** *The Journal of clinical endocrinology and metabolism* 2004, **89**(1):114-120.
15. Grabowska W, Sikora E, Bielak-Zmijewska A: **Sirtuins, a promising target in slowing down the ageing process.** *Biogerontology* 2017, **18**(4):447-476.
16. Razi S, Cogger VC, Kennerson M, Benson VL, McMahon AC, Blyth FM, Handelsman DJ, Seibel MJ, Hirani V, Naganathan V *et al*: **SIRT1 Polymorphisms and Serum-Induced SIRT1 Protein Expression in Aging and Frailty: The CHAMP Study.** *The journals of gerontology Series A, Biological sciences and medical sciences* 2017, **72**(7):870-876.
17. Friedrich U, Griesse E, Schwab M, Fritz P, Thon K, Klotz U: **Telomere length in different tissues of elderly patients.** *Mechanisms of ageing and development* 2000, **119**(3):89-99.

18. Wilson WR, Herbert KE, Mistry Y, Stevens SE, Patel HR, Hastings RA, Thompson MM, Williams B: **Blood leucocyte telomere DNA content predicts vascular telomere DNA content in humans with and without vascular disease.** *European heart journal* 2008, **29**(21):2689-2694.
19. Okuda K, Bardeguet A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, Skurnick J, Awad G, Aviv A: **Telomere length in the newborn.** *Pediatric research* 2002, **52**(3):377-381.
20. Booth SA, Charchar FJ: **Cardiac telomere length in heart development, function, and disease.** *Physiol Genomics* 2017, **49**(7):368-384.
21. Niccoli T, Partridge L: **Ageing as a risk factor for disease.** *Current biology : CB* 2012, **22**(17):R741-752.
22. Pettersen AA, Seljeflot I, Abdelnoor M, Arnesen H: **High On-Aspirin Platelet Reactivity and Clinical Outcome in Patients With Stable Coronary Artery Disease: Results From ASCET (Aspirin Nonresponsiveness and Clopidogrel Endpoint Trial).** *J Am Heart Assoc* 2012, **1**(3):e000703.
23. Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, Williams GR: **Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults.** *Circulation* 2004, **110**(10):1245-1250.
24. Cawthon RM: **Telomere measurement by quantitative PCR.** *Nucleic acids research* 2002, **30**(10):e47.
25. Opstad TB, Pettersen AA, Weiss TW, Akra S, Ovstebo R, Arnesen H, Seljeflot I: **Genetic variation, gene-expression and circulating levels of matrix metalloproteinase-9 in patients with stable coronary artery disease.** *Clin Chim Acta* 2012, **413**(1-2):113-120.
26. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method.** *Methods* 2001, **25**(4):402-408.
27. Vestergaard PF, Hansen M, Frystyk J, Espelund U, Christiansen JS, Jorgensen JO, Fisker S: **Serum levels of bioactive IGF1 and physiological markers of ageing in healthy adults.** *European journal of endocrinology* 2014, **170**(2):229-236.
28. Benetos A, Okuda K, Lajemi M, Kimura M, Thomas F, Skurnick J, Labat C, Bean K, Aviv A: **Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity.** *Hypertension* 2001, **37**(2 Pt 2):381-385.
29. Brouillette S, Singh RK, Thompson JR, Goodall AH, Samani NJ: **White cell telomere length and risk of premature myocardial infarction.** *Arteriosclerosis, thrombosis, and vascular biology* 2003, **23**(5):842-846.
30. Olson KA, Beatty AL, Heidecker B, Regan MC, Brody EN, Foreman T, Kato S, Mehler RE, Singer BS, Hveem K *et al*: **Association of growth differentiation factor 11/8, putative anti-ageing factor, with cardiovascular outcomes and overall mortality in humans: analysis of the Heart and Soul and HUNT3 cohorts.** *European heart journal* 2015, **36**(48):3426-3434.
31. Schafer MJ, Atkinson EJ, Vanderboom PM, Kotajarvi B, White TA, Moore MM, Bruce CJ, Greason KL, Suri RM, Khosla S *et al*: **Quantification of GDF11 and Myostatin in Human Aging and Cardiovascular Disease.** *Cell metabolism* 2016, **23**(6):1207-1215.
32. van Bunderen CC, Oosterwerff MM, van Schoor NM, Deeg DJ, Lips P, Drent ML: **Serum IGF1, metabolic syndrome, and incident cardiovascular disease in older people: a population-based study.** *European journal of endocrinology* 2013, **168**(3):393-401.
33. Wennberg AMV, Hagen CE, Petersen RC, Mielke MM: **Trajectories of plasma IGF-1, IGFBP-3, and their ratio in the Mayo Clinic Study of Aging.** *Experimental gerontology* 2018.
34. Tran D, Bergholz J, Zhang H, He H, Wang Y, Zhang Y, Li Q, Kirkland JL, Xiao ZX: **Insulin-like growth factor-1 regulates the SIRT1-p53 pathway in cellular senescence.** *Aging cell* 2014, **13**(4):669-678.
35. O'Donovan A, Pantell MS, Puterman E, Dhabhar FS, Blackburn EH, Yaffe K, Cawthon RM, Opresko PL, Hsueh WC, Satterfield S *et al*: **Cumulative inflammatory load is associated with**

- short leukocyte telomere length in the Health, Aging and Body Composition Study.** *PloS one* 2011, **6**(5):e19687.
36. Tower J: **Sex-Specific Gene Expression and Life Span Regulation.** *Trends in endocrinology and metabolism: TEM* 2017, **28**(10):735-747.
 37. Bruderlein S, Muller K, Melzner J, Hogel J, Wiegand P, Moller P: **Different rates of telomere attrition in peripheral lymphocytes in a pair of dizygotic twins with hematopoietic chimerism.** *Aging cell* 2008, **7**(5):663-666.
 38. Mendes KL, Lelis DF, Santos SHS: **Nuclear sirtuins and inflammatory signaling pathways.** *Cytokine Growth Factor Rev* 2017, **38**:98-105.
 39. de Kreutzenberg SV, Ceolotto G, Papparella I, Bortoluzzi A, Semplicini A, Dalla Man C, Cobelli C, Fadini GP, Avogaro A: **Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: potential biochemical mechanisms.** *Diabetes* 2010, **59**(4):1006-1015.
 40. Renehan AG, Atkin WS, O'Dwyer S T, Shalet SM: **The effect of cigarette smoking use and cessation on serum insulin-like growth factors.** *British journal of cancer* 2004, **91**(8):1525-1531.

Supplementary Tables

Supplementary Table 1 Primer sequences for the telomeres and SCG analyses

PCR primers	Oligomer sequences (5' to 3')
Telomere fw.	CAG CAA GTG GGA AGG TGT AAT CC
Telomere rev.	GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT
SCG (36B4) fw	CAG CAA GTG GGA AGG TGT AAT CC
SCG (36B4) rev	CCC ATT CTA TCA TCA ACG GGT ACA A

fw; forward, rev; reverse, SCG: single copy gene

Supplementary Table 2 Distribution of LTLs, genetically expressed GDF11, IGF1 and SIRT1 through quartiles (Qs) of age in the total population

	Age years Q1 $\leq$ 54	Age years Q2 54-61	Age years Q3 61-69	Age years Q4 $\geq$ 69	<i>p</i>
LTL RQ	0.78 (0.52, 1.09)	0.57 (0.44, 0.93)	0.65 (0.44, 1.00)	0.57 (0.43, 0.80)	0.003
GDF11 RQ	1.02 (0.75, 1.22)	1.02 (0.71, 1.44)	0.89 (0.70, 1.57)	0.83 (0.62, 1.13)	0.027
IGF1RQ	0.61 (0.33, 0.98)	0.45 (0.25, 1.34)	0.44 (0.27, 1.14)	0.48 (0.21, 0.72)	0.18
SIRT1RQ	1.64 (1.33, 1.84)	1.64 (1.20, 1.93)	1.46 (1.19, 1.82)	1.48 (1.19, 1.95)	0.33

RQ: relative quantification of gene-expression ; median values (25, 75 percentiles)

P-values represent difference in variables through quartiles of age (Kruskal Wallis test).

After Bonferroni correction, p-values in bold remained statistically significant ($p = 0.0125$ by 4 performed associations)

Supplementary Table 3 Gene-expression of GDF11, IGF1, SIRT1, IL-12, IL-18, and IFN γ , and circulating levels of the measured pro-inflammatory markers in the total cohort and in relation to gender

Variable	In total	In men (n = 240)	In women (n = 60)	<i>p</i>
GDF11RQ	0.93 (0.70, 1.22)	0.91 (0.68, 1.21)	1.06 (0.83, 1.38)	0.044
IGF1RQ	0.49 (0.27, 0.89)	0.45 (0.24, 0.80)	0.72 (0.43, 1.34)	0.001
SIRT1RQ	1.52 (1.22, 1.90)	1.51 (1.21, 1.87)	1.77 (1.30, 2.06)	0.082
IL-12RQ	2.52 (1.95, 3.38)	2.50 (1.94, 3.38)	2.53 (2.04, 3.55)	0.556
IL-18RQ	1.42 (1.11, 1.84)	1.42 (1.12, 1.84)	1.41 (1.01, 1.88)	0.710
IFN γ RQ	2.24 (1.51, 3.14)	2.27 (1.52, 3.15)	2.12 (1.41, 3.07)	0.698
sIL-12 pg/mL	86.4 (60.2, 135.2)	86.4 (59.8, 133.0)	81.0 (63.0, 167.7)	0.964
sIL-18 pg/mL	217.3 (160.0, 280.8)	225.3 (167.6, 275.5)	195.7 (131.1, 293.4)	0.161
sIL-6 pg/mL	2.58 (1.86, 4.28)	2.56 (1.86, 4.29)	2.88 (1.92, 4.36)	0.943
sTNF α pg/ml	1.28 (0.94, 1.79)	1.21 (0.94, 1.76)	1.41 (0.99, 1.98)	0.225

RQ: relative quantification of gene-expression

s = serum values

Values are median (25, 75 percentiles)

p-values indicate differences in the variables between gender

After Bonferroni correction, p-values in bold remained statistically significant ($p = 0.005$ by 10 performed associations)

Supplementary Table 4 Correlations between genetically expressed GDF11, IGF1, SIRT1 and cytokines in the total population.

	IL-12RQ	IL-18RQ	IFN γ RQ	sIL-12	sIL-18	sTNF α	sIL-6
GDF11RQ	.51 p<0.0001	.29 p<0.0001	.14 p=0.033	<0.1 p=0.111	<0.1 p=0.902	<0.1 p=0.387	-.35 p<0.0001
IGF1RQ	.17 p=0.031	.13 p=0.107	<0.1 p=0.379	-.19 p=0.007	<0.1 p=0.418	<0.1 p=0.811	<0.1 p=0.324
SIRT1RQ	.42 p<0.0001	.35 p<0.0001	<0.1 p=0.572	<0.1 p=0.127	-.14 p=0.020	<0.1 p=0.715	-.26 p<0.0001

Coefficient of correlation; Spearman Rho

RQ: relative quantification of gene-expression

s: serum

After Bonferroni correction, p-values in bold remained statistically significant (p =0.0024 by 21 performed correlations)

Highlights

- Long LTLs associate with 64 % lower frequency of previous MI in patients with CAD
- LTLs correlate to age, exclusively in women with CAD
- GDF11 and SIRT1 strongly correlate, indicating common intracellular pathways
- Long LTLs associate with high GDF11/SIRT1 expression in women and less inflammation in both gender
- Possible sex-dimorphism in LTL dynamics and regulation

Graphical abstract

Observed associations between LTL and GDF11, IGF1, SIRT1 and pro-inflammatory cytokines in women

Grey lines indicate observed favorable effects on telomere lengths, red arrows indicate unfavorable effects on telomere lengths and stiplet lines indicate a possible link between IGF1 and telomere lengths, although not observed in this study. Light blue lines indicate literature reports. Lines with double arrows indicate the observed associations between SIRT1 and GDF11 with IL-6 and TNF α .

GDF11: growth differentiating factor 11, SIRT1: sirtuin1, IGF1: insulin growth factor1

ROS: reactive oxygen species, IL- interleukin