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Cell-free DNA as a Novel Biomarker of Aging

Characterization and genetic regulation

ACADEMIC DISSERTATION
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To my family

Contents

List of original communications

This dissertation is based on the following original communications, which are referred in the text by their Roman numerals (I-III).

I Jylhävä J, Jylhä M, Lehtimäki T, Hervonen A, Hurme M. Circulating cell-free DNA is associated with mortality and inflammatory markers in nonagenarians: the Vitality 90+ Study. Exp Gerontol. 2012 May;47(5):372-8.

II Jylhävä J*, Lyytikäinen LP*, Kähönen M, Hutri-Kähönen N, Kettunen J, Viikari J, Raitakari OT, Lehtimäki T, Hurme M. A genome-wide association study identifies UGT1A1 as a regulator of serum cell-free DNA in young adults: The Cardiovascular Risk in Young Finns Study. PLoS One. 2012;7(4):e35426.

III Jylhävä J, Nevalainen T, Marttila S, Jylhä M, Hervonen A, Hurme M. Characterization of the role of distinct plasma cell-free DNA (cf-DNA) species in age-associated inflammation and frailty. Aging Cell, in press, DOI: 10.1111/acel.12058.

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Abbreviations

ANA	antinuclear antibodies
BMI	body mass index
CAMP/LL-37	cathelicidin antimicrobial peptide
cf-DNA	cell-free DNA
cf-RNA	cell-free RNA
CMV	cytomegalovirus
CNAs	circulating nucleic acids
CRP	C-reactive protein
CVD	cardiovascular disease
DC	dendritic cell
EBV	Epstein-Barr virus
GWAS	genome-wide association study
HMGB	high motility group box
IC	immunocomplex
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
miRNA	micro RNA
MMSE	Mini-Mental State Examination
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa B
PBMC	peripheral blood mononuclear cell
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species

SAA	serum amyloid A
SAP	serum amyloid P
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
UGT1A1	UDP-glucuronosyltransferase 1 polypeptide A1

Abstract

Cell-free DNA (cf-DNA), which is released into circulation following cellular death and tissue injury, has recently emerged as a promising new indicator of the degree of the damage. In addition to assessing the quantities of circulating cf-DNA, qualitative features, such as the cf-DNA methylation level and fragment size distribution have been demonstrated to be useful diagnostic and prognostic markers in various pathologies. The fact that virtually every cell in the body can release cf-DNA makes this measure a highly sensitive indicator of homeostatic disturbances and alterations in the rate of cellular death. However, despite the growing utility of cf-DNA assessment, many aspects regarding the regulation of circulating cf-DNA levels and alterations in the composition of the total cf-DNA pool in physiological conditions, such as aging, are currently unknown. Likewise, it is unclear whether cf-DNA is merely a risk-indicating biomarker or whether it can acquire a pathogenic role in certain conditions.

In the studies comprising this dissertation, we aimed to examine the role of cf-DNA as a biomarker of different age-related phenomena using the Vitality 90+ study cohorts (n=258 and n=144). We investigated the utility of plasma total cf-DNA level to indicate age-associated inflammation and as a predictor of mortality in old age. To assess the composition of cf-DNA, the following different cf-DNA species were quantified: total, unmethylated, *RNase P*-coding and *Alu* repeat cf-DNA and the mitochondrial DNA (mtDNA) copy number. These cf-DNA species were also analyzed for their relationship to age-associated changes in the immune system and functional performance. In addition, we determined the immunological responses associated with plasma cf-DNA levels using the Vitality 90+ study nonagenarians and young control individuals. To elucidate the mechanisms that are responsible for cf-DNA metabolism and to determine the role of genetics in the regulation of serum cf-DNA levels, a genome-wide association study (GWAS) was preformed in the Cardiovascular Risk in Young Finns study cohort (n=1841).

Except for mtDNA copy number, the plasma levels of all of the cf-DNA species, exhibited age-associated increase, and the total cf-DNA level was an independent 4-year mortality predictor in the nonagenarians. The plasma levels of total and unmethylated cf-DNA correlated directly with the level of systemic inflammation and impairments in cognitive and physical performance. Moreover, the plasma mtDNA copy number correlated directly with the markers of physical impairment in the nonagenarians. The immune cell responses that were associated with the plasma levels of total and unmethylated cf-DNAs included the activation of various immunoinflammatory pathways with increasing concentrations of these cf-DNA species in the nonagenarians but not in the young controls. In the GWAS, single nucleotide polymorphisms (SNPs) in the *UDP-glucuronosyltransferase 1 polypeptide A1* gene region were associated with serum cf-DNA levels among the Young Finns study subjects. The result identifies a previously unrecognized mechanism in the regulation of cf-DNA levels.

In conclusion, plasma levels of total cf-DNA and unmethylated cf-DNA and the mtDNA copy number appear to be compelling biomarkers for various essential age-accompanied impairments. However, additional research will be required to establish the immunostimulatory potential of plasma cf-DNA in aged individuals and to elucidate the basis of the genetic regulation of cf-DNA levels.

Tiivistelmä

Elimistössä tapahtuvan solukuoleman ja kudostuhon seurauksena verenkiertoon vapautuu DNA:ta. Tämä soluvapaan DNA:n pitoisuus on osoittautunut lupaavaksi vaurioiden laajuutta kuvastavaksi indikaattoriksi. Pitoisuuden lisäksi, soluvapaan DNA:n tietyt laadulliset ominaisuudet, kuten metylaatioaste ja juosteiden pituuden vaihtelu ovat osoittautuneet käyttökelpoisiksi biomarkkereiksi useiden sairauksien diagnoosia ja ennustetta määritettäessä. Koska soluvapaata DNA:ta voi vapautua miltei kaikista elimistön soluista, sen määrä verenkierossa osoittaa herkästi muutoksia kudosten homeostaattisessa tasapainossa ja solukuoleman vauhdissa. Huolimatta siitä, että soluvapaan DNA:n käyttöalueiden määrä on suuri, tällä hetkellä ei tarkasti tiedetä mitkä tekijät osallistuvat soluvapaan DNA:n pitoisuuden säätelyyn ja miten soluvapaan DNA:n koostumus muuttuu erilaisissa fysiologisissa tiloissa, kuten vanhenemisessa. Lisäksi on epäselvää onko soluvapaa DNA vain riskiä indikoiva biomarkkeri vai onko sillä myös itsenäinen, patogeeninen rooli tietyissä tilanteissa.

Väitöskirjatyön tavoite oli tutkia soluvapaan DNA:n kykyä toimia ikääntymiseen liittyvien tapahtumien biomarkkerina Tervaskannot 90+ tutkimusaineistoissa (n=258 ja n=144). Analysoimme soluvapaan DNA:n kokonaispitoisuuden yhteyttä vanhusten matala-asteiseen tulehdukseen sekä sen kykyä ennustaa kuolleisuutta 90-91 -vuotiailla henkilöillä. Kartoitimme soluvapaan DNA:n koostumusta määrittämällä siitä kokonaispitoisuuden lisäksi metyloimattoman DNA:n, *RNase P*-geeniä koodaavan DNA:n, *Alu* toistojaksoja koodaavan DNA:n ja mitokondrioperäisen DNA:n (mtDNA) määrän, ja analysoimme näiden soluvapaan DNA:n komponenttien yhteyttä immuunisysteemin vanhenemiseen ja vanhusten toimintakykyyn. Plasman soluvapaan DNA:n määrään yhteydessä olevaa immuunisolujen vastetta tutkimme Tervaskannot 90+ -tutkimushenkilöillä sekä nuorilla kontrollihenkilöillä. Soluvapaan DNA:n metaboliaan osallistuvia tekijöitä ja genetiikan osuutta seerumipitoisuuden säätelyssä kartoitimme genominlaajuisen

assosiaatiotutkimuksen avulla Lasten Sepelvaltimotaudin Riskitekijät -tutkimusaineistossa (n=1841).

mtDNA:n määrää lukuun ottamatta, kaikkien soluvapaan DNA:n komponenttien plasmapitoisuudet olivat vanhuksilla kohonneet nuoriin verrattuna, ja soluvapaan DNA:n kokonaispitoisuuden havaittiin ennustavan vanhusten kuolleisuutta 4-vuotisseurannassa. Soluvapaan DNA:n kokonaispitoisuus ja metyloimattoman DNA:n määrä korreloivat suoraan vanhusten systeemisen tulehdustason ja heikentyneen kognitiivisen ja fyysisen toimintakyvyn kanssa. mtDNA:n määrä plasmassa oli myös suoraan yhteydessä huonompaan fyysiseen toimintakykyyn. Korkea soluvapaan DNA:n kokonaispitoisuus sekä korkea metyloimattoman DNA:n määrä olivat myös yhteydessä useiden tulehdus- ja immuunireaktiota välittävien signalointireittien aktivaatioon vanhusten immuunisoluissa. Nuorilla vastaavaa ilmiötä ei havaittu. Genominlaajuisessa assosiaatiotutkimuksessa havaitsimme, että geneettiset variaatiokohdat *UDP-glukuronosyylitransferaasi 1 polypeptidi A1* -geenin alueella yhdistyivät soluvapaan DNA:n seerumitasoihin. Tuloksemme osoittaa aiemmin tunnistamattoman mekanismin toimivan soluvapaan DNA:n pitoisuuden säätelyssä.

Yhteenvetona voidaan todeta, että plasman soluvapaan DNA:n kokonaispitoisuus, metyloimattoman DNA:n määrä ja mtDNA:n määrä vaikuttavat lupaavilta vanhenemismuutoksia kuvaavilta biomarkkerilta. Lisää tutkimuksia kuitenkin tarvitaan kartoittamaan soluvapaan DNA:n roolia immuunisysteemiä stimuloivana tekijänä vanhuksilla sekä valottamaan geneettisen säätelyn taustamekanismeja.

Introduction

The quantity of circulating cell-free DNA (cf-DNA) in plasma/serum has proven to serve as a potent marker for indicating the extent of tissue damage, cellular death and inflammation in various pathologies. Elevated cf-DNA levels have been observed in multiple acute and chronic diseases, ranging from autoimmune diseases to myocardial infarction (Wagner 2012). The increase in cf-DNA quantity has also been demonstrated to have a predictive value in the outcomes of sepsis, certain cancers and acute cardiovascular disease (Butt and Swaminathan 2008, Mittra et al. 2012, Wagner 2012). Moreover, characterization of cf-DNA for its methylation patterns, mutations and fragment size length has offered a non-invasive "liquid biopsy" for the diagnostics of several types of cancer (Schwarzenbach et al. 2011). With respect to aging, data regarding the alterations in the level and composition of cf-DNA are very scarce. Nevertheless, as aging is accompanied with increased systemic inflammation, cellular senescence and death, it represents an amenable condition for which to assess cf-DNA as a candidate biomarker.

The quest for aging biomarkers arises from the observed discrepancy between the calendar age and biological age in the elderly individuals; certain individuals live in relatively good health and maintain their functional capabilities up to the age of 90 years, whereas others suffer from multimorbidity and functional impairments in their 70s (Steinhagen-Thiessen and Borchelt 1993). Identifying the markers reflecting the biological aging rate would serve in delineating the key mechanisms that underlie the aging process and provide means by which to counteract age-associated impairments.

The immune system plays a central role in determining the late-life health. Common phenomena in elderly individuals, such as susceptibility to infections, autoimmune diseases and inefficient vaccine responses, originate form age-accompanied deficits in the immunoinflammatory functions, which are collectively termed

immunosenescence (Agarwal and Busse 2009, McElhaney and Effros 2009). Systemic low-grade inflammation ("inflammaging") is likewise a manifestation of immunosenescence (Franceschi et al. 2007), and certain inflammatory mediators, such as C-reactive protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), have even been proposed as causal factors in age-related functional decline and frailty (Roubenoff 2003, Hubbard and Woodhouse 2010).

The present dissertation focuses on the role of plasma cf-DNA as an aging biomarker and a potential immunomodulatory agent. In addition, it elucidates the previously unaddressed issue regarding the genetic regulation of cf-DNA levels. In the first study, we analyzed the relationship between plasma cf-DNA and inflammaging and mortality rate in the nonagenarians participating in the Vitality 90+ study. In the second study, we wished to ascertain the mechanisms that are involved in cf-DNA turnover and to establish the contribution of genetics to the variation in baseline serum cf-DNA levels. To this end, a genome-wide genome association study (GWAS) was performed using the Cardiovascular Risk in the Young Finns study cohort. Following the observations in the first study, the third study, which was performed using another Vitality 90+ study cohort, aimed to characterize the different cf-DNA species that constitute the plasma "cf-DNA pool" and to expand the areas of utilization of cf-DNA as a biomarker of age-associated impairments. The third study also assessed the potential of cf-DNA as an immunomodulatory agent in aged (i.e., the Vitality 90+ study nonagenarians) and young individuals (healthy control subjects between 19 and 30 years of age).

Review of the literature

1. Circulating cell-free nucleic acids

The initial discovery of cell-free nucleic acids (CNAs) was made in 1948 when Mandel and Metais detected circulating DNA and RNA in the plasma of healthy individuals and patients (Mandel and Metais 1948). However, due to the lack of understanding of the basis of the finding, this study remained largely overlooked at the time. The second finding on CNAs was reported by Tan et al. (1966), who observed an anomalous pattern of cf-DNA in patients who suffered from systemic lupus erythematosus (SLE) (Tan et al. 1966) – an autoimmune disease in which the major antigen is nucleosomal self-DNA (Rumore and Steinman 1990, Decker et al. 2005). The relevance of CNAs did not begin to be unraveled until the 1990s when the presence of tumor-derived oncogenic DNA was observed in the plasma of patients with cancer (Sorenson et al. 1994) and DNA of fetal origin was detected in the maternal circulation (Lo et al. 1997). It was subsequently discovered that cf-DNA levels were significantly increased in patients with chronic and acute pathologies, including autoimmune diseases, stroke and trauma (Butt and Swaminathan 2008, Wagner 2012). It was therefore concluded that the concentration of cf-DNA could serve as a non-invasive blood biomarker to reflect the rate of tissue damage, cellular death and turnover. Research into CNAs has also contributed to a paradigm shift in the behavior of genetic material; horizontally transferred endogenous DNA and RNA have been demonstrated to internalize and exhibit biological activity in the recipient cells (Garcia-Olmo and Garcia-Olmo 2001, Valadi et al. 2007, Garcia-Olmo et al. 2010). However, despite the accumulating body of data, several fundamental issues that are related to the origin, metabolism and inherent properties of CNAs have remained unclear and controversial.

1.2 Circulating cf-DNA

1.2.1. Forms and features of cf-DNA

The nature of the cf-DNA molecules in the circulation, also termed the nucleome, has been a subject of controversy. It has been debated whether cf-DNA appears as free, "naked" DNA, as nucleosomes, in which the DNA is wrapped around a double set of the histones H2A, H2B, H3, and H4 (Luger 2003), or bound to particulate structures, such as apoptotic bodies, serum carrier proteins or anti-DNA antibodies (van der Vaart and Pretorius 2008, Peters and Pretorius 2011). A secreted lipoprotein structure termed the virtosome has also been identified as a DNA-containing structure, although the biological relevance and amount of DNA it contains is less clear (Gahan and Stroun 2010, Peters and Pretorius 2011). Somewhat contrastingly, Chiu et al. (2003) have demonstrated that cf-DNA of nuclear genomic origin circulates as a free entity whereas circulating mitochondrial DNA (mtDNA) can exist in both free and particle-bound forms (Chiu et al. 2003).

Nevertheless, a rather consistent view exists in that the predominant form of cf-DNA can vary with the condition of the organism (Holdenrieder et al. 2008). Using gel electrophoresis, cf-DNA in children with diabetes and in patients with rheumatoid arthritis, lymphoma and myeloma has been demonstrated to acquire a nucleosomal or a ladder-like pattern, resembling that seen in apoptotic cells (Deligezer et al. 2006, Langford et al. 2007). The quantification of circulating nucleosomes has revealed that their number in healthy individuals is fairly low, whereas higher levels are observed in individuals with various autoimmune diseases and cancer (Holdenrieder et al. 2006, Holdenrieder et al. 2008). It has also been demonstrated that in several types of cancer, the patients exhibit increased cf-DNA integrity (size) compared to healthy individuals (Schwarzenbach et al. 2011). Lastly, quasi-genomic-sized cf-DNA fragments have been observed in both healthy subjects (Gormally et al. 2007) and in individuals with diverse malignancies (Schwarzenbach et al. 2011).

Circulating cf-DNA has been analyzed with regard to the representation and distribution of specific sequences and epigenetic features, such as methylation patterns. The opportunities that have been provided by the recent advent of massively parallel sequencing technologies (Schuster 2008) have also been utilized to identify the sequences of cf-DNA (Lo and Chiu 2011). Beck et al. (2009) have demonstrated that the plasma nucleome in healthy individuals largely mirrors that of the cellular nuclear genome, although with an increased representation of short interspersed *Alu* repeat sequences and a lower representation of chromosome 9 and L1 and L2 long interspersed nuclear element sequences (Beck et al. 2009). As regards the correlation between methylation status in cf-DNA and in disease-affected tissue, various pathologies from infectious diseases to psychiatric disorders have been investigated in this respect (Levenson and Melnikov 2012). Generally, a high degree of concordance between the cf-DNA methylation pattern and that of the target tissue has been observed, although contradictory findings also exist (Lo and Chiu 2011, Levenson and Melnikov 2012).

1.2.2. The origin and the release of cf-DNA

In pathological conditions, the primary sources of cf-DNA are apoptotic and necrotic cells (Jahr et al. 2001, Pisetsky 2012), although the degree of the input of the other cell death type over the other has been a subject of controversy. In a murine model of induced liver cell death, Jahr et al. (2001) demonstrated an apparent involvement of necrosis in the generation of cf-DNA (Jahr et al. 2001), whereas Pachl et al. (2005) have reported that in critically ill intensive care unit patients, the contribution of apoptosis to the total cf-DNA pool is 16-fold greater than that of necrosis (Pachl et al. 2005). However, in various pathologies, the predominance of an archetypical apoptotic laddering or nucleosomal appearance of cf-DNA has been considered to be evidence for apoptosis-derived cf-DNA (Gormally et al. 2007, Langford et al. 2007). Cell death contributes to the pool of circulating cf-DNA in healthy individuals as well given that several hundred billions of cells undergo apoptosis daily (Nagata et al. 2010). An additional mechanism that contributes to the circulating nucleome is NETosis, a newly identified mode of neutrophil antimicrobial defense (Wartha et al. 2007). During NETosis, the

neutrophils discharge their extracellular traps (NETs) that contain DNA, both nuclear and mitochondrial, histones and antimicrobial peptides (Wartha et al. 2007, Keshari et al. 2011). Although NETosis is typically associated with pathogen clearance, it has also been implicated in the pathogenesis of SLE (Bouts et al. 2012) and thrombosis (Brill et al. 2012).

Certain viable cells can also release cf-DNA. In the course of erythrocyte maturation, the erythroblasts expel their nuclei in a process termed enucleation (Nagata et al. 2010) and the expelled chromatin is subjected to macrophage-mediated processing (Yoshida et al. 2005a). The precise amount of thus-generated DNA that is released to the circulation is uncertain; however, the total amount of DNA to be degraded in the macrophages is estimated to be more than 1 g daily (Nagata and Kawane 2011). Evidence for active cellular secretion of newly synthesized DNA in the form of (lipo)nucleoprotein complexes also exists. As early as in the 1970s, Anker et al. (1975) and Rogers et al. (1972) reported that cultured viable lymphocytes secreted DNA-protein complexes into the culture medium (Rogers et al. 1972, Anker et al. 1975). More recently, actively secreted structures termed virtosomes have been demonstrated to contain DNA, RNA, lipoproteins and DNA and RNA polymerases (Gahan and Stroun 2010, Peters and Pretorius 2011). However, the magnitude and significance of active cf-DNA secretion in humans is currently unknown.

1.2.3. The clearance of cf-DNA

The molecular mechanisms that are responsible for the cf-DNA clearance are enigmatic, although it has been assumed that plasma nucleases are responsible for degradation of cf-DNA (van der Vaart and Pretorius 2008). However, it has been demonstrated that even though the mean half-life for postpartum fetal cf-DNA in the maternal circulation is only 16.3 min, plasma nucleases play only a partial role in its clearance (Lo et al. 1999). On the other hand, in patients with prostate, colon and stomach cancer, plasma DNase activity has been observed to be decreased with increased cf-DNA levels (Tamkovich et al. 2006). The essential role of nucleases in the degradation of self-DNA has been observed in SLE, in which the accumulation

of DNA-immunocomplexes (DNA-ICs) leads to tissue damage at the affected sites (Frese and Diamond 2011). Furthermore, the impairment of serum DNase I-mediated degradation of NETs has been implicated in aggravating SLE (Leffler et al. 2012) and lupus nephritis (Hakkim et al. 2010). However, the direct function of nucleases in cf-DNA turnover in healthy individuals has not been explicitly demonstrated.

Considering that cf-DNA, particularly in its nucleosomal form, appears to be protected from DNase-mediated degradation (Luger 2003, Holdenrieder et al. 2005), the involvement of other cf-DNA-clearance mechanisms appears plausible. Indeed, studies with mice have demonstrated that injected nucleosomes primarily localized to the liver, which accounted for as much as 84.7% of the clearance (Gauthier et al. 1996). Only a minority of the injected nucleosomes were detected in the kidneys. However, with prior injection of histones that bind to renal glomeruli, the localization of nucleosomes to renal sites is increased threefold (Gauthier et al. 1996). Burlingame et al. (1996) utilized a similar approach and observed that the injected chromatin localized predominantly in the liver and to a lesser extent in spleen and the kidneys (Burlingame et al. 1996). These authors also observed that acute phase response caused a delay in the chromatin clearance and even decreased its extrahepatic localization (Burlingame et al. 1996). Detailed findings by Du Clos et al. (1999) have demonstrated that the hepatic chromatin clearance entails interactions with cell surface heparan sulphate proteoglycans and (unidentified) DNA receptors on Kupffer cells (Du Clos et al. 1999). The authors suggested that chromatin fragmentation is primarily intracellular and that the DNA breakdown products are subsequently released to circulation, thus arguing against the role for plasma nucleases in chromatin degradation.

Liver-mediated clearance has also been observed for DNA-ICs and free double-stranded DNA (dsDNA) in nonhuman primates. Cosio et al. (1987) reported that injected dsDNA-ICs bound rapidly to erythrocytes, whereas free dsDNA did not; however, the liver was the primary site of uptake for both of the cf-DNA forms (Cosio et al. 1987). The erythrocyte complement receptor 1 (CR1) appears to bind to the ICs that are opsonized by the complement component 3b. The internalization and degradation of these ICs is likely accomplished by the resident macrophages via

their Fc receptors (FcRs) in a manner that disintegrates the CR1 but preserves the carrier erythrocyte (Nardin et al. 1999, Henderson et al. 2002). The role of the erythrocyte CR1-facilitated cf-DNA clearance is highlighted by the fact that SLE patients display reduced levels of erythrocyte CR1 compared to healthy controls (Iida et al. 1982). Moreover, the CR1 number has been observed to correlate inversely both with the SLE disease activity and the number of circulating ICs (Yen et al. 1989). Leukocytes are likewise known to possess receptors for DNA and chromatin-ICs uptake, and the plasma proteins CRP and serum amyloid P (SAP) also mediate chromatin disposal. These molecules and their pathways are discussed in more detail in section 2. "The biological effects of cf-DNA". Lastly, plasma cf-DNA can also reach the urine via renal excretion (Zhang et al. 1999, Su et al. 2004). However, according to the estimates by Botezatu et al. (2000) this amount is normally very low, ranging from 0.5 to 2% of the total circulating cf-DNA (Botezatu et al. 2000).

1.2.4. cf-DNA in other body fluids

In addition to plasma/serum, detectable levels of endogenous cf-DNA can be quantified in other body fluids, such as urine, synovial fluid, saliva and cerebrospinal fluid (Wagner 2012). Of these, urinary cf-DNA analysis has attracted much attention in examining both the transrenal cf-DNA originating from the circulation as well as the cf-DNA generated in the urinary tract and renal tissues. Although only trace levels of circulatory cf-DNA pass through the kidneys (Botezatu et al. 2000), it has been demonstrated that this cf-DNA contains sequences that originate from non-renal and non-urinary tract tissues in sufficient amounts for diagnostic applications in colorectal cancer (Su et al. 2004). Moreover, fetal Y-chromosomal cf-DNA has been detected in maternal urine, and sex-mismatched hematopoietic stem cell transplant recipients display donor-derived cf-DNA in their urine (Hung et al. 2009). Hence, screening the urinary cf-DNA may provide a noninvasive diagnostic alternative in certain cases (Umansky and Tomei 2006).

1.2.5. The assessment of cf-DNA

Throughout the history of cf-DNA, the methods that were used to assess its plasma/serum concentration have been diverse, and no universal protocol standardizations have been established (Wagner 2012). The impact of different pre-analytical and analytical practices is indicated by the substantial variation in the reported cf-DNA values for different patient materials and healthy individuals (Gormally et al. 2004, Schwarzenbach et al. 2011, Wagner 2012). Gormally et al (2007) have measured cf-DNA concentrations in healthy individuals whose plasma was collected in 23 different centers in Europe and observed that the greatest variation in cf-DNA levels was between the recruitment centers, demonstrating the impact of heterogeneous sampling procedures on the measurement outcome (Gormally et al. 2007). Nevertheless, emphasis has now been placed on creating uniform cf-DNA monitoring protocols, especially in prenatal diagnostics and in other fields where cf-DNA assessment is anticipated to enter clinical practice (Butt and Swaminathan 2008, Wagner 2012).

Previous cf-DNA quantification methods typically relied on quantitative PCR (qPCR) – a method that requires a preanalytical DNA extraction step. However, each of the commercial DNA-extraction captures and recovers only a specific size range of DNA, typically genomic-sized DNA or low-molecular weight DNA. Such biases may affect the results of the assay, especially in case-control studies in which the cases and the controls may possess differential cf-DNA size profiles. However, commercial kits that employ a fluorescent DNA-intercalating dye were recently adopted for broader use to directly quantify the total cf-DNA level in the plasma/serum. These kits have a very low detection limit (typically 20-25 pg/ml) and are tolerable to contaminants, such as salts and proteins (Gormally et al. 2007). As no prior DNA extraction step is required, such assays are anticipated to quantify all of the accessible cf-DNA ("total cf-DNA"), regardless of its sequence, fragment size or structure.

With respect to the pre-analytical standardization, Sozzi et al. (2005) have reported that when quantified using PCR, 30% of the cf-DNA in the plasma or in extracted samples degrades every year (Sozzi et al. 2005). In contrast, Sjöholm et al. (2005)

have demonstrated that plasma and serum samples that are stored for 10-30 years produce reliable results when the cf-DNA extracted and amplified using PCR (Sjoholm et al. 2005). Pertaining to the use of plasma versus serum as the source material for cf-DNA assessment, Lee et al. (2001) have observed a 20-fold higher cf-DNA level in fresh serum compared to fresh plasma with a PCR-based method, attributing the difference to the DNA that released from leukocytes during blood clotting (Lee et al. 2001). In contrast, Umetani and coworkers (2006) have reported that the elevated level of cf-DNA in serum compared to plasma cannot be essentially ascribed to the leukocyte rupture during serum preparation (Umetani et al. 2006). Likewise, by analyzing Epstein-Barr virus (EBV) -specific sequences in cf-DNA, Jones et al. (2011) have reported that plasma and serum produce equivalent results (Jones et al. 2012). Hence, no consensus exists as to whether plasma or serum provides a more appropriate specimen type.

1.3. cf-DNA in diagnostics and disease

Currently, the fields with the greatest interest in performing cf-DNA assessments in clinical practice are oncology and prenatal diagnostics. However, the list of biomedical areas in which cf-DNA monitoring has been considered to hold value is growing. These areas include transplantation medicine, cardiovascular care, traumatology and monitoring of certain autoimmune and microbial diseases (Tsang and Lo 2007, Wagner 2012). In the majority of the pathologies that fall into the above-mentioned categories, elevated cf-DNA levels have been observed to be a hallmark feature of disease severity (Butt and Swaminathan 2008, Wagner 2012), whereas the qualitative characteristics of cf-DNA, especially the methylation patterns, better serve diagnostic purposes (Schwarzenbach et al. 2011).

1.3.1. Prenatal diagnostics

Fetus-derived cf-DNA, originating from placental trophoblast cells, is detectable in maternal plasma after 4-5 weeks of gestation (Hill et al. 2012). Although the vast majority of the cf-DNA in pregnant women is of maternal origin, it has been

demonstrated that the entire fetal genome is present in the maternal nucleome (Fan et al. 2012). Currently, certain European countries already practice noninvasive fetal sex determination via Y-chromosome sequence assessment from maternal plasma to aid in the diagnosis of sex-linked disorders in women who are at risk of having a child with a severe sex-linked genetic disorder (Hill et al. 2012). A meta-analysis on the test performance of fetal sex determination in maternal cf-DNA has shown a good overall sensitivity (95.4%) and specificity (98.6%) for the testing; however, an adequate sensitivity can be achieved only after 7 gestational weeks (Devaney et al. 2011). Prenatal applications using maternal cf-DNA are expected to expand to the clinical monitoring of chromosomal aneuploidies, paternally inherited single-gene disorders and fetal RhD status (Hill et al. 2012).

1.3.2. Cancer screening

Patients with various types of tumors, including those of breast, ovarian, colonic, lung, prostate, pancreatic and leukemia have been observed to possess increased cf-DNA levels in relation to healthy controls (Jung et al. 2010, Schwarzenbach et al. 2011). However, the data are not entirely unequivocal, and elevated cf-DNA levels have also been detected in patients with benign lesions (Jung et al. 2010, Schwarzenbach et al. 2011). Nevertheless, certain studies have reported that elevated cf-DNA concentrations are typically attributed to metastatic cancers, and others have observed correlations between cf-DNA level and tumor size, stage, location and aggressiveness (Jung et al. 2010). Some of these discrepancies may be related to the fact that, especially in the initial phase of tumor establishment, a large number of non-tumor DNA is released into the circulation, suggesting an interaction between the tumor and adjacent non-tumor cells (Garcia-Olmo et al. 2008). The proportion of tumor-originating cf-DNA has been reported to range from as low as 0.2% to as high as 90% (Gormally et al. 2007) with the highest percentages being observed in situations with low total plasma cf-DNA concentrations (Jahr et al. 2001). As regards the effectiveness of treatment in patients who undergo surgery or chemo- or radiotherapy, cf-DNA monitoring has provided clues regarding the status and/or subsequent progression of the cancer. In most cases, decreased post-treatment cf-DNA values reflect the successfulness of the treatment and predict

remission, whereas persistently high cf-DNA levels indicate a presence of residual cancer cells, unresponsiveness to treatment or a systemic spread of the disease (Jung et al. 2010, Schwarzenbach et al. 2011).

One of the milestones in the history of cf-DNA research was the discovery (Sorenson et al. 1994) and subsequent use of cf-DNA to monitor oncogene and tumor suppressor mutations in tumor cell DNA. Genes with a high mutation frequency – i.e., *KRAS* and *TP53* – in several cancers exhibit a concordance of 0-75% between target tissue DNA and cf-DNA, indicative of nonexistent to moderate diagnostic potential (Jung et al. 2010, Schwarzenbach et al. 2011). An acceptable level of concordance between cancer DNA and cf-DNA mutation status hence appears to be obtained only in more advanced tumors (Jung et al. 2010, Schwarzenbach et al. 2011). With regard to cancer-specific methylation markers, certain "prototypical" genes that display aberrant methylation, e.g., *p16* in lung and breast cancer, and *SEPT9* and *APC* in colon cancer, have shown promise as diagnostic and prognostic tools. However, the maximal diagnostic sensitivity (≥80%) is obtained with the concomitant assessment of multiple methylation markers (Jung et al. 2010, Schwarzenbach et al. 2011). A potential conflicting factor in cf-DNA methylation analysis is that 8-20% of healthy individuals have also been reported to exhibit abnormal methylation markers (Jung et al. 2010). In a study by Hoque et al. (2004) such aberrant cf-DNA methylation patterns were attributed to smoking (Hoque et al. 2004).

Lastly, circulating viral DNA has been a detected in the patient nucleome in cancers that have a viral etiological contribution. Specifically, the occurrence of EBV sequences in cf-DNA has been demonstrated to be associated with nasopharyngeal carcinoma, Burkitt's lymphoma and Hodgkings's disease (Schwarzenbach et al. 2011). Human hepatitis B virus cf-DNA has similarly been related to certain forms of hepatocellular carcinoma, whereas human papilloma virus cf-DNA has been ascribed to cervical, head and neck and hepatocellular cancers (Schwarzenbach et al. 2011). The treatment response of nasopharyngeal carcinoma is now also being monitored though blood EBV DNA assessment in certain Asian countries where the occurrence of this cancer is high (Leung et al. 2006).

1.3.3. Autoimmune diseases

The list of autoimmune diseases that have been reported to be associated with elevated cf-DNA levels or qualitative alterations in cf-DNA is extensive. The list includes SLE, rheumatoid arthritis, type I diabetes, Sjögren's syndrome, multiple sclerosis and hepatic autoimmune diseases (Holdenrieder et al. 2006, Levenson and Melnikov 2012, Wagner 2012). The most exhaustively studied autoimmune disease in relation to cf-DNA is SLE, in which certain diseases manifestations are causally related to cf-DNA or nucleosome ICs (Deligezer et al. 2006, Frese and Diamond 2011). Nevertheless, conflicting results have been obtained regarding the association between circulating nucleosome levels and the activity and extent of SLE. In a study by Williams et al. (2001), elevated nucleosome levels were associated with higher disease activity (Williams et al. 2001), whereas another study by Amoura et al. (1997) did not observe such an association (Amoura et al. 1997).

1.3.4. Acute cardiovascular conditions

Markedly elevated cf-DNA levels have been unequivocally observed in patients with various acute conditions, such as myocardial infarction (MI), stroke, burn injuries and blunt trauma (Butt and Swaminathan 2008, Wagner 2012). In the two first-mentioned conditions, plasma nucleosomes or cf-DNA levels have been demonstrated to correlate with certain traditional risk markers and to serve as useful predictors of post-attack mortality and/or other complications, although negative findings in this regard also exist (Butt and Swaminathan 2008, Wagner 2012). The usefulness of cf-DNA measurement in these conditions allegedly is due to its release from the affected tissues: the myocardium and central nervous system. In MI, prolonged ischemia has been proposed to be the cause of the elevated cf-DNA levels (Chang et al. 2003). Increased levels of extracellular DNA and histones, and a correlation between disease activity and the plasma level of cf-DNA, have also been reported in thrombotic complications (Fuchs et al. 2012b). Supporting its sensitivity in outcome prediction, plasma cf-DNA has been demonstrated to predict post-stroke mortality and morbidity in patients with a negative neuroimaging result (Lam et al. 2006).

1.3.5. Infectious diseases

Increased levels of cf-DNA have been detected both in cases of viral and bacterial infections. Septic bacterial infections that characteristically involve massive cell death and inflammation have been reported to be accompanied by extremely high cf-DNA levels with the cf-DNA concentration serving as an independent predictor of mortality from the disease (Rhodes et al. 2006, Huttunen et al. 2011). Massive apoptosis and necrosis of the host cells are presumed to account for the peak in cf-DNA levels, as indicated by the apoptotic size appearance of cf-DNA in the patients' plasma (Huttunen et al. 2011). However, the contribution of bacterial lysis to the increase in cf-DNA is unknown. Viral infections that have been studied in relation to alterations in cf-DNA concentration are currently limited to those of dengue virus and nephropathia epidemica caused by Puumala hantavirus. In both of these infections, cf-DNA levels were observed to be elevated compared to healthy controls or baseline values, respectively (Ha et al. 2011, Outinen et al. 2012) . In the dengue patients, cf-DNA levels correlated with several parameters that reflect infection severity (Ha et al. 2011), whereas in Puumala hantavirus patients, cf-DNA levels associated with blood platelet and leukocyte counts and with the length of hospital stay but not with renal function or chest radiograph findings (Outinen et al. 2012).

1.4. Cell-free RNA

Although the presence of cell-free RNA (cf-RNA) was demonstrated concomitantly with cf-DNA in the pioneering study by Mandel and Metais in 1948 (Mandel and Metais 1948), the biology of cf-RNA has remained perhaps even more elusive than that of cf-DNA. A peculiar feature of circulating cf-RNA compared to its cellular counterpart is its remarkable stability (Tsui et al. 2002). Its protection from RNase-mediated degradation has been attributed to its presence in circulation as a particle-associated form (Ng et al. 2002). Cancer research and prenatal diagnostics have been among the fields to most intensively use cf-RNA in diagnostic purposes. Analogously to cf-DNA, cf-RNA monitoring may likewise have diagnostic potential in the aforementioned conditions (Tong and Lo 2006, Tsang and Lo 2007).

Furthermore, a broad range of distinct micro RNAs (miRNAs) have been detected in plasma, and specific circulatory miRNA signatures have been assigned to several cancers (Qu et al. 2011) and cardiovascular disease (CVD) (Creemers et al. 2012).

Studies of cf-RNA have largely focused on quantifying distinct messenger RNA (mRNA) species rather than the total cf-RNA concentration in plasma; hence, data regarding the amount of circulatory RNA in relation to cf-DNA is limited. Tamkovich et al. (2005) have reported that following extraction, the respective mean concentrations of plasma cf-DNA and cf-RNA are 15 ng/ml and 36 ng/ml in women and 16 ng/ml and 126 ng/ml in men (Tamkovich et al. 2005). However, these authors also observed that significant quantities of circulating RNA and DNA were bound to erythrocyte and leukocyte surfaces; when these fractions were counted in the total levels of circulating DNA and RNA in blood, the corresponding values were 430 ng/ml and 100 ng/ml for women, and 1030 ng/ml and 770 ng/ml for men (Tamkovich et al. 2005). Moreover, an undetermined portion of cf-RNA (both mRNA and miRNA) is enclosed in vesicular structures, termed exosomes (Peters and Pretorius 2011). The exosomes have been advocated as the means of paracrine communication between cells in which the enclosed mRNA can be translated in the recipient cells, whereas the enclosed miRNAs can target and regulate mRNA processing (Valadi et al. 2007). This phenomenon has been identified for many cell types, including endothelial, immune and cancer cells (Valadi et al. 2007). However, it is unclear how different RNA extraction protocols capture and preserve the distinct cf-RNA species.

2. The biological effects of cf-DNA

The functions of circulating DNA can be broadly divided into the following categories: i) immunological sensing and subsequent signaling, ii) robust phenotypical outcomes (e.g., genometastasis) following the cf-DNA uptake into cells, and iii) intravascular consequences resulting from cf-DNA that is released from NETs. Although it was reported in 1963 that nucleic acids are capable of inducing interferon (IFN) production in chicken fibroblasts (Jensen et al. 1963), it

was long held that only microbial DNA or unmethylated CpG oligodeoxynucleotides (ODNs, which are used as immune-boosting vaccine adjuvants) were able to generate a DNA-driven immunoinflammatory response (Krieg 2002). However, it was later demonstrated that dsDNA without the CpG motifs is also a potent immunostimulant, and nowadays it is widely established that endogenous DNA that escapes from apoptotic degradation can serve as a danger- (or damage)-associated molecular pattern (DAMP) (Pisetsky 2012). The different types of DNA-induced immunomodulatory functions are essentially dictated by the DNA-embedded sequence motifs. Whereas both CpG and AT-rich sequences can serve as immune activators (Frese and Diamond 2011, Keating et al. 2011), G tetrads or telomere-like motifs have been demonstrated to act as suppressors (Gursel et al. 2003). Interestingly, DNA-driven immunostimulation is not restricted to immune cells; non-immune cells can mount immunoinflammatory responses upon encountering cytoplasmic DNA (Kawashima et al. 2011). Moreover, non-immune cells can and become antigen-presenting cells with the subsequent induction of autoimmunity via the actions of bystander immune cells (Suzuki et al. 1999).

However, the sequence-based functions of extracellular DNA can be masked by several molecules that associate with it. The factors that are known to bind to and convert extracellular DNA to an even more potent immune activator/DAMP include cathelicidin antimicrobial peptide (CAMP; also referred to as LL37), high motility group box (HMGB) proteins and anti-DNA antibodies (Pisetsky 2012). The HMGB proteins have been proposed to serve as universal nucleic acid sentinels, of which the best-studied family member, the HMGB1, is known for its dysregulation in many pathologies, including sepsis, SLE and rheumatoid arthritis (Harris et al. 2012). In normal conditions, HMGB1 functions as a nuclear transcription factor and a nucleosome stabilizer, whereas upon necrosis, inflammation or infection, HMGB1 is released from immune cells to the serum, where it can bind to DNA and propagate DNA-associated immune responses (Harris et al. 2012, Pisetsky 2012). The antimicrobial peptide LL37 has been discovered to convert otherwise inert self DNA into an inducer of type I IFN (i.e., IFN-α and IFN-β) production in plasmacytoid dendritic cells (pDCs) in psoriasis (Lande et al. 2007) and atherosclerosis (Doring et al. 2012). LL37 has also been implicated in SLE (Sun et al. 2011). Lastly, certain subgroups of anti-DNA antibodies have pathogenic effects.

Whereas most healthy individuals possess natural, low-affinity IgM antibodies to single-stranded DNA (ssDNA), patients with SLE often have high affinity IgG class antibodies that can bind ssDNA, dsDNA and nucleosomes (Hahn 1998). The pathogenicity of high affinity DNA antibodies is likely increased if they acquire the capability of complement fixation or cross-reactivity with other antigens (Hahn 1998).

Among DNA binding proteins, two plasma proteins have been identified that have a beneficial impact on circulating DNA metabolism and disposal. CRP and SAP possess the capacity to bind to chromatin that is released from damaged cells and facilitate its removal to prevent autoimmunity (Robey et al. 1984, Bickerstaff et al. 1999). Indeed, SAP-deficient mice have been shown to develop glomerulonephritis with high levels of antibodies to nuclear material, such as chromatin, naked DNA and histones (Bickerstaff et al. 1999). Somewhat puzzlingly, the SAP-deficient mice displayed accelerated chromatin degradation, for which the authors concluded that SAP is required to stabilize and solubilize extracellular chromatin, and perhaps to facilitate its transfer to the liver for non-immunological processing (Bickerstaff et al. 1999). In keeping with this hypothesis, it has been theorized that the physiological role of SAP is to function as an extracellular chromatin scavenger, whereas CRP may take over this role during the acute phase response in which the CRP level can exceed that of SAP by 2- to 10-fold (Hicks et al. 1992).

2.1. DNA-sensing molecules and pathways

The immunological effects of various types of endogenous DNA has been discovered to overlap with those of immunostimulatory ODNs (Keating et al. 2011). In view of these findings, it has been suggested that once DNA gains access to the cytoplasm, DNA sensors are unable to discriminate between self- and non-self DNA (Barbalat et al. 2011). Thins notion is supported by the finding that mice lacking lysosomal DNase II die prenatally due to anemia that is caused by accumulation of undigested, erythroid precursor-derived DNA in the macrophage cytoplasm (Kawane et al. 2001). Subsequent activation of IFN-β-induced gene expression has been shown to account for the lethality of these mice (Yoshida et al. 2005b).

However, even if the DNase-deficient mice are rescued from lethality by type I IFN receptor deficiency, they later develop chronic polyarthritis and display increased production of IL-6, IL-1β, IFN-β and TNF-α (Kawane et al. 2006, Nagata and Kawane 2011).

A ubiquitous receptor for endocytosed DNA is endosomal toll-like receptor 9 (TLR9), expressed in pDCs, B cells and macrophages (Barbalat et al. 2011). The TLRs belong the repertoire of pattern recognition receptors (PPRs) that sense conserved microbial moieties, including nucleic acids (Barbalat et al. 2011). Although unmethylated CpG DNA is the classical, strong activator of TLR9, various forms of endogenous DNA have been demonstrated to engage it as well (Barbalat et al. 2011). For example, self-DNA complexed with LL37 and in the form of DNA-ICs triggers TLR9-dependent immune activation (Marshak-Rothstein et al. 2004, Lande et al. 2007). Moreover, DNA-ICs can be bound and internalized via the B cell receptor and FcγRIIa on DCs, with subsequent engagement of TLR9-dependent or -independent pathways (Leadbetter et al. 2002, Boule et al. 2004). Intracellular signal transduction of the DNA-induced TLR9-pathway is mediated through the adaptor protein myeloid differentiation factor 88 and a series activations events, resulting in an IFN-regulatory factor 7 (IRF7)-mediated type I IFN response, or in nuclear factor kappa B (NF-κB) or mitogen-activated protein kinase -dependent cytokine and chemokine production (Barbalat et al. 2011).

TLR9-independent, cytoplasmic DNA sensing has been demonstrated for AT-rich B-DNA (the normal right-handed form of dsDNA) in both immune and non-immune cells. The process involves DNA-sensing by the DNA-dependent activator of IRF and the subsequent signaling of the stimulator of interferon genes (STING) and TANK-binding kinase 1 (TBK1), as well as the activation of the transcription factors IRF3, IRF7 and NF-κB (Barbalat et al. 2011, Keating et al. 2011). Another group demonstrated that this pathway can also be triggered by RNA polymerase III after it has bound and converted AT-rich DNA to into an RNA intermediate that is capable of activating a PRR referred to as retinoic acid-induced gene I (Bauernfeind et al. 2010). Several cytoplasmic sensors for other types of ds DNA, such as viral genomes have likewise been identified as inducers of the IRF3 or NF-κB -mediated

signaling (Desmet and Ishii 2012). For example, an interferon-inducible IFI16 protein has been demonstrated to signal through STING, TBK1 and IRF3 leading to type I interferon production (Unterholzner et al. 2010). Lastly, a PRR with the capacity to sense cytoplasmic dsDNA independently of TLR is the absent in melanoma 2-inflammasome (AIM2). In contrast to the other pathways, AIM2 can activate caspase 1 and the subsequent IL-1β production and pyroptosis – an inflammatory form of cell death that involves cellular lysis (Frese and Diamond 2011, Keating et al. 2011). However, the existence of as yet uncharacterized DNA-sensing molecules and pathways is likely.

2.2. Genometastasis

In keeping with the data regarding the internalization of extracellular nucleic acids by various cell types, cancer-derived cf-DNA that contains dominant oncogenes has been demonstrated to transform recipient cells in a process termed genometastasis (Garcia-Olmo and Garcia-Olmo 2001). Such horizontal transfer of oncogenicity has been demonstrated in an experiment in which cell-depleted plasma that contained mutated *K-ras* sequences (obtained from metastasis-free patients with colorectal cancer) was able to transform cultured murine NIH-3T3 cells (Garcia-Olmo et al. 2010). Immunodeficient mice that were injected with these cells developed carcinomas (Garcia-Olmo et al. 2010). A subsequent study that was performed 2 years later with the same colorectal cancer patients, revealed that following the surgical removal of the primary tumor, two of the three patients whose plasma induced the NIH-3T3 cells transformation had developed metastases (Garcia-Olmo et al. 2012). In another similar experiment, the NIH-3T3 cells that were transformed with human colorectal cancer patient plasma, were observed to give rise to murine tumors that phenotypically resembled those of the original cancer patients (Serrano-Heras et al. 2012). The authors concluded that the NIH-3T3 fibroblasts were converted into malignant epithelial-like cells during human cancer plasma-induced transformation, suggesting a mesenchymal to epithelial transition (Serrano-Heras et al. 2012).

The uptake of apoptotic bodies derived from malignant cells has also been

demonstrated to give rise to a horizontal oncogene transfer. Using endothelial cells and fibroblasts as recipient cells, it has been demonstrated that chromosome fragments and even whole chromosomes can be transferred in this manner, suggesting that tumor propagation-related aneuploidy could take occur via lateral gene transfer (Holmgren et al. 2002). In addition, this group reported i) that internalized DNA will integrate and propagate only if the p53/p21 pathway is deficient in the target cells and ii) that DNase II-mediated fragmentation of the incoming DNA and the activation of checkpoint kinase 2 is crucial in preventing replication of the incoming DNA (Bergsmedh et al. 2002, Bergsmedh et al. 2006). Hence, these authors suggested that such a mechanism could trigger the DNA-damage response in healthy cells and inhibit propagation of the possibly harmful DNA. The same group also reported that apoptotic bodies that contained EBV and human immunodeficiency virus 1 DNA can likewise be internalized and expressed in cell types that are normally resistant to these viruses (Holmgren et al. 1999, Bergsmedh et al. 2002).

2.3. The intravascular effects of NETs

NETs, which are composed of intact DNA fibers complexed with histones and antimicrobial peptides (Wartha et al. 2007), have also been implicated in sterile inflammatory conditions, such as SLE (Bouts et al. 2012, Leffler et al. 2012) and small-vessel vasculitis (Kessenbrock et al. 2009). The mesh-like structure of released NETs is ideal for trapping microbes but has also been shown to acquire prothrombic and procoagulant properties *in vitro*, a finding that was demonstrated by their ability to facilitate platelet and red blood cell adherence (Fuchs et al. 2010, Fuchs et al. 2012a). Indeed, extracellular chromatin has been identified as a structural component of experimental venous thrombi in animals, and treatment with DNase prevents thrombus formation (Brill et al. 2012). NET formation has also been observed in cocultures of activated endothelial cells and neutrophils, and the released NETs can induce endothelial cell death (Gupta et al. 2010). Lastly, it has been proposed that NETs could serve as the inflammatory second hit in thrombotic microangiopathies and in patients with multiple trauma (Margraf et al. 2008). In

conclusion, cf-DNA in the form of NETs, in co-operation of concurrently released proteins, may acquire a pathogenic role in certain thrombotic vasculopathies.

3. Aging of the immune system

Age-accompanied changes in immunoinflammatory functions – collectively termed immunosenescence – entail both innate and adaptive arms of the immune system (Agarwal and Busse 2009). However, rather than undergoing explicit impairment, the aging immune system is considered to undergo remodeling and deregulation, in which certain functions are decreased, some are increased and others remain essentially unchanged (Agarwal and Busse 2009, Shaw et al. 2010). A central characteristic of immunosenescence is that co-operation between the different immunoinflammatory players is debilitated (Agarwal and Busse 2009). Beyond the immune system itself, declined endocrine output in other homeostatic systems can contribute to immunosenescence. For example, decreased production of sex steroids, adrenal hormones and somatotropic hormones (growth hormone and insulin-like growth factor 1), all of which have essential immunomodulatory functions, account for certain immune aging deficits, in particular weakened anti-inflammatory functions (Arlt and Hewison 2004).

Phenotypically, immunosenescence manifests as increased self-reactivity, systemic low-grade inflammation, increased susceptibility to infections and cancer and defective vaccine responses (Agarwal and Busse 2009, McElhaney and Effros 2009). The adaptive immune branch is often argued to be more profoundly affected by aging, yet notable changes are observed also in innate immune functions (Agarwal and Busse 2009, Derhovanessian et al. 2009). However, it is not entirely clear which of the changes are causally related and which are non-causally associated. Population-based studies have identified chronic antigenic stress, including the contribution of cytomegalovirus (CMV), as a driver of immunosenescence (Koch et al. 2007, Derhovanessian et al. 2009). At the individual level, however, the situation is more complex. That is, several genetic, stochastic and environmental factors are known to shape immune aging, which in turn have

consequences on late-life health and remaining life time, i.e., longevity (Montesanto et al. 2012).

To assess changes that are truly inherent to aging and distinct from underlying pathology or pharmacological interference due to medication, a stringent set of enrollment criteria that are termed the SENIEUR protocol has been established. Briefly, the SENIEUR protocol excludes individuals with abnormal/deviating values in certain hematological, metabolic, urinary and liver enzyme parameters, individuals with malignancies or any condition that affects the immune system, and individuals who use any prescription drug(s) for a defined disease (Ligthart et al. 1984). The use of the SENIEUR protocol has been criticized for that it excludes ≥70% of community-dwelling subjects who are older than 65 years of age and as many as 90% of elderly nursing home residents, creating a potential bias by including only exceptionally healthy subjects (Wick and Grubeck-Loebenstein 1997, Bruunsgaard 2006). Nevertheless, certain studies using the SENIEUR criteria have not reported the typical hallmarks of immunosenescence in the elderly (Ahluwalia et al. 2001, Wu et al. 2012).

3.1. Aging-related changes in immune cells and inflammatory mediators

With advancing age, the output of bone marrow and the number of hematopoietic stem cells decrease along with a predominance of myeloid commitment and a relative reduction in lymphoid progenitors (Shaw et al. 2010). Whereas the numbers of innate immune cells of myeloid origin, i.e., monocytes/macrophages, neutrophils, natural killer (NK) cells, NK T (NKT) cells and DCs, are known to increase or remain unchanged with aging, notable functional changes are observed among these cell types (Agarwal and Busse 2009, Shaw et al. 2010). For example, the chemotaxis and phagocytic capability of neutrophils and monocytes/macrophages is reduced, the production of chemokines and IFN-γ in NK and NKT cells is decreased (Agarwal and Busse 2009, Shaw et al. 2010) and the reactivity of DCs to self-DNA is increased (Agrawal et al. 2009). Adaptive immune cells, T and B lymphocytes, likewise undergo profound alterations with aging, most notably a loss of the CD28 costimulatory molecule on CD8+ and CD4+ lymphocytes (Derhovanessian et al.

2009). Moreover, together with a reduced replenishment capacity of naïve T lymphocytes, thymic atrophy has been suggested to underlie age-accompanied immunoincompetence (Agarwal and Busse 2009). Collectively, these alterations bring about the cell-related manifestations of immunosenescence.

Findings regarding the proinflammatory (occasionally also referred to as hyperinflammatory) status in elderly individuals demonstrate a clear pattern towards elevated baseline plasma CRP, IL-6, and TNF-α levels (Krabbe et al. 2004, Bruunsgaard 2006). However, it appears that inflammaging and the cellular capability to produce cytokines and to mount an effective inflammatory response are unrelated, a finding that was demonstrated by Wijsman et al. (2011). These authors also observed that a high, unopposed *ex vivo* proinflammatory response in the very elderly is beneficial in terms of survival (Wijsman et al. 2011). Nevertheless, the findings on cytokine production of the immune cells of aged individuals upon immunostimulation with lipopolysaccharide (LPS) or phorbol ester compounds have been very heterogeneous; decreased, unaltered or increased production of IL-6, TNF-α and IL-1β in comparison to the immune cells of young individuals have been reported (Krabbe et al. 2004, Bruunsgaard 2006). Certain discrepancies can be explained by the use of different cell types (e.g., whole blood vs. mononuclear cells or monocytes) or different measurement times in these studies. In fact, it has been demonstrated that while the peak values of cytokine production do not differ between the elderly and the young, release kinetics do. Upon stimulation, peak levels are obtained earlier in aged cell cultures, and there may also be a prolonged cytokine production by aged cells compared to the young (Bruunsgaard 2006). A similar phenomenon has also been observed in a human sepsis model (Krabbe et al. 2001) and further supported by the observation of a lengthened cytokine production time in elderly patients with pneumococcal infections (Bruunsgaard et al. 1999).

3.2. Immunosenescence, frailty and longevity

Age-related impairments in immune functions are closely related to functional capabilities, both physical and cognitive, and longevity. Inflammation has even been proposed to be one of the driving forces of frailty (Roubenoff 2003, Hubbard and

Woodhouse 2010), which is defined as a state of increased vulnerability and shortness of physiological reserves (Flicker 2008). Frailty also identifies older individuals who are at increased risk of mortality (Fried et al. 2001); however, the involvement of inflammatory mediators to this association is multifaceted. An inflammatory contribution has been proposed to occur either via i) direct catabolic effects of TNF-α and IL-6 on skeletal muscle, which manifests as sarcopenia (Roubenoff 2003, Schaap et al. 2009), ii) the deregulation of the hypothalamic-pituitary-adrenal axis and the induction of sickness behavior by IL-1β, IL-6 and TNF-α (Sparkman and Johnson 2008) or iii) the aggravation of existing pathologies, such as atherosclerosis and diabetes by continuous inflammatory stimuli (Bruunsgaard 2006). Regardless of the mode of action, the general consensus is that cross-talk between immune and other bodily systems plays a central role in aging and age-related phenomena, although the detailed mechanisms are currently unclear (Arlt and Hewison 2004). Nevertheless, inflammaging may also represent, or be an attempt, of a compensatory response, especially as regards IL-6, which also has an anti-inflammatory role in limiting and resolving inflammation (Maggio et al. 2006). Despite the firm link between systemic inflammation and aging, it is possible that inflammaging is merely an epiphenomenon that indicates underlying pathophysiology and not an active player in the outcomes. In several large epidemiological studies, however, plasma levels of IL-6, CRP and TNF-α have been shown to be independent predictors of frailty and functional capabilities (Barbieri et al. 2003, Cappola et al. 2003, Cesari et al. 2004, Barzilay et al. 2007).

3.3. Genetics of immunosenescence

The impact of genetic variation on immunosenescence is recognized, although its extent is uncertain (Franceschi et al. 2007). The assumption that genetic variation in the genes that code for inflammatory mediators participates in innate immunity senescence is based on the findings that baseline levels of certain cytokines are at least in partially genetically determined (Naumova et al. 2011). Thus, it has been theorized that genetics could differentially predispose individuals to inflammaging and therefore affect an individual's ability to achieve old ages (Naumova et al. 2011). The best-studied genetic variants in this regard are the single nucleotide

polymorphisms (SNPs) in the promoter regions of *IL6* (-174G/C) and *TNFA* (-308G/A) genes. In certain studies, the SNPs that result in higher production of these cytokines (*IL6* -174G and *TNFA* -308A, respectively) have been demonstrated to be associated with adverse aging phenotypes and susceptibility to age-related diseases, such as CVD, as well as to be less frequent among long-living individuals (Bruunsgaard 2006, Naumova et al. 2011). Similarly, a high-producing variant of the anti-inflammatory gene *IL10* (-1082GG homozygosity or -1082G/-819C/-592C haplotype) has been attributed to healthy aging and longevity (Naumova et al. 2011) and to be less frequent in individuals with age-related comorbidities, such as Alzheimer's disease (Di Bona et al. 2012). Generally, however, the findings have been population or sex-specific and hence do not support a robust association between cytokine gene polymorphisms and immunosenescence *per se*.

With regard to adaptive immunity senescence, Derhovanessian et al. (2010) observed that individuals who belong to families with long-lived and thus who are likely to be genetically predisposed to longevity, display different immune signatures in relation to CMV-associated parameters than their partners who represent the general population. Specifically, CMV seropositive individuals belonging to the long-lived families were observed to be devoid of the age/CMV-associated reduction in the number of naïve T cells and enrichment of late-differentiated effector memory T cells, i.e., the hallmark features of immunosenescence that were observed in the control individuals. Moreover, the control individuals exhibit a higher proinflammatory status as measured by plasma CRP levels compared to the individuals from families with long-lived members. Although this study did not assess genetics as such, it provides a certain degree of evidence for a genetic component of immunosenescence, specifically with regard to the immunological control of CMV (Derhovanessian et al. 2010).

4. Biomarkers of aging and longevity

The proportion of elderly individuals in the population is continuously increasing. It has been estimated that by 2050, individuals aged 65 years or over will constitute

between 25 and 33% the population in several developed courtiers and in certain developing countries (World Population Aging, 2009, United Nations, available at: http://www.un.org/esa/population/publications/WPA2009/WPA2009_WorkingPaper.pdf). Increased age is a major risk factor for many diseases and disabilities; despite the tremendous increase in life expectancy during the past decades, it is uncertain whether the health span, which is defined as the lifetime spent without major comorbidities or functional deficits, has increased or will increase with the life span (Sierra et al. 2009). The objective to identify biomarkers of aging arises from the observations that individuals with the same chronological age may possess dissimilar biomarker signatures that reflect their biological age (Simm et al. 2008). The pace of biological aging can be defined as changes in functional capacity and stress resistance and susceptibility to age-related diseases or disabilities (Simm et al. 2008, Davinelli et al. 2012). Identifying a set of aging biomarkers would assist in understanding the mechanisms of aging and ultimately aid in the development of strategies to counteract age-accompanied deficits and to prevent or postpone the onset of accompanying morbidities (Johnson 2006, Simm et al. 2008). Lastly, biomarker assessment in the elderly would also allow for the identification of the key determinants of longevity.

4.1. The definition of an aging biomarker

Detailed criteria for an aging biomarker have been formulated by The American Federation for Aging Research (Johnson 2006). The criteria state that an aging biomarker must have the following qualities:

1. Predict the rate of aging. That is, determine precisely where an individual is in their total life span. The marker must be a better predictor of life span than chronological age.
2. Monitor a basic process that underlies the aging process, not the effects of disease.
3. Be capable of being tested repeatedly without harming the individual. For example, a blood test or an imaging technique.
4. Can function as a marker in both humans and laboratory animals, such as mice. This is so that it can be tested in lab animals before being validated in humans.

Additionally, once a set of aging biomarkers has been identified, the markers should ideally adhere to the major theories of aging. The theories of aging can be broadly grouped to two categories: i) aging as a programmed series of developmental periods that ultimately result in senescence and ii) aging as a consequence of damage and random errors in vital processes that are primarily caused by external factors (Simm et al. 2008). Despite enormous efforts, no such aging biomarker has been able to fulfill all of these criteria. However, many marker candidates are now under investigation, and several potential candidates have been presented (Simm et al. 2008, Davinelli et al. 2012).

4.2. Aging biomarker candidates

The term "aging biomarker" has been used quite liberally for many types of markers, including biological variables (measurable from cellular samples or biological fluids) (Simm et al. 2008, Davinelli et al. 2012), genetic (Naumova et al. 2011) and epigenetic (Bell et al. 2012) markers, and indices of functional performance, physiology and disease (Crimmins et al. 2008). The most intensively studied aging biomarkers fall into the categories that indicate oxidative stress, inflammation, hormonal changes, glycation products and cellular (replicative) senescence (Simm et al. 2008). Certain studies have analyzed changes in a given biomarker quantity in relation to age (Table 1), whereas others have assessed biomarkers in relation to a distinct age-associated parameter, such as a functional capability or an indicator of healthy aging or disease (Table 2). Generally, many of the markers fluctuate with underlying pathologies and thus cannot be considered to be true aging biomarkers. However, certain markers that are directly related to CVD and diabetes have been reported to be good predictors of healthy aging (Crimmins et al. 2008). Lastly, it is not entirely clear if and to what extent the marker candidates are age-specific; certain markers that indicate the aging rate or healthy aging in septuagenarians may not perform equally well in nonagenarians.

Table 1. Examples of aging biomarker candidates

Marker category	Assessed marker	Change with age	Reference
Inflammation	CRP	Increase	(Krabbe et al. 2004, Bruunsgaard 2006)
	IL-6	Increase	
	TNF-α	Increase	
	SAA	Increase	
Oxidative stress	8-Ohdg	Increase	(Levine 2002, Simm et al. 2008)
	oxLDL-cholesterol	Increase	
	Protein carbonyls	Increase	
Hormones	GH and IGF-1	Decrease	(Arlt and Hewison 2004, Sherlock and Toogood 2007)
	Testosterone and estrogen	Decrease	
	DHEA(S)	Decrease	
	Cortisol	Increase/ no change	
	Melatonin	Decrease	
Replicative senescence	Telomere length	Decrease	(Kim and Sharpless 2006, Simm et al. 2008)
	$p16^{INK4a}$	Increase	
Glycation products	HbA1c	Increase/ no change	(Wiener and Roberts 1999, Ramasamy et al. 2005, Simm et al. 2008)
	Pentosidine	Increase	
	CML	Increase	

Abbreviations: CRP, C-reactive protein; IL-6, interleukin 6; SAA, serum amyloid A; TNF-α, tumor necrosis factor alpha, 8-OHdg, 8-hydroxy-2´deoxyguanosine; oxLDL, oxidated low-density lipoprotein; GH, growth hormone; IGF-1, insulin-like growth factor 1; DHEA(S), dehydroepiandrosterone(sulphate); $p16^{INK4a}$, Cyclin-dependent kinase inhibitor 2A; HbA1c, glycated hemoglobin; CML, carboxymethyllysine

Table 2. Examples of biomarkers of age-related phenotypes and diseases

Biomarker*	Association with the outcome	Reference
CRP	Direct association with physical and cognitive decline, frailty, mortality and CVD	(Crimmins et al. 2008, Hubbard and Woodhouse 2010)
IL-6	Direct association with functional and cognitive disability, sarcopenia, mortality, CVD and AD	(Roubenoff et al. 2003, Cesari et al. 2004, Crimmins et al. 2008)
SAA	Direct association with CVD, mild cognitive impairment and cancer	(Crimmins et al. 2008, Trollor et al. 2010)
TNF-α	Direct association with mortality, sarcopenia, mild cognitive impairment, AD, diabetes and stroke	(Roubenoff et al. 2003, Crimmins et al. 2008, Trollor et al. 2010)
Fasting glucose	Direct association with cognitive decline, mortality, diabetes and CVD	(Crimmins et al. 2008)
HbA1c	Direct association with cognitive impairment and diabetes-associated complications	(Crimmins et al. 2008)
DHEA(S)	Direct association with mortality, mental and physical performance, inverse association with CVD	(Crimmins et al. 2008)
Cortisol	Direct association with mortality, CVD and cognitive and physical decline	(Crimmins et al. 2008)
IGF-1	Inverse association with CVD and mortality, direct association with cancer and good cognitive function	(Roubenoff et al. 2003, Landi et al. 2007, Crimmins et al. 2008)

*Biomarkers measured in blood or other body fluid
Abbreviations: CRP, C-reactive protein; IL-6, interleukin 6; SAA, serum amyloid A; TNF-α, tumor necrosis factor alpha; HbA1c, glycated hemoglobin DHEA(S), dehydroepiandrosterone(sulphate); ROS, reactive oxygen species; GH, growth hormone; IGF-1, insulin-like growth factor; CVD, cardiovascular disease; AD, Alzheimer's disease

Some of the aforementioned biomarker candidates have also been proposed to causally act upon aging phenotypes and longevity. The mechanisms that are responsible for these "anti-" or "pro-aging" effects are, however, incompletely understood, and the markers may share common target pathways. For example,

oxidative stress in the form of cellular respiration-induced ROS formation is known to damage DNA, proteins and membranes. The formation of advanced glycation end products in a non-enzymatic reaction between sugars and the free amino groups in DNA, lipids and proteins in turn induces cellular ROS production and the activation of the NF-κB-mediated inflammatory cascade. Low-grade inflammation in the elderly can also be triggered by the decreased production of sex steroids and aberrant metabolic regulation, such as insulin resistance and adipose tissue dysfunction, with the latter two also being induced by inflammatory stimuli. Lastly, a major determinant for cellular senescence, telomere shortening, occurs in each cell division as well as in the course of ROS-induced chromatin damage. Inflammatory conditions can also aggravate and be aggravated by telomere shortening and the subsequent chromatin instability.

Human life span is also known to be partially under genetic control. Estimates based on twin studies indicate that the total heritability of life span is approximately 25%; the impact of genetic variation is modest before the age of 60 years, after it becomes increasingly important in determining the remaining life span (Christensen et al. 2006). Although it has been estimated that approximately 7,000 genes could theoretically participate in human life span regulation (Martin 1997), the number of identified "true" longevity genes is quite small. Only a small number of genetic variants have consistently, across population and sex boundaries, been associated with longevity. The most robust allelic associations with longevity have been observed in the *FOXO3A* and *AKT1* genes that belong to the metabolic pathway responding to insulin and insulin-like growth factor 1 stimuli (Chung et al. 2010). Allelic variants in the gene *APOE* gene have also been shown to associate with longevity in several distinct cohorts (Christensen et al. 2006, Chung et al. 2010). However, due to the association of the *APOE* variants with plasma cholesterol levels, CVD and Alzheimer's disease, these variants likely exert their effect on longevity through predisposition/protection from age-related diseases (Christensen et al. 2006). Nevertheless, genetic studies into longevity propose that nutrient metabolism pathways likewise play part in determining the human life span.

Aims of the Study

The present study was undertaken with the following aims:

1. Elucidate the potential of plasma cf-DNA as a biomarker of aging and old age mortality
2. Investigate the distribution of different cf-DNA species in very elderly and young individuals and to examine the relationship between the abundance of cf-DNA and age-associated changes in the immune system and functional capabilities
3. Characterize the role of the different cf-DNA species as immunomodulatory agents
4. Study the involvement of genetics in the regulation of circulating total cf-DNA levels

Subjects and methods

1. Subjects

1.1. Studies I and III

The study cohorts consisted of home-dwelling and institutionalized subjects participating to the ongoing Vitality 90+ study, which is a prospective population-based study that involves nonagenarians who live in the city of Tampere, Finland. Subjects in Study I (n=258, 196 women and 62 men) were nonagenarians who were born in 1909 and 1910 and who were living in Tampere in the year 2000. Those in Study III (n=144, 101 women and 43 men) were individuals who were born in 1920 and who were living in the city of Tampere in 2010. The examination of the participants included interviews, physical examination and blood-drawing by a home-visiting nurse or a trained medical student. For Study I, the subjects' medical diagnoses were obtained from health center records and classified according to the International Classification of Diseases, 10th revision (ICD-10). Mortality data for this cohort (all-cause deaths during the 4-year follow-up) were collected from the National Population register Center. The young control individuals in Study III (n=30, 21 women and 9 men) consisted of healthy laboratory personnel without medically diagnosed diseases.

1.2. Study II

The subjects in Study II were participants in the ongoing Cardiovascular Risk in Young Finns Study, which is a multi-center follow-up study being executed by five university hospitals in Finland. This study was initiated in 1980 when the participants (n=3596; aged between 3 and 18 years) were randomly selected from the national population register of the study centers (Helsinki, Turku, Tampere,

Kuopio and Oulu and their rural surroundings). The cohort in the current study (n=1841; 1018 women and 823 men) was drawn from the follow-up that was executed in 2001, when the participants were between 24 and 39 years of age.

2. Methods

2.1. The assessment of cf-DNA

2.1.1. The quantification of cf-DNA

Total cf-DNA concentrations were measured directly in plasma/serum with the fluorescence-based Quant-iT™ high-sensitivity DNA assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA). The amount of unmethylated cf-DNA in plasma was determined using a Methylation EIA kit (Cayman Chemicals, Ann Arbor, MI, USA) that measures the amount of 5-methyl-2-deoxy cytidine in DNA. This result was then subtracted from the amount of total cf-DNA. For the quantification of the circulating *RNase P* gene coding cf-DNA (a representative of single-copy gene-coding DNA), Alu repeat-containing cf-DNA and the mtDNA copy number, cf-DNA was extracted from plasma with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The levels of these cf-DNA species were then quantified using TaqMan qPCR. The amount of *RNase P* gene-coding cf-DNA was determined using *RNase P* detection reagents (Applied Biosystems, Foster City, CA, USA). The primer sequences for the plasma *Alu* repeats were F 5′-GGAGGCTGAGGCAGGAGAA-3′ and R 5′-ATCTCGGCTCACTGCAACCT-3′, and the probe sequence was 5′-(FAM)CGCCTCCCGGGTTCAAGCG-3′. For mtDNA copy number, the primer sequences were F 5′-CTTCTGGCCACAGCACTTAAAC-3′ and R 5′-GCTGGTGTTAGGGTTCTTTGTTTT-3′, and the probe sequence was 5′-(FAM)ATCTCTGCCAAACCCC-3′. All of the qPCR assays were performed with an ABI PRISM® 7900 HT Sequence Detection System with 40 cycles of amplification in the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C.

2.1.2. The analysis of low-molecular weight cf-DNA

In Study I, the presence of low-molecular-weight cf-DNA as a putative representative of nucleosomal DNA originating from apoptosis, was assessed in cf-DNA that was extracted from plasma with the NucleoSpin Plasma XS kit (MACHEREY-NAGEL, GmbH &Co., Düren, Germany). An Agilent 2100 Bioanalyzer and a high-sensitivity lab-on-a-chip DNA assay (Agilent Technologies Inc., Santa Clara, CA, USA) were used to analyze the presence of this low-molecular-weight, i.e., 150-200 base pairs (bp), cf-DNA band. The results were coded as a dichotomous variable. If such a band was visible the sample was coded "1"; if not, the sample was coded "0".

2.2. Other biochemical measurements

In Study I, plasma high-sensitivity CRP (hs-CRP) was performed using a particle-enhanced immunoturbidometric assay with Cobas Integra 700 automatic analyzer (Hoffman-La Roche, Ltd., Basel, Switzerland). In Study II, serum hs-CRP was measured using a latex turbidometric immunoassay (Wako Chemicals GmbH, Neuss, Germany in Study II). In Study III, CRP levels were measured using a Human CRP Immunoassay kit (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA). In the Study I, plasma IL-1ra was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine). In Study III, plasma IL-6 and IL-10 concentrations were determined using ELISAs (PeliKine human IL-6 ELISA kit and PeliKine Compact™ human IL-10 ELISA, Sanquin Reagents, Amsterdam, The Netherlands). Plasma IDO activity was assessed as the kynurenine (kyn) to tryptophan (trp) ratio. Both kyn and trp levels were measured using a reverse-phase high-performance liquid chromatography; kyn was separated using a Hewlett Packard 1100 liquid chromatograph (Palo Alto, CA, USA), and trp was separated using a Shimadzu liquid chromatograph LC-10AD VP (Shimadzu Co., Kyoto, Japan). The plasma antinuclear antibody (ANA) titer was determined using indirect immunofluorescence staining in Hep-2 cells (Inova Diagnostic Inc., San Diego, CA, USA).

2.3. The determination of immune cell proportions

Immune cell type distributions (CD3+, CD4+, CD8+ and CD14+ cells, and the expression of the CD28 costimulatory molecule on the CD4+ and CD8+ cells) were analyzed using flow cytometry (BD FACSCanto II and BD FACS DIVA software, version 6.1.3, BD Biosciences, Franklin Lakes, NJ, USA) from peripheral blood mononuclear cells (PBMCs) that were isolated from whole blood with a Ficoll-Paque density gradient (Ficoll-Paque Premium, GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.4. The assessment of functional capabilities and frailty

Handgrip strength was determined in kilograms using a hand-held dynamometer (The Martin Vigorimeter, Gebrüder Martin, Tuttlingen, Germany). Chair-stand test time was assessed as the time taken to stand up and sit down five times without help and with the arms crossed across the chest. Cognitive status was assessed using the Mini-Mental State Examination (MMSE) test, and the level of subjects' daily functioning was assessed using the Barthel index. The frailty score (min 0, max 5) was assessed according to Fried et al. (2001) as follows: if the individual met the criteria/threshold value in any of the five assessment steps, he/she was given one point for criteria met and the points were summed to yield the frailty score (Fried et al. 2001). The criteria for the points were 1) MMSE score $\leq$ 22.2) weight loss of $\geq$ 10% of body weight in the previous two years or body mass index (BMI) < 18.5 kg/m^2, 3) self-reported fatigue (the individual reported that he/she felt fatigued 'often', 4) low hand grip strength (the maximum hand grip strength of the prefereed hand was in the lowest sex- and BMI-specific quartile according to Fried et al. (2001), and 5) low mobility (the individual was unable to walk independently on a level surface or on stairs) (Fried et al. 2001).

2.5. GWAS

The genotyping was performed using custom-made Illumina Human 670 k BeadChips, and the genotypes were determined using the Illumina clustering algorithm and genotype imputations with MACH 1.0. After quality control, 546,677

genotyped and 2,543,887 imputed SNPs were available for further analysis. The GWAS was performed using linear regression with an assumption of an additive genetic effect. True genotyped SNPs were assessed using the PLINK tool set (Purcell et al. 2007), and the imputed SNPs were assessed using the ProbABEL package (Aulchenko et al. 2010). The validity of the analysis was confirmed based on Manhattan and Q-Q plots. Haploview (Barrett et al. 2005) was used to identify tag SNPs and haploblocks; the SNP with the lowest p-value in each haploblock was selected as the tag SNP.

2.6. Statistical analyses

In Study I, the data were analyzed using SPSS for Windows (version 17.0, SPSS Inc., Chicago, IL, USA). In Study III, IBM SPSS Statistics for Windows (version 19) was used. For non-parametric variables, the Mann-Whitney test and Spearman's rank correlation tests were used. A Chi-squared test with Fisher's exact 2-sided significance level was used to assess the distribution of variables between the groups. The survival analyses were performed using Kaplan-Meier and Cox regression analyses. For all of the analyses (excluding the GWAS), a level of $p<0.05$ was considered to be statistically significant.

In Study II (GWAS), the statistical analyses were performed using an R statistics package. A stepwise backward model (Akaike information criterion) was used to determine the covariate set that best explained the variation in the serum cf-DNA levels. These factors were used as adjustment covariates in the GWAS. The threshold for genome-wide statistical significance level was set at $p<5\times10^{-8}$.

2.7. Transcriptomic analysis

In Study III, the transcriptomic analysis was performed using PBMCs, which were separated from whole blood using a Ficoll-Paque density gradient (Ficoll-Paque Premium, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and which were subjected to RNA extraction with the miRNeasy Mini Kit (Qiagen, Hilden, Germany). For biotin labeling of the RNA (330 ng) an Illumina TotalPrep RNA

amplification Kit (Ambion Inc., Austin, TX, USA) was used. The quality of the biotinylated complementary RNA (cRNA) products was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA), and a total of 1,500 ng of labeled cRNA was hybridized to HumanHT-12 v4 Expression BeadChips (Illumina Inc., CA, USA). The chips were scanned using Beadscan (Illumina Inc., CA, USA).

The bioinformatic microarray data analysis was performed using Chipster v2.0 (Kallio et al. 2011). The quality of the data was confirmed using box plot, density plot and principal component analyses. The bgAdjust.affy package was used for background correction, and the data were log2-transformed to normality. The transcripts whose expression level correlated with the concentrations of total cf-DNA, unmethylated cf-DNA and plasma mtDNA copy number were identified with the Correlate with phenodata-tool in Chipster. Ingenuity Pathway Analysis (IPA) was used to identify the canonical pathways that were most significant to each of the data sets (i.e., the correlated transcripts for plasma total cf-DNA and unmethylated cf-DNA levels, and the mtDNA copy number).

2.8. Ethics

For Studies I and III, the study protocol was reviewed and accepted by the Ethics Committee of the Pirkanmaa Hospital District and the Tampere City Ethical Committee. For Study II, the study protocol was approved by the local ethics committees. The subjects gave their written informed consent for all of the studies, which were conducted following the guidelines of the Declaration of Helsinki.

Results

1. Plasma cf-DNA as a biomarker of aging and predictor of mortality in old age (Studies I and III)

We analyzed whether the plasma levels of the different cf-DNA species were associated with age. In Study III, which consisted of n=144 nonagenarians and n=30 young control individuals, we observed that the plasma levels of total cf-DNA, unmethylated cf-DNA, *RNase P*-coding cf-DNA and *Alu* repeat cf-DNA, but not the mtDNA copy number were elevated in the nonagenarians compared to the controls (Mann-Whitney, $p<0.05$). In Study I, we observed that the plasma level of total cf-DNA was an independent predictor of a 4-year mortality in n=258 nonagenarians (Table 3). When the cf-DNA values were divided into quartiles for the Kaplan-Meier survival analysis, we observed that those individuals with cf-DNA levels in the highest quartile exhibited the most prominently increased mortality rate compared to the other three quartiles (log rank significance test, $p<0.05$). In contrast, the presence of low-molecular weight (apoptotic) cf-DNA was not associated with mortality; however a predominance of this form of cf-DNA was observed in ANA-seropositive subjects (Chi-squared test, $p=0.02$).

Table 3. Cox regression analysis for the 4-year all-cause mortality in n=258 nonagenarians. All of the statistically significant predictors are given in bold.

	Beta	**p**	**HR**	**95% CI**
BMI	-0.040	0.083	0.960	0.918-1.005
Cancer	0.375	0.165	1.455	0.857-2.471
CVD	**0.641**	**0.042**	**1.897**	**1.024-3.515**
CRP	-0.001	0.949	0.999	0.979-1.021
Diabetes	-0.011	0.980	0.989	0.408-2.395
Existence of apoptotic cf-DNA	0.217	0.348	1.242	0.790-1.954
IDO	2.580	0.463	13.196	$0.014\text{-}1.3\text{x}10^4$
IgA	0.019	0.834	1.019	0.856-1.212
IL-1ra	**0.001**	**0.025**	**1.001**	**1.000-1.001**
MMSE	**-0.042**	**0.043**	**0.959**	**0.920-0.999**
Total cf-DNA level	**1.136**	**0.017**	**3.114**	**1.228-7.901**
Sex	**0.580**	**0.022**	**1.786**	**1.086-2.939**

Abbreviations: BMI, body mass index; CI, confidence interval; CRP, C-reactive protein; CVD, cardiovascular disease; HR, hazard ratio; IDO, indoleamine 2,3- dioxygenase enzyme activity; IgA, immunoglobulin A; IL-1ra, interleukin-1 receptor antagonist; MMSE, Mini-Mental Score Examination

2. Associations of the cf-DNA species with immunoinflammatory parameters and functional performance (Studies I and III)

In this study, we investigated whether the total plasma cf-DNA level was associated with indicators of inflammation, physiological measures or functional performance in n=258 nonagenarians. We observed that the total plasma cf-DNA level correlated directly with plasma levels of CRP, SAA, IL-1ra, IDO activity, body weight and chair-stand test time. An inverse correlation was observed between total plasma cf-DNA level and the plasma level of high-density lipoprotein cholesterol (Spearman's correlation, $p<0.05$).

In Study III, which was composed of n=144 nonagenarians and n=30 young control individuals, we assessed the plasma levels of total cf-DNA, unmethylated cf-DNA and the mtDNA copy number and investigated the relationship of these metrics with age-associated changes in the immune system, parameters of functional capacity and frailty. We observed that among the cf-DNA species, only the plasma levels of total and unmethylated cf-DNAs were directly correlated with the plasma levels of CRP and IL-6 in the nonagenarians (Spearman's correlation, $p<0.05$) but not in the young controls. The plasma levels of the cf-DNA species were not associated with immune cell proportions (CD4+, CD8+ and CD14+ cells) or markers of adaptive immunity immunosenescence (the proportions of CD4+CD28- and CD8+CD28- cells). The plasma levels of total cf-DNA, unmethylated cf-DNA and the mtDNA copy number correlated directly with the frailty score, with higher score indicating increased frailty (Spearman's correlation, $p<0.05$). When the cf-DNA species were analyzed for their association with the measures of functional performance, a dichotomy was seen; the levels of total and unmethylated cf-DNAs reflected the overall frailty, including the cognitive capabilities, whereas the mtDNA copy number reflected only the physical aspect of frailty. Specifically, the level of total cf-DNA correlated directly with chair-stand test time and inversely with the MMSE score and the Barthel index (Spearman's correlation, $p<0.05$), and the unmethylated cf-DNA level correlated inversely with the handgrip strength, MMSE score and Barthel index (Spearman's correlation, $p<0.05$), whereas the mtDNA copy number correlated inversely with BMI and directly with the chair-stand test time (Spearman's correlation, $p<0.05$). The plasma quantities of *RNase P*-coding cf-DNA and *Alu* repeat cf-DNA did not correlate with any of the examined variables.

3. Plasma cf-DNA as an immunomodulatory agent (Study III)

In the light of findings that have demonstrated increased reactivity to self-DNA in aged subjects *in vitro* (Agrawal et al. 2009), we asked in Study III whether the physiological levels cf-DNA were associated with immunoinflammatory responses in the n=144 nonagenarians and n=30 young controls. We performed a whole genome-covering transcriptomic analysis using the subjects' PBMCs and correlated

the transcript expression levels with the plasma levels of total cf-DNA, unmethylated cf-DNA and the mtDNA copy number *in vivo*. These cf-DNA species were chosen for this analysis due to their observed associations with inflammaging, functional impairments and frailty in the nonagenarians. To identify the cellular pathways and processes that were associated with the plasma levels of these cf-DNA species, the 250 most significant transcripts from each of the data sets were subjected to pathway analysis using IPA. The IPA canonical pathways revealed that increased plasma levels of total and unmethylated cf-DNA were associated with immunoinflammatory activation in the PBMCs. Specifically, among the ten most significant pathways for these data sets, the majority of the canonical pathways were involved in interleukin or cytoskeleton/integrin-related cellular signaling. The opposite was observed for the corresponding canonical pathways in the young controls; higher unmethylated cf-DNA levels were associated with the downregulation of pathways that are involved in immune signaling. The corresponding pathways for total cf-DNA primarily encompassed processes that are associated with cellular metabolism and turnover. The ten most significant canonical pathways that comprised mtDNA copy number-correlated transcripts in both the nonagenarians and the young controls were not associated with immunological processes; instead, these pathways were related to cellular metabolism and maintenance.

4. The role of genetics in the regulation of serum total cf-DNA levels (Study II)

To investigate the involvement of genetics in the regulation of serum cf-DNA levels, a GWAS was performed for the participants (n=1841) of the Cardiovascular Risk in Young Finns study – a sample size that is sufficiently large to permit such analysis. The analysis covered 546,677 genotyped and 2,543,887 imputed SNPs and was adjusted for sex, age, systolic blood pressure, fasting glucose, triglycerides, CRP, serum homocysteine levels, daily smoking, the use of alcohol, the use of combined oral contraceptives, the main identity-by-descent components and the following interaction terms: gender*age and daily smoking*use of alcohol.

Together, these variables explained 30.5% of the variation in the serum cf-DNA concentration. Only one peak region emerged; chromosome 2q37 was observed to contain 110 SNPs that were associated with serum cf-DNA levels, with a genome wide significance level ($p<5\times10^{-8}$). Of the 110 SNPs, the most significant were localized to the *UDP-glucuronosyltransferase 1 polypeptide A1* (*UGT1A1)* gene region. The tag SNP rs4148324 that identifies the sub-haploblock no. 8, which contained the four most significant SNPs, was observed to explain 5.3% of the total variation in serum cf-DNA levels. To identify additional SNPs that are potentially associated with serum cf-DNA levels, the analysis was adjusted for the top SNP, rs4148324; however, no additional SNPs were observed to associate with serum cf-DNA levels.

Discussion

1. Plasma cf-DNA as an aging biomarker

1.1. Associations between the abundance of the different cf-DNA species and age

The plasma levels of total cf-DNA, unmethylated cf-DNA, *RNase P*-coding cf-DNA and *Alu* repeat cf-DNA were observed to be significantly elevated in the nonagenarians compared to the young controls; no difference was observed in the plasma mtDNA copy number between the nonagenarians and young controls (Study III). These findings suggest that all of the cf-DNA species, except the mtDNA copy number, serve as biomarkers that are associated with chronological age. However, whether the increase in cf-DNA occurs linearly with aging and at which age the difference becomes significant cannot be addressed by our data. Nevertheless, we speculate that the increase in plasma cf-DNA levels occurs concomitantly with increased cellular senescence and death and decreased clearance and phagocytic capabilities. With respect to the cell death and senescence hypothesis in the generation of plasma cf-DNA, the lack of an observed difference in the mtDNA copy number between the nonagenarians and young controls may be related to observations that the mtDNA content declines with age in various tissues, such as the skeletal muscle (Short et al. 2005), the brain (Blokhin et al. 2008) and pancreatic islets (Cree et al. 2008). Cellular mtDNA depletion would thus lead to proportionally decreased plasma mtDNA content.

In addition to our current analysis and an earlier pilot study, in which we observed an increase in plasma total cf-DNA and β-globin encoding cf-DNA in nonagenarian women compared to young women, three studies have assessed the age-associated changes in plasma cf-DNA content. Zhong et al. (2007) analyzed the plasma cf-DNA level using qPCR in healthy blood donors in various age categories and

reported that women who were older than 60 years, but not men, had elevated cf-DNA levels compared 20-40 years and 41-60 year year-old women (Zhong et al. 2007). Fournie et al. (1993) have reported that elderly (aged >68 years) institutionalized subjects without chronic disease have elevated cf-DNA levels compared to middle-aged subjects (Fournie et al. 1993). Although not directly comparable due to the differences in the age-ranges of the study samples, our findings appear to be consistent with those that were reported in these previous studies. However, in a study by Wu et al. (2002) the authors reported a tendency of a u-shaped distribution in the cf-DNA levels with age (Wu et al. 2002). Although no statistical testing was applied, the highest cf-DNA levels were observed in the youngest individuals (aged <20 years, for both men and women) and in the oldest individuals (aged >70 years for women and 60-70 years for men) compared to other age groups (Wu et al. 2002).

In a majority of the studies that assessed cf-DNA quantities, the cf-DNA was first been extracted from plasma and then quantified using qPCR for a distinct amplicon. It has been demonstrated that considerable inter-individual variation exists in the abundances of different amplicons present in cf-DNA with such an approach (Puszyk et al. 2009). Another approach using high-throughput cf-DNA sequencing has been demonstrated to give concordant results with respect to the representation of serum cf-DNA sequences and chromosome size, with the exception of the underrepresentation of chromosome 19 and the overrepresentation of *Alu* repeat sequences (Beck et al. 2009). In view of these data, we analyzed the role and utility of each of the quantified cf-DNA species as an aging biomarker. Given that the total cf-DNA and unmethylated cf-DNA were quantified directly in the plasma, without a prior DNA extraction or qPCR, we were able to obtain a measure of the total plasma cf-DNA content, irrespective of its sequences and length of the fragments. Although we did not assess the levels of the cf-DNA species in relation to cellular nuclear DNA, we observed a significant direct correlation between the quantities of the *Alu* repeat cf-DNA and the total cf-DNA only in the young controls but not in the nonagenarians. Thus, it appears that the *Alu* repeat number is disproportionate to the total cf-DNA in elderly individuals; whether this finding is related to an inherent age-associated feature in cf-DNA is unknown.

1.2. Plasma levels of the cf-DNA species as indicators of age-associated inflammation, functional impairment and frailty

In Study I, we observed that the total cf-DNA level correlated directly with the plasma levels of CRP, SAA, IL-1ra and IDO activity. In the Study II we observed that both the total cf-DNA level and the amount of unmethylated cf-DNA were directly correlated with CRP and IL-6 levels in nonagenarians. These results indicate that the total cf-DNA level and the unmethylated cf-DNA content, which are very strongly correlated with each other, are linked with the rate of inflammaging. Nevertheless, the nature of the relationship between the levels of inflammatory mediators and cf-DNA appears complex. Elevated cf-DNA levels have been shown to be associated with higher CRP levels in cases of sepsis (Rhodes et al. 2006, Huttunen et al. 2011), in patients admitted to intensive care unit (Wijeratne et al. 2004) and in febrile patients of which a subset developed sepsis and septic shock (Moreira et al. 2010) but not in patients with Puumala hantavirus infection (Outinen et al. 2012), individuals with aseptic inflammation due to exercise overtraining (Fatouros et al. 2006) or in our young control individuals (Study III). Based on these data, a tentative overall conclusion is that elevated cf-DNA levels are more likely to be associated with systemic inflammation in conditions for which there is a greater possibility of a fatal outcome.

With regard to age-associated functional impairments, higher levels of total-cf-DNA were associated with lower body strength (increased chair-stand test time) (Studies I and III), cognitive impairment (lower MMSE score) and decreased capabilities in daily functioning and mobility (lower Barthel index) (Study III). Elevated unmethylated cf-DNA levels were likewise associated with cognitive impairment, decreased daily functioning and mobility, and decreased general muscle strength (handgrip strength). When both the physical and cognitive aspects of functional performance were assessed as a frailty score to indicate overall frailty, an association between increased overall frailty and higher total and unmethylated cf-DNA levels were observed (Study III). Although the increased mtDNA copy number was also correlated directly with the frailty score, inspecting the associations between the individual frailty components and mtDNA copy number revealed that the mtDNA copy number reflected only the physical aspect of frailty

(i.e., an inverse correlation with BMI and a direct correlation with the chair-stand test time) (Study III). Instead, the plasma quantities of *RNase P*-coding cf-DNA and *Alu* repeat cf-DNA did not correlate with any of the study variables in either the nonagenarians or young controls. One possible explanation for this result is that the fraction of total cf-DNA that contained the short (oligonucleotide-sized) fragments became lost during the cf-DNA extraction and hence was not quantified with the qPCR. This hypothesis is based on the assumption that the short fragments are relevant in the context of total cf-DNA and unmethylated cf-DNA levels in reflecting the aging phenotypes, such as inflammaging.

Our studies are the first to assess cf-DNA characteristics in relation to aging-associated changes. However, the phenomenon of aging-associated global DNA demethylation is recognized, and the methylation level in leukocytes in relation to cognitive functions and frailty has been previously addressed by two studies. Bellizi et al. (2012) reported a decrease in the global DNA methylation status along with increasing frailty in a 7-year follow-up study that involved 65-85 -year-old individuals (Bellizzi et al. 2012). However, similar association was not reported in ultranonagenarians in the same study (Bellizzi et al. 2012). Schiepers et al. (2012) observed no association between global DNA methylation and cognitive performance in healthy adults who were aged between 50 and 70 years (Schiepers et al. 2012). Nevertheless, as cf-DNA can be derived from various tissues, the amount of unmethylated cf-DNA in plasma is likely to represent the methylation status in a wider range of cell types. However, whether the unmethylated plasma cf-DNA represents methylation changes in the primary target tissues of frailty (e.g., brain and skeletal muscle) or is derived from these tissues cannot be resolved from our data. Similarly, we cannot definitely conclude whether the observed increase in the plasma mtDNA copy number with decreasing body strength (Study III) is due to mtDNA depletion particularly in the skeletal muscle or due to a more generalized catabolism of mitochondria in various tissues. However, Swarup et al. (2011) observed elevated cf-DNA levels in individuals with spinocerebellar ataxia and Friedreich's ataxia compared to the controls, and attributed the increased cf-DNA levels to neuronal and muscular degeneration in the patients (Swarup et al. 2011).

1.3. Plasma cf-DNA as a predictor of mortality in old age

The total cf-DNA level was observed to be a predictor of 4-year all-cause mortality among the nonagenarians in Study I. Of the inflammatory mediators, in addition to the total cf-DNA level, only the IL-1ra level was independently associated with mortality, whereas the CRP level and IDO activity were not. However, the presence of the low-molecular-weight (apoptotic) cf-DNA was not associated with mortality; instead it was predominantly confined to ANA-positive individuals. These findings are consistent with previous observations that the ANA serostatus is unrelated to mortality in this Vitality 90+ cohort (Hurme et al. 2007). However, the basis for the association between the ANA serostatus and apoptotic cf-DNA is unknown. This association may involve the prolonged presence of apoptotic cells or nuclei in the circulation that have the capability to induce the production of various autoantibodies, such as ANA. Nevertheless, as the amount of the total cf-DNA was unrelated to the presence of apoptotic cf-DNA, these findings raise the question of whether the mortality-predicting capacity of the total cf-DNA level is primarily related to a phenomenon other than increased apoptosis. In addition to the results of our study, the role of plasma cf-DNA as an indicator of elderly mortality has been demonstrated by Fournie et al (1993). These authors observed reduced survival with increasing plasma cf-DNA concentrations among elderly institutionalized patients over 68 years of age.

2. Immunoinflammatory activity in relation to cf-DNA levels

Immunoinflammatory reactivity to self-DNA has been reported to be increased *in vitro* in monocyte-derived DCs from elderly individuals (Agrawal et al. 2009). This reactivity has been attributed to the age-associated decrease in the global DNA methylation level, which renders the DNA more immunogenic and proposedly leads to a gradual loss of peripheral self tolerance (Agrawal et al. 2010). In Study III, we aimed to address the issue of the potential self-DNA-associated immunoinflammatory activation in the PBMCs. This analysis was performed by i) correlating the transcript expression levels with the plasma levels of total cf-DNA,

unmethylated cf-DNA and mtDNA copy number and ii) subjecting the identified transcripts to pathway analysis. In the pathways that were identified for total cf-DNA and unmethylated cf-DNA levels, an abundance of immunoinflammatory and cytoskeleton/integrin-related signaling pathways was observed in the nonagenarians. The majority of transcript expression levels in these pathways were upregulated with increasing levels of total and unmethylated cf-DNA. A similar phenomenon was not observed in the corresponding pathways of the young controls. These findings suggest that plasma levels of total and unmethylated cf-DNA are inherently linked with aggravated immunoinflammatory activation in the nonagenarians, whereas in the young controls, these cf-DNA species appear to be inert in this respect. The pathways for plasma mtDNA copy number demonstrated that fluctuation of this cf-DNA species was mainly associated with cellular turnover, metabolism, and maintenance both in the nonagenarians and young controls. Nevertheless, given that the cell population used in this analysis (PBMCs) was a mixed population that consisted of several distinct cell types, the identified pathways describe the "overall" activity of the immune cells in relation to cf-DNA levels. Moreover, certain of the pathways may be non-causally or indirectly related to cf-DNA levels. However, the *HMGB1 Signaling* pathway, which is known to be involved in immunoinflammatory sensing of extracellular DNA (Pisetsky 2012), was associated with total and unmethylated cf-DNA levels in the nonagenarians.

3. The genetic regulation of cf-DNA levels

In the GWAS (Study II), the gene region coding for the UGT1A1 enzyme was identified as the locus that contained genetic variants associated with serum cf-DNA levels among the participants of the Cardiovascular Risk in Yung Finns study. The top SNP accounted for 5.3% of the variation in serum cf-DNA concentration. Given that no other chromosome regions with genome-wide significance, or even near significance, emerged in the analysis, the finding suggests that UGT1A1-mediated processes regulate serum cf-DNA levels in young adults. However, the relationship and biological connection between UGT1A1 and cf-DNA is enigmatic. The UGT1A1 enzyme, which is primarily expressed in the liver, is best known for its

role in facilitating the elimination of bilirubin as well as various drugs and endogenous compounds. Whether UGT1A1 controls cf-DNA levels directly via glucuronidation or closely related processes is unknown. A physical interaction between UGT1A1 and triphosphate nucleosides has been demonstrated; ATP, CTP and GTP were reported to be allosteric inhibitors of UGT1A1 (Nishimura et al. 2007). However, we are currently unable to determine whether this finding is related to the turnover of cf-DNA. Nevertheless, the fact that the observed genetic associations involved none of the traditional scavenger molecules or DNases suggests that a previously unrecognized mechanism operates in the regulation of cf-DNA levels.

Considering that the directionality of the *UGT1A1* allelic effects on cf-DNA levels is the same as that reported for bilirubin, the co-regulation of the bilirubin formation and glucuronidation and cf-DNA metabolism may be one explanation for the observed effect. The majority of the bilirubin in the body forms as a breakdown product of heme in dying red blood cells, and given that erythrocytes can carry cf-DNA on their surface to the liver (Cosio et al. 1987), and release it during their enucleation (Yoshida et al. 2005a, Nagata et al. 2010), the erythrocyte half-life may contribute to the rate of cf-DNA elimination. Other uncharacterized mechanisms, such as those involved in the cellular uptake of bilirubin and cf-DNA, can also underlie the observed association. Likewise, in addition to the identified statistical covariates (sex, age, systolic blood pressure, fasting glucose, triglycerides, CRP, homocysteine, daily smoking, use of alcohol, use of combined oral contraceptives, main identity-by-descent components and the interaction terms gender*age and daily smoking*use of alcohol), which explain 30.5 % of the variation in the serum cf-DNA levels, identifying the factors that explain the remaining variation in requires additional investigation. Lastly, the results do not permit us to conclude whether the fluctuation of cf-DNA in the young individuals is controlled more by clearance-associated mechanisms or input (cellular release)-related ones. Further research is also required to ascertain the role of genetics in the regulation of cf-DNA levels in elderly individuals and in pathological states.

Summary and Conclusions

The results of this thesis delineate the characteristics and immunoinflammatory aspects of cf-DNA that are essential to its utility as an aging biomarker. While it appears to provide a potent candidate marker for reflecting systemic inflammation and predicting survival in the old age, more large-scale longitudinal studies covering wider age ranges will be required to determine whether the total cf-DNA level is a valid predictor of longevity. If so, the molecular mechanisms that underlie its correlation to old age survival, and perhaps more essentially, to the aging process itself, will need to be determined. However, as the cf-DNA level is also known to be elevated in several age-related pathologies, it is unlikely to be a genuine biomarker of aging according to the strict criteria (Johnson 2006).

Establishing the detailed nature of the relationship between the increased total and unmethylated cf-DNA levels and immunoinflammatory activation in nonagenarians warrants more in-depth analysis of the sequences and structural motifs in the total cf-DNA pool. Likewise, cf-DNA assessment in larger cohorts of young and middle-aged will ultimately reveal whether cf-DNA is immunologically inert, or even suppressive, in physiological concentrations before the old age. The role of genetics in the regulation of the cf-DNA levels is also an area of uncertainty, as no other study than the one that is presently described (with the Young Finns cohort adults), has addressed the issue. Considering the age-associated differences in the characteristics of cf-DNA, genetic variants can be expected to play a differential role in very elderly individuals compared to young and healthy individuals. Lastly, overcoming the heterogeneity in the assessment methods and preanalytical procedures is a prerequisite for the establishment of reference values for given conditions and for enabling a straightforward comparison of the results that are obtained in different studies.

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Circulating cell-free DNA is associated with mortality and inflammatory markers in nonagenarians: The Vitality 90+ Study

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Highlights:

- cell-free DNA plasma level (cf-DNA) is an independent predictor of all-cause 4-year mortality in very old individuals
- cf-DNA concentration reflects systemic inflammation, analogously to C-reactive protein
- Presence of low-molecular-weight/nucleosomal cf-DNA associates with serum antinuclear antibody seropositivity.

Key words : cell-free DNA, mortality, elderly, inflammation, biomarker
Running title: cf-DNA in nonagenarians

Abstract

Aging is characteristically accompanied by a low-grade inflammatory state and increased cellular death. In this study, we analyzed the associations between the plasma cell-free DNA (cf-DNA) level and immunoinflammatory factors and 4-year mortality. The distribution of qualitative cf-DNA patterns in relation to the total cf-DNA concentration and the antinuclear antibody (ANA) serostatus was also evaluated. The study population consisted of n=258 nonagenarians who were participants in the Vitality 90+ Study. Cf-DNA levels were positively correlated with the C-reactive protein (CRP) level, the interleukin-1 receptor antagonist (IL-1ra) level, the serum amyloid A (SAA) level and the indoleamine 2,3-dioxygenase enzyme activity (IDO), weight and chair-stand test time and inversely correlated with the HDL cholesterol level. The total cf-DNA concentration also remained as an independent predictor of 4-year all-cause mortality. A predominance of low-molecular-weight cf-DNA was observed in ANA-seropositive subjects but this cf-DNA pattern was not associated with mortality. The cf-DNA concentration could thus represent a novel biomarker for systemic inflammation and mortality in the elderly.

1. Introduction

A chronic low-grade proinflammatory state or "inflammaging" is often the hallmark of aging and is associated with adverse health effects, such as disability, frailty and age-related comorbidities (Bruunsgaard 2006; Franceschi et al. 2007). The cause-effect relationships between the aging process, age-related pathologies and inflammaging are currently unclear, yet systemic inflammation has been demonstrated to contribute directly to mortality in old age (Krabbe et al. 2004; Bruunsgaard 2006). Various individual inflammatory markers and marker combinations have been used to measure the proinflammatory status of the elderly individuals, and the most frequently applied markers include C-reactive protein (CRP) and various interleukins (IL), primarly IL-6. In several studies, these inflammatory markers have also predicted elderly mortality independent of pre-existing morbidity, although some controversy exists (Krabbe et al. 2004; Bruunsgaard 2006; Bandeen-Roche et al. 2009). Nevertheless, because inflammaging can represent multiple aspects of generalized inflammation and cellular senescence and death, it is likely that not all of its characteristics are covered by current markers.

Recently, circulating cell-free DNA (cf-DNA) has become an attractive candidate for assessing cell death- and tissue damage-related phenomena in individuals with several acute and chronic disorders, such as bacteremia, cardiovascular disease, cancer and autoimmune disorders (Tong & Lo 2006; Tsang & Lo 2007). It has also been demonstrated that elderly patients suffering from various chronic and acute diseases that are accompanied with apoptosis or necrosis have higher cf-DNA plasma levels and increased 9-month mortality than clinically healthy elderly subjects (Fournie et al. 1993). In addition to the quantity of circulating cf-DNA in the plasma, the actual physical size of cf-DNA molecules has also been investigated; compared with healthy controls, diabetic individuals (Langford et al. 2007) and cancer patients (Deligezer et al. 2003; Deligezer et al. 2006) have a predominant pattern of fragmented or low-molecular-weight cf-DNA. The observations of cf-DNA laddering and fragment sizes consistent with nucleosomes support the involvement of apoptosis in cf-DNA release (Fournie et al. 1995; Giacona et al. 1998; Jahr et al. 2001). It has also been demonstrated *in vitro* that late apoptotic cells release their chromatin in the form nucleosomes (van Nieuwenhuijze et al. 2003). However, other mechanisms, such as necrosis and active cellular secretion have also been demonstrated to contribute to the pool of circulating cf-DNA (Stroun et al. 2000; Stroun et al. 2001; Gormally et al. 2007; van der Vaart & Pretorius 2008). From this perspective, cf-DNA appears to be an intriguing biomarker candidate because practically every cell type can release it and its production is not limited to liver or blood cells.

Although the current literature indicates that elevated cf-DNA levels are inherently linked with systemic inflammation, data regarding the relationships between cf-DNA quantity and other inflammatory markers, particularly in elderly individuals, are scarce. In our previous pilot study, we demonstrated that nonagenarian women have higher plasma cf-DNA levels than young controls, and that the cf-DNA in nonagenarians displayed a predominance of low-molecular-weight (apoptotic) cf-DNA bands (Jylhava et al. 2011). In the current study, we wished to expand our previous work and assess the clinical relevance of cf-DNA quantity and quality in a large cohort of nonagenarian men and women. Specifically, we evaluated the use of cf-DNA quantity as a marker of inflammaging and examined the relationship between the plasma cf-DNA concentration and all-cause 4-year mortality. We also examined the distribution of discrete qualitative cf-DNA patterns and analyzed whether the appearance of low-molecular-weight cf-DNA (150-200 bp) was associated with age-associated autoimmunity and 4-year mortality.

2. Materials and methods

2.1 Study population

The study population consisted of n=258 nonagenarians (n=196 women, n=62 men) who were participants in the Vitality 90+ Study. The Vitality 90+ Study is a prospective population-based study that includes both home-dwelling and institutionalized individuals who are aged 90 years and older and living in Tampere, Finland. Blood tests, physiological measurements, interviews and performance tests were conducted by a home-visiting nurse. The subjects' medical diagnoses were obtained from health center records and coded according to the International Classification of Diseases, 10^{th} revision (ICD-10). Cardiovascular disease (CVD) included coronary heart disease, myocardial infarction, chronic heart failure, hypertension and atrial fibrillation (ICD codes 0-50). The diagnosis of cancer (former or present) involved all types of cancers except basal cell carcinoma. Morbidity data were available from n=233 individuals and the prevalences of the disease entities were as follows: CVD, 78.5% (185/233); diabetes, 10.3% (24/233); and cancer, 18.5% (43/233).

All-cause deaths during the 4-year follow-up were collected from the national Population Register Center. The total 4-year all-cause mortality in this cohort was 56.2 % (145/258).

Written informed consent was obtained from each participant and the study protocol followed the guidelines of the Declaration of Helsinki. The study protocol was approved by the

Ethics Committee of the Pirkanmaa Hospital District and the Ethics Committee of the Tampere Health Center (for detailed cohort description, see (Goebeler et al. 2003)).

2.2 Biochemical measurements

Fasting EDTA-blood was collected in the morning and the plasma was separated by centrifugation for 15 min at 700 g and then stored at -80°C. Total cf-DNA was measured directly in previously unthawed plasma samples using a Quant-iT^{TM} DNA High-Sensitivity Assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All of the samples were analyzed in duplicate, and the mean of the two measurements was used as the final value. At the mean cf-DNA level of 0.593 μg/ml, the intra- and inter-day variation coefficients for the Quant-iT^{TM} DNA High-Sensitivity Assay were 1.9% and 4.7%, respectively, and at the mean level of 1.007 μg/ml, the corresponding values were 2.3% and 5.2%, respectively. The Quant-iT^{TM} DNA High-Sensitivity Assay kit was chosen as the method for quantifying total cf-DNA due to its reproducibility; because this assay is based on a DNA-intercalating dye, it measures total cf-DNA regardless of its sequence, fragment size or integrity. In addition, because no prior DNA extraction step is performed, the difficulties related to capturing and recovering cf-DNA fragments of all sizes with one extraction kit can be avoided.

Regarding to the effect of different blood-processing protocols on cf-DNA concentration, it has been reported that PCR-based cf-DNA quantification methods can be affected by the presence of residual cells if a low-speed centrifugation (800g) is used to separate the plasma (Chiu et al. 2001). Even though The Quant-iT^{TM} DNA High-Sensitivity Assay does not include a preprocessing step in which the residual plasma leukocytes would lyze and release their chromatin, we assessed the effect of different centrifugation protocols (700g, 700g + 1600g and 700g +1600g +1600) on the total cf-DNA quantity in 30 randomly chosen samples.

The serum amyloid A (SAA) level was measured using a human SAA ELISA kit with a detection limit of <0.004 mg/L (Human SAA, Biosource International, Camarillo, CA). The Anti-cytomegalovirus IgG titer was measured using a commercial enzyme immunoassay (Enzygnost Anti-CMV/IgG, Siemens Healthcare Diagnostics Products Gmbh, Marburg, Germany). The methods for the assessment of CRP, IL-6, IL-1ra, indoleamine 2,3-dioxygenase enzyme activity (IDO), plasma lipids, antinuclear antibodies (ANA) immunoglobulin A (IgA), IgG and IgM have been previously described (Hurme et al. 2005; Pertovaara et al. 2006; Rontu et al. 2006; Hurme et al. 2007; Jylha et al. 2007).

2.3 Physiological measurements and functional performance

The methods for the assessment of BMI, weight, blood pressure, Mini-Mental Score Examination (MMSE), chair-stand test, handgrip strength and Barthel index have been previously described (Jylha et al. 2007; Tiainen et al. 2010).

2.4 Qualitative analysis of cf-DNA

For the qualitative analysis of the plasma cf-DNA patterns, the NucleoSpin® Plasma XS kit (MACHEREY-NAGEL GmbH & Co., Düren, Germany) was used to isolate cf-DNA from 230 µl of EDTA-plasma. The kit has high a specificity in recovering fragmented DNA, especially in the range of 50-1000 bp. The cf-DNA isolation was performed according to the manufacturer's instructions, following the high-sensitivity DNA extraction protocol.

The high-sensitivity lab-on-a-chip DNA assay and an Agilent 2100 Bioanalyzer with Expert 2100 software (Agilent Technologies Inc., Santa Clara, CA) were used to perform the qualitative cf-DNA analysis. The samples were analyzed in duplicate, and 1 µl of each sample was applied to the gel-loaded chip, which was prepared and run according to the manufacturer's instructions (Agilent Technologies Inc., Santa Clara, CA). During the assay, cf-DNA molecules were separated electrophoretically, analogous to capillary electrophoresis, and detected by laser-induced fluorescence from the DNA-intercalating dye. Cf-DNA fragment sizes were determined by normalizing the samples to the low- and high-molecular-weight internal DNA markers (35 and 10380 base pairs, respectively) and to a DNA ladder. For each sample, the cf-DNA pattern was analyzed in the Expert 2100 software sample electropherogram and coded as follows: if no cf-DNA was observed in the chip detection range (i.e., no valid peaks), then the sample was coded "0", if a low-molecular-weight cf-DNA band or multiple cf-DNA fragments were clearly observable in replicate samples, the sample was coded "1." A few samples that displayed high-molecular-weight cf-DNA within the 10380 bp marker area, but no smaller cf-DNA fragments, were coded "0", because the DNA of this size (≥7000 bp) typically represents intact genomic DNA. Samples displaying deviations in cf-DNA patterns in duplicate assays were rerun at least once more to verify the result. As a methodological control for the cf-DNA extraction, n=52 (~20%) plasma samples were subjected to re-extraction followed by the high-sensitivity lab-on-a-chip DNA assay.

2.5 Statistical analyses

Correlates of cf-DNA were analyzed with Spearman's rho. Kaplan-Meier survival analysis with the log rank significance test was used to evaluate the association between the cf-DNA level and survival time during the 4-year follow-up period. In this analysis, the subjects were divided as

follows: (a) based on median cf-DNA into 1 = low cf-DNA level (≤0.707 μg/ml) and 2 = high cf-DNA level (>0.707 μg/ml) categories and (b) based on 25% cut-off cf-DNA values into quartiles (1= <0.651 μg/ml, 2= 0.651-0.707 μg/ml, 3= 0.708-0.811 μg/ml, 4= ≥0.812 μg/ml).

Cox regression analysis, adjusted for sex, MMSE, IL-1ra, CRP, IgA, IDO, CVD, diabetes, BMI and qualitative cf-DNA pattern (the presence of low-molecular-weight cf-DNA), was used to determine the relationship between the cf-DNA levels and 4-year all-cause mortality. The adjustment covariates were chosen as follows: i) for their established role as a mortality predictor of elderly individuals (sex, CVD, cancer, diabetes, BMI, CRP and MMSE) (Korten et al. 1999; Hurd et al. 2001; Bruunsgaard 2006) or ii) based on their previously reported independent association with mortality in this cohort (IL-1ra level, IDO activity and IgA level). In the Cox regression model, only the subjects with complete covariate data (i.e., all covariate parameters available) were included; this inclusion criterion explains the reduced number of deaths/individuals (87/171) in this analysis. Differences in the distribution of qualitative cf-DNA features (0 = no low-molecular-weight or fragmented cf-DNA, 1 = low-molecular-weight cf-DNA band or multiple fragments) in relation to the total cf-DNA concentration (dichotomized as low and high cf-DNA level categories in which the median cf-DNA level was the cut-off value) and the ANA serostatus (negative = S-ANA titer ≤160, positive = S-ANA titer ≥160, additionally tested as 1 = S-ANA titer ≥ 80, 0 = S-ANA titer < 80) were compared using the chi-Square test with Fisher's exact 2-sided test. Mann-Whitney U -test was used to assess the association between the total cf-DNA quantity and the different centrifugation protocols (700g, 700g + 1600g and 700g +1600g +1600; all the three "centrifugation groups" were tested pairwise). All of the statistical analyses were conducted using SPSS Statistics version 17.0 (SPSS Inc, Chicago, IL, USA). P-values <0.05 were considered statistically significant.

3. Results

Men had a slightly but significantly higher median cf-DNA level than women (men: 0.729 μg/ml, interquartile range 0.679-0.827 μg/ml, vs. women: 0.699 μg/ml, interquartile range 0.637-0.795 μg/ml; Mann-Whitney test for difference p=0.018). The median cf-DNA level for the entire cohort was 0.707 μg/ml (minimum 0.496 μg/ml, maximum 1.930 μg/ml, interquartile range 0.651-0.851 μg/ml). Because there were few men in this cohort, the men and women were analyzed together to obtain maximal statistical power when assessing the correlates of cf-DNA. The correlation

coefficients among significantly correlated variables in men and women were essentially in the same directions.

The cf-DNA levels were positively correlated ($p<0.05$) with the CRP level, the interleukin-1 receptor antagonist (IL-1ra) level, the serum amyloid A (SAA) level, the indoleamine 2,3-dioxygenase enzyme activity (IDO), weight and the chair-stand test time. An inverse correlation ($p<0.05$) was observed between the cf-DNA level and the HDL cholesterol (Table 1).

The total 4-year all-cause mortality in this cohort was 56.2% (145/258). In the adjusted Cox regression analysis, cf-DNA remained an independent predictor of 4-year all-cause mortality together with sex, MMSE, IL-1ra and CVD (Table 2). The Kaplan-Meier survival analysis revealed that categorized total cf-DNA values were significantly associated ($p<0.05$) with the 4-year mortality rate (Figure 1).

Assessing the total cf-DNA quantity after the different centrifugation protocols (700g, 700g + 1600g and 700g +1600g +1600) revealed no statistically significant associations between the total cf-DNA concentration and the different centrifugation protocols (supplementary Figure 1), indicating that the cf-DNA quantity measured with the Quant-iT^{TM} DNA High-Sensitivity Assay kit is not significantly affected by the applied centrifugation protocol.

Analysis of the qualitative cf-DNA patterns was successful in n=236 individuals. A low-molecular-weight band or cf-DNA fragments were observed in 44.9% of the subjects. Examples of sample coding for cf-DNA patterns are displayed as sample electropherograms in Figure 2. The distribution of the cf-DNA patterns did not differ between the low and high cf-DNA level categories ($p=0.682$, median cf-DNA level as the cut-off value). ANA-positivity in this cohort was 12.3% when an S-ANA titer $\geq$160 was used as the cut-off value, and 22.9% when an S-ANA titer $\geq$80 was used as the cut-off value. The distribution of cf-DNA patterns in relation to ANA status displayed a trend toward significance ($p=0.072$) when S-ANA titers $\geq$160 were regarded as ANA positive. However, when S-ANA titer $\geq$80 were regarded as ANA positive, the difference became statistically significant: among the ANA-positive subjects, the majority had low-molecular-weight or fragmented cf-DNA, whereas among ANA-negative subjects this distribution was reversed ($p=0.020$, Table 3). In the vast majority of cases who scored positive for low-molecular-weight cf-DNA, the size of the cf-DNA band corresponded to that of apoptotic, nucleosomal DNA i.e., 150-200 bp. The assessment of the methodological quality for cf-DNA extraction by repeated extraction and qualitative analysis revealed that the consistency of the cf-DNA pattern was 47/52 (90%).

5. Discussion

In this study, we present a novel biomarker candidate, cf-DNA, for measuring systemic inflammation in very old individuals. We demonstrated that the plasma cf-DNA level is positively associated with the plasma levels of CRP, IL-1ra and SAA and IDO activity and is inversely associated with the HDL cholesterol level. The cf-DNA concentration was also positively correlated with the chair-stand test time and weight. When the cf-DNA values were categorized for the Kaplan-Meier survival analysis, individuals with cf-DNA level in the highest quartile had the most prominently increased mortality rate. In the Cox regression model, we observed that cf-DNA remained an independent determinant for all-cause mortality together with sex, CVD, MMSE and IL-1ra. Although the model is likely to contain some collinearity between the variables, the effect of cf-DNA on mortality was found to be the most significant among the immunoinflammatory factors tested (e.g., CRP, IL-1ra, IDO and IgA). Moreover, the association between cf-DNA and mortality was independent of the major age-associated comorbidities, such as CVD, cancer, diabetes and cognitive decline (MMSE), indicating that elevated plasma cf-DNA levels are not merely a by-product of these clinically defined morbidities. Interestingly, the presence of low-molecular-weight cf-DNA band was not associated with mortality, indicating that in contrast to the total cf-DNA concentration, this particular qualitative cf-DNA pattern is not relevant with regard to elderly mortality.

We can only speculate on the mechanisms that connect the increased total cf-DNA levels with the all-cause mortality in very old individuals, but we assume that these mechanisms are related to disturbances in tissue homeostasis, accelerated rate of catabolism and apoptosis and cellular senescence. Total cf-DNA may also contain some specific features − other that the low-molecular-weight fragment − that render it proinflammatory and potentially harmful. Conversely, the increase in the total plasma cf-DNA level may be a direct consequence of generalized systemic deterioration that precedes death in the elderly. However, because our population consisted of nonagenarian subjects, we were unable determine whether cf-DNA predicts mortality in younger elderly individuals as well. Nevertheless, reaching the age of 90 is not exceptional in the present, and hence relatively large nonagenarian study cohorts are available. Analyzing the biomarkers and survival predictors in individuals 90+ years of age can help us to identify novel biological determinants for successful aging and longevity.

In the qualitative cf-DNA analysis, we observed that the predominance of low-molecular-weight cf-DNA band, corresponding to the size of nucleosomal DNA (150-200 bp), was

confined to ANA-positive individuals. Hence, this low-molecular-weight cf-DNA may be mainly composed of nucleosomal DNA, which has the potential to induce autoreactivity (Bruns et al. 2000; Decker et al. 2005). Regarding to autoreactivity and aging, the production of ANA is known to increase with age (Xavier et al. 1995), although it does not appear to compromise longevity or to be associated with an increased rate of inflammation (Hurme et al. 2007). Further studies, however, are needed to explicate the clinical relevance and interconnections between apoptotic/nucleosomal plasma cf-DNA and systemic late-onset autoimmunity. Nevertheless, the distribution of the low-molecular-weight/nucleosomal cf-DNA pattern did not differ between the low and high total cf-DNA level groups, indicating that the individuals possessing the low-molecular-weight cf-DNA/nucleosomal cf-DNA did not have overall higher total cf-DNA levels. Likewise, the lack of correlation between the total cf-DNA concentration and the S-ANA titer suggests that cf-DNA is not directly related to autoreactivity in contrast with its low-molecular-weight/nucleosomal form which does appear to be directly related to autoreactivity.

Inflammatory factors play a major role in age-related processes and pathologies, although the underlying molecular mechanisms connecting system dysfunction and inflammaging are unclear. The observed positive correlations between the cf-DNA level and both the chair-stand test time and the levels of inflammatory factors (CRP, IL-1ra, IDO activity and SAA) could be explained by the loss of proliferative homeostasis with aging that can manifest as a deregulated inflammatory response, muscle atrophy and a decline in the number of T cells. The observed inverse correlation between the cf-DNA level and the HDL cholesterol level and the positive correlation between the cf-DNA level and weight may indicate that an increase in the plasma cf-DNA level is also related to a disadvantageous metabolic profile. This hypothesis, however, warrants further investigation because no correlation was observed between the cf-DNA level and the levels of other plasma lipids or BMI.

The functionality and potential pathological mechanisms of cf-DNA are currently elusive. However, tumor-derived circulating DNA may mediate genometastasis, i.e., induce the transformation of normal cells even in distant target organs, via transfection-like uptake of the malignant circulating DNA (Garcia-Olmo & Garcia-Olmo 2001; Garcia-Olmo et al. 2010). Several *in vitro* studies with divergent approaches have also demonstrated that cells that take up free DNA can subsequently exhibit the biological activity encoded by the transferred sequences (reviewed in (Gahan et al. 2008)). Details of the horizontal transfer of genetic material are unclear, yet in addition to transmembrane toll-like receptor 9 (TLR9), several intracellular receptors, such as the high-mobility group box (HMGB) proteins, have been identified for endogenous double-stranded DNA (dsDNA) (Muruve et al. 2008; Yanai et al. 2009; Hornung & Latz 2010). Upon sensing

dsDNA, these receptors can trigger multiple pathways, including those leading to the production of proinflammatory cytokines, chemokines and type I interferons, and thus activate the innate and adaptive immune functions (Muruve et al. 2008; Yanai et al. 2009; Hornung & Latz 2010). Recently, this scenario has been tentatively ascribed to the development of autoimmune thyroid dysfunction through a mechanism that involves the recognition of cytosolic dsDNA by extrachromosomal histone H2B followed by an intrinsic immune activation in the thyroid (Kawashima et al. 2011). Nevertheless, additional research is required to determine whether the aforementioned phenomena also underlie the observed alterations in cf-DNA quantity and quality regarding to mortality and autoimmunity, respectively, in the elderly.

We conclude that cf-DNA may be a potential all-cause mortality predictor in very old individuals and a novel marker of inflammaging, analogous to CRP, IL-1ra and SAA. Our results also shed light on the relationship between the total cf-DNA level and cf-DNA quality and their differential associations with elderly mortality and systemic autoreactivity. However, the establishment of universal cut-off values and reference ranges for plasma cf-DNA concentration and the standardization of the assessment methods are necessary to develop cf-DNA as a useful biomarker.

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Tables

Table 1. Correlates of plasma cf-DNA in n= 258 nonagenarians. Statistically significant correlations are shown in bold.

	Spearman's rho	p-value
Immunoinflammatory factors		
anti-cytomegalovirus IgG	-0.008	0.912
CRP	**0.294**	**<0.001**
IDO	**0.208**	**0.001**
IgA	0.084	0.180
IgG	0.069	0.271
IgM	-0.077	0.215
IL-1ra	**0.153**	**0.014**
IL-6	0.078	0.210
SAA	**0.187**	**0.003**
S-ANA	-0.040	0.519
Plasma lipids		
HDL cholesterol	**-0.196**	**0.002**
LDL cholesterol	0.017	0.780
Triglycerides	-0.031	0.615
Total cholesterol	-0.073	0.245
Physiological factors		
BMI	0.074	0.258
Weight	**0.195**	**0.003**
Diastolic blood pressure	-0.048	0.500
Systolic blood pressure	0.004	0.961
Functional performance		
Chair-stand test time	**0.267**	**<0.001**
MMSE	-0.062	0.332
Barthel Index	-0.075	0.243
Handgrip strength	0.108	0.104

Abbreviations: BMI, body mass index; CRP, C-reactive protein; IDO, indoleamine 2,3-dioxygenase enzyme activity; Ig, immunoglobulin; IL-1ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; MMSE, Mini-Mental Score Examination; SAA, serum amyloid A; S-ANA, serum antinuclear antibodies

Table 2. Assessment of 4-year all-cause mortality in n=171 nonagenarians with Cox regression analysis. The total number of deaths was 87 (50.9%). Statistically significant predictors are shown in bold.

	Beta	p-value	HR	95% CI
total cf-DNA concentration	**1.136**	**0.017**	**3.114**	**1.228-7.901**
CVD	**0.641**	**0.042**	**1.897**	**1.024-3.515**
sex	**0.580**	**0.022**	**1.786**	**1.086-2.939**
MMSE	**-0.042**	**0.043**	**0.959**	**0.920-0.999**
IL-1ra	**0.001**	**0.025**	**1.001**	**1.000-1.001**
presence of low-molecular-weight cf-DNA	0.217	0.348	1.242	0.790-1.954
CRP	-0.001	0.949	0.999	0.979-1.021
IgA	0.019	0.834	1.019	0.856-1.212
IDO	2.580	0.463	13.196	$0.014\text{-}1.3x10^4$
Diabetes	-0.011	0.980	0.989	0.408-2.395
Cancer	0.375	0.165	1.455	0.857-2.471
BMI	-0.040	0.083	0.960	0.918-1.005

Abbreviations: BMI, body mass index; CI, confidence interval; CRP, C reactive protein; CVD, cardiovascular disease; HR, hazard ratio; IDO, indoleamine 2,3- dioxygenase enzyme activity; IgA, immunoglobulin A; IL-1ra, interleukin-1 receptor antagonist; MMSE, Mini-Mental Score Examination

Table 3. Distribution of qualitative cf-DNA patterns in relation to ANA status.

	ANA serostatus*		
cf-DNA pattern	Negative n (%)	Positive n (%)	
No low-molecular-weight or fragmented cf-DNA	107 (59.1)	22 (40.7)	
Low-molecular-weight and/or cf-DNA fragments observed	74 (40.9)	32 (59.3)	p**= 0.020
Total n (100%)	181	54	

* S-ANA titer ≥80 was regarded as the cut-off value for ANA-positivity
* *Chi-square test with Fisher's exact 2-sided significance level

Figure legends

Figure 1. Kaplan-Meier survival analysis for 4-year all-cause mortality in n=258 nonagenarians. Subjects were divided into categories 1 and 2 using the median cf-DNA concentration as a cut-off as follows: 1 = low cf-DNA level category (≤0.707 µg/ml) and 2 = high cf-DNA level category (>0.707 µg/ml) (Fig A). The total numbers of deaths/individuals were 63/130 in category 1 and 82/128 in category 2 (Fig A). Subjects were also dived into quartile categories 1, 2, 3, and 4 based on 25% cut-offs for the cf-DNA concentration as follows: 1: <0.651 µg/ml, 2: 0.651-0.707 µg/ml, 3: 0.708-0.811 µg/ml, 4: ≥0.812 µg/ml (Fig B). The total numbers of deaths/individuals were 31/64 in category 1, 32/65 in category 2, 35/65 in category 3 and 47/64 in category 4 (Fig B).

* Log rank significance test for pairwise comparison between categories

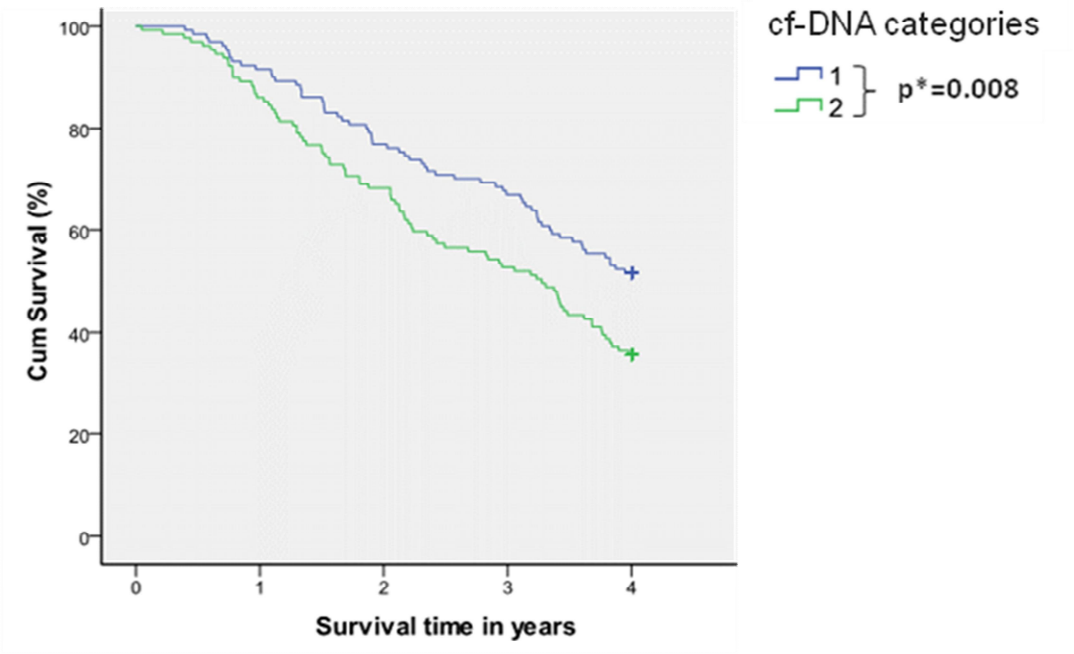
Figure 2. Analysis of the qualitative cf-DNA patterns. Examples of sample coding. Thecf-DNA patterns are displayed as sample electropherograms and were coded as follows: "0"= no low molecular-weight cf-DNA or fragments observable (A) and "1"= a low-molecular-weight cf-DNA band or cf-DNA fragments observable (B). Samples were assayed with Agilent's High Sensitivity Lab-on-a-chip DNA assay and with Expert 2100 software. The 35 bp peak represents the low DNA marker and the 10380 bp peak represents the high DNA marker.

Abbreviations: L, ladder; bp, base pairs; FU; fluorescence unit; S, sample

Supplementary Figure 1 (S1). Assessment the total cf-DNA quantity in 30 randomly chosen samples with the Quant-iT^{TM} DNA High-Sensitivity Assay kit after the different centrifugation protocols (700g, 700g + 1600g and 700g +1600g +1600). No statistically significant associations we observed between the total cf-DNA concentration and the different centrifugation protocols (Mann-Whitney U-test, all centrifugation protocols tested pairwise).

Figures

A.



B.

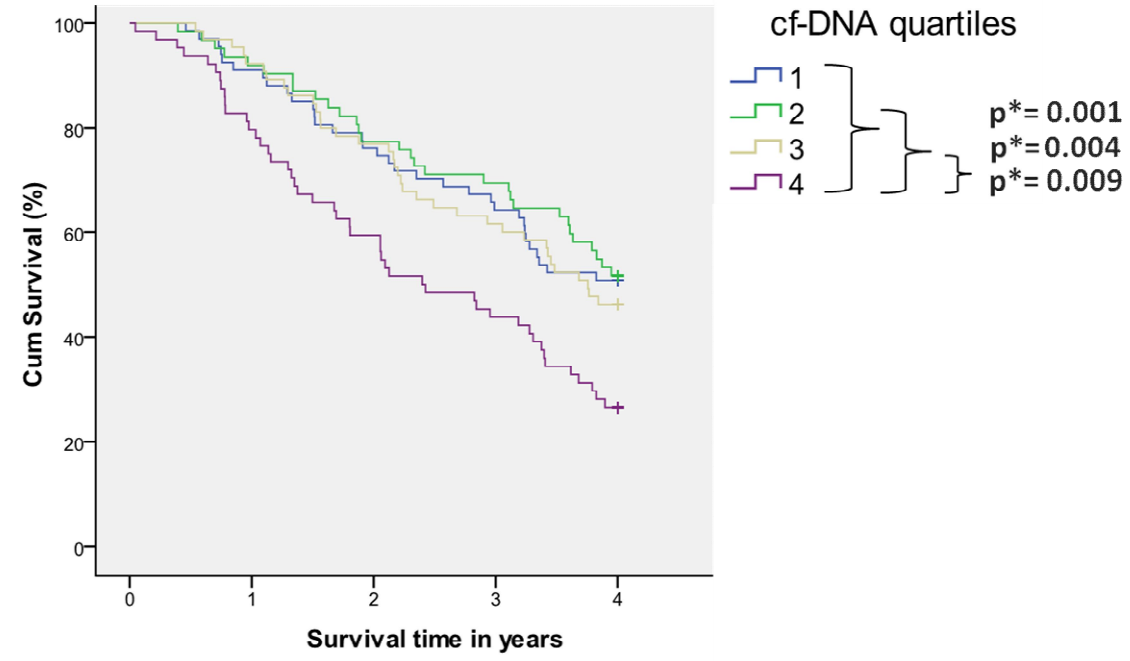
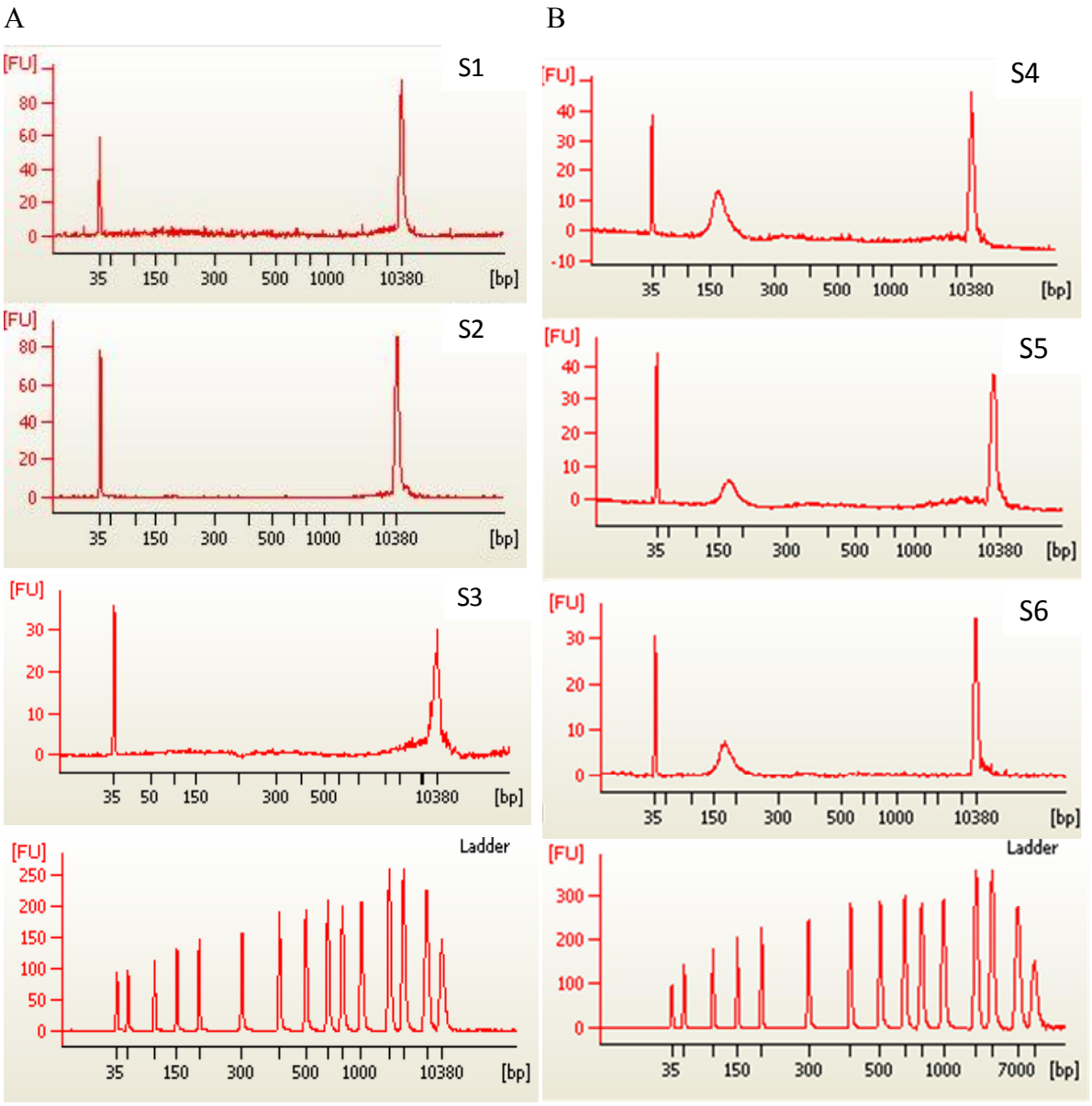


Figure 1.


A
B
S1
S2
S3
S4
S5
S6
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35
50
150
300
500
1000
7000
10380

Figure 2.

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A Genome-Wide Association Study Identifies *UGT1A1* as a Regulator of Serum Cell-Free DNA in Young Adults: The Cardiovascular Risk in Young Finns Study

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Abstract

Introduction: Circulating cell-free DNA (cf-DNA) is a useful indicator of cell death, and it can also be used to predict outcomes in various clinical disorders. Several innate immune mechanisms are known to be involved in eliminating DNA and chromatin-related material as part of the inhibition of potentially harmful autoimmune responses. However, the exact molecular mechanism underlying the clearance of circulating cf-DNA is currently unclear.

Methods: To examine the mechanisms controlling serum levels of cf-DNA, we carried out a genome-wide association analysis (GWA) in a cohort of young adults (aged 24–39 years; n = 1841; 1018 women and 823 men) participating in the Cardiovascular Risk in Young Finns Study. Genotyping was performed with a custom-built Illumina Human 670 k BeadChip. The Quant-iT^{TM} high sensitivity DNA assay was used to measure cf-DNA directly from serum.

Results: The results revealed that 110 single nucleotide polymorphisms (SNPs) were associated with serum cf-DNA with genome-wide significance ($p < 5 \times 10^{-8}$). All of these significant SNPs were localised to chromosome 2q37, near the *UDP-glucuronosyltransferase 1 (UGT1)* family locus, and the most significant SNPs localised within the *UGT1 polypeptide A1* (*UGT1A1*) gene region.

Conclusion: The UGT1A1 enzyme catalyses the detoxification of several drugs and the turnover of many xenobiotic and endogenous compounds by glucuronidating its substrates. These data indicate that UGT1A1-associated processes are also involved in the regulation of serum cf-DNA concentrations.

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Introduction

Circulating cell-free DNA (cf-DNA) was first described in 1940s [1], and it has frequently been used as a convenient indicator of cell death and tissue damage in various acute and chronic disorders [2–4]. Detectable amounts of cf-DNA are present in healthy individuals, yet significantly increased plasma/serum levels of cf-DNA have been reported in patients with acute cardiovascular disease, sepsis, cancer and pre-eclampsia [2–4]. Circulating nucleosomal DNA has also been proposed as a major autoantigen in systemic lupus erythematosus [5] and as a potential player in lupus nephritis [6]. Additionally, genomic DNA fragments released during sterile thyroid injury may also trigger autoreactivity-related thyroid dysfunction [7]. Other potentially detrimental functions implicated for cf-DNA include the horizontal transfer of oncogenic sequences (i.e. genometastasis) [8] and the creation of a proinflammatory milieu in the circulation [9]. Endogenous or microbial DNA are also known to act as alerting signals to the host immune system, and various soluble, membrane-bound and intracellular receptors that can recognise DNA have been

Table 1. Characteristics of the study population.

Variable	Women (n = 1018)		Men (n = 823)		p for difference
	Mean	S.D.	Mean	S.D.	
Age (years)	31.7	5.0	31.7	5.0	N.S.
BMI (kg/m^2)	24.4	4.5	25.7	4.2	<0.001
Waist circumference (cm)	79.2	11.2	90.0	11.0	<0.001
Systolic blood pressure (mmHg)	113	12	121	13	<0.001
Diastolic blood pressure (mmHg)	69	10	73	11	<0.001
Total cholesterol (mmol/L)	5.02	0.87	5.20	1.00	<0.001
HDL-cholesterol (mmol/L)	1.39	0.29	1.16	0.28	<0.001
LDL-cholesterol (mmol/L)	3.11	0.76	3.40	0.89	<0.001
Triglycerides (mmol/L)*	1.0	0.8–1.3	1.2	0.9–1.8	<0.001
Insulin (mU/L)*	6	5–9	6	4–9	N.S.
Glucose (mmol/L)*	4.9	4.6–5.1	5.1	4.9–5.4	<0.001
Homocysteine (μmol/L)*	8.5	7.2–10.1	10.1	8.7–11.7	<0.001
cf-DNA (μg/ml)	0.755	0.135	0.862	0.111	<0.001
CRP (mg/L)*	0.84	0.35–2.30	0.59	0.30–1.42	<0.001
Alcohol (drinks per week)*	2	0–5	6	1–13	<0.001
Smoking daily (% of total)†	19.3		29.4		<0.001
Use of COCs (% of total)	24.9				

*Median values and interquartile range (IQR), Mann-Whitney test for between-group differences.
†Chi-squared test for differences between groups.
Abbreviations: BMI,body mass index; cf-DNA, cell-free DNA; COCs, combined oral contraceptives; CRP, C-reactive protein; N.S., not significant.
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identified. These receptors include pattern recognition molecules such as collectins, ficolins, pentraxins, soluble CD14 and C1q [10]; Toll-like receptors (TLRs) [11]; the high-mobility group box (HMGB) proteins [12]; components of the inflammasome [13]; and extrachromosomal histone H2B [7]. Upon sensing DNA, these receptors can mount an immuno-inflammatory response to eliminate the circulating DNA and prevent anti-DNA autoimmunity.

Despite its proven utility in diagnosis and in determining various clinical conditions, many aspects ofthe origin, metabolism and non-pathological fluctuations of cf-DNA remain unresolved. According to the current understanding, the majority of cf-DNA is derived from apoptotic or necrotic cells; however, active secretion by leukocytes may also contribute to the pool of circulatory DNA [4]. Serum cf-DNA is hypothesised to consist of both free DNA and particle-bound forms [4], yet the relationships between cf-DNA composition, originating cell types and the given clinical conditions are unclear.

Studies in mice have demonstrated that injected DNA, including single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), nucleosomal core particles and oligonucleotides, is cleared from the system very rapidly, in less than 40 minutes, and that the major organ responsible for the solubilisation and generation of DNA degradation products is the liver [14–16]. Nevertheless, the clearance kinetics of these distinct DNA speciesare somewhat different, and the induction of the acute phase response or co-injection of the acute phase reactants serum amyloid P (SAP) and C-reactive protein (CRP) causes a delay in chromatin clearance, concomitant with an increased localisation of cf-DNA to the liver [17]. However, it has also been demonstrated that macrophages play an essential role in processing the cf-DNA released from dead or dying cells [18,19]. Data regarding the corresponding cf-DNA dynamics in humans are very scarce, but it has been reported that foetal cf-DNA has a mean half-life of 16.3 minutes in the maternal plasma and that plasma nucleases have only a partial role in the removal of foetal DNA [20].

To identify the genetic determinants of serum cf-DNA levels and thus elucidate the mechanisms involved in cf-DNA turnover, we performed a genome-wide association analysis (GWA) in a cohort of young adults participating in the Cardiovascular Risk of Young Finns study.

Results

The characteristics of the study population are presented in Table 1. In addition to age and insulin concentration, all of the variables tested differed significantly between women and men (Table 1). In the GWA, a total of 110 single nucleotide polymorphisms (SNPs) were associated with serum cf-DNA levels with a genome wide significance level of $p<5\times10^{-8}$. All 110 SNPs localised to the *UDP-glucuronosyltransferase 1 (UGT1)* family locus on chromosome 2q37, and the most significant SNPs resided in the *UGT1 polypeptide A1* (*UGT1A1)* gene region (Figure 1 and Figure 2). In the *UGT1* gene family region, we observed a major haploblock that could be further divided into nine sub-blocks (using the four gamete rule in HaploView). The four top SNPs on *UGT1A1* belong to the sub-block no. 8, which spans approximately 11 kb, and these SNPs appeared to be in virtually perfect linkage ($r^2 = 0.9991-1.0$). None of the significant SNPs in the sub-block no. 8 resided in exonic regions. However, among the significant SNPs outside this haploblock, three non-synonymous SNPs (rs6759892, rs2070959, rs1105879, $p = 8.2\times10^{-18}-3.4\times10^{-14}$) were identified in the coding region of *UGT1A6* (Figure 2). In addition, three significant SNPs (rs17864701, rs4663967, rs6741669, $p = 1.2\times10^{-21}-1.1\times10^{-17}$) were detected in the

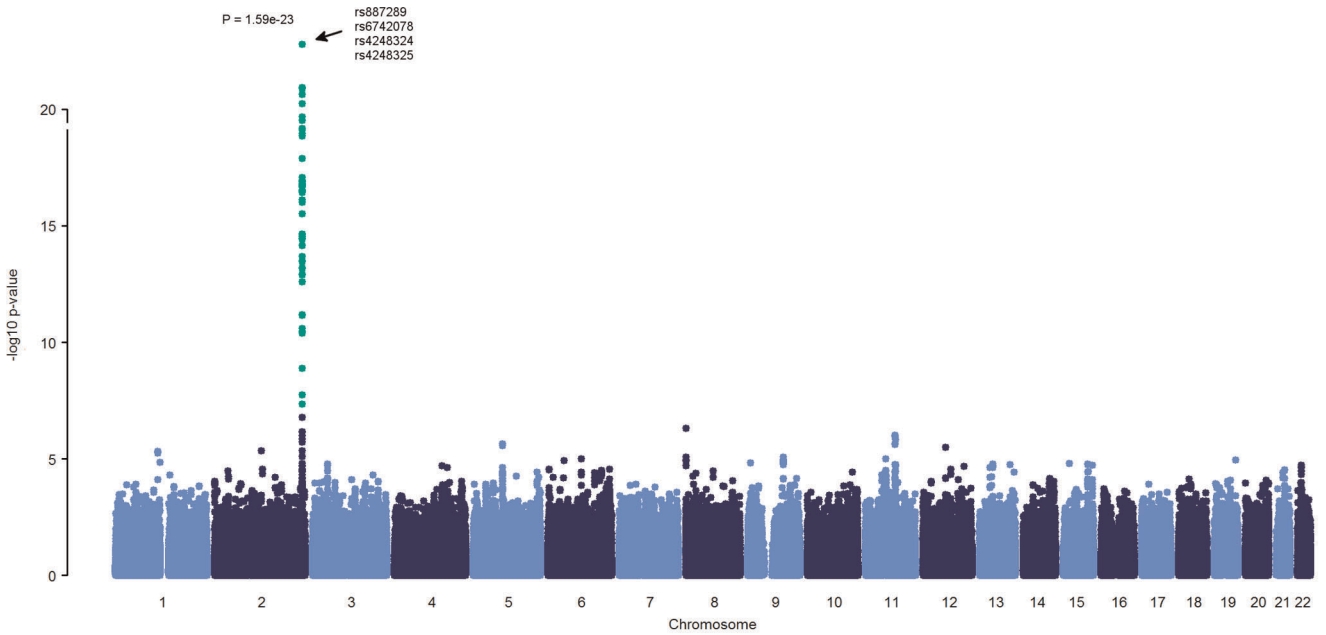

Figure 1. Manhattan plot of the genome-wide analysis of SNPs associated with serum cf-DNA levels. SNPs with significant genome-wide p-values ($p<5\times10^{-8}$) are indicated in green.
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promoter region of *DNAJB3* (Figure 2). The top SNPs in each of the nine sub-blocks are presented in Table 2.

When the selected tag SNPs from each sub-block were analysed using the stepwise AIC model selection algorithm, only the tag SNP rs4148324 which identifies the sub-haploblock no. 8 containing the four top SNPs, remained in the model. This SNP was found to explain 5.3% of the total variation in serum cf-DNA levels. In addition, conditioning the analysis on the top SNP rs4148324 (i.e. additional adjustment of the model with the top SNP) did not reveal any other SNPs associated with cf-DNA levels at a genome-wide significance level (all p-values>0.4).

An interaction network for UGT1A1 constructed with IPA (Ingenuity Pathway Analysis) revealed that several molecules in the UGT1A1 interaction network are connected to DNA metabolism, quantity, fragmentation and synthesis of DNA (Figure 3). In addition, molecular connections to HMGB signalling and TLR signalling were observed for UGT1A1 in the IPA Canonical Pathway analysis (Figure 3)

Discussion

The circulating cf-DNA concentration has been shown to increase during pathological processes, reflecting the rate of cellular death and tissue damage. However, the regulation and metabolism of cf-DNA have received much less attention, and data regarding the turnover of cf-DNA are scarce. In this study, we demonstrate that a novel candidate gene, UGT1A1, may contribute to the regulation of serum cf-DNA in young adults. The UGTs comprise a family of enzymes that primarily transfer glucuronic acid to hydroxyl, carboxylic acid and amine group-bearing compounds to facilitate the catabolism and clearance of these compounds [21]. Because a wide variety of compounds contain these groups, glucuronidation provides a common mechanism for the elimination of several endobiotics, xenobiotics and drugs. The *UGT1A* locus encodes several isoforms of the enzyme, each with a tissue-specific expression pattern [21]. Although the liver is the major producer of UGT1A1, the expression of this isoform has also been detected in the bile duct, the stomach, the small intestine, the kidneys, the bladder, the uterus and the colon [21,22]. Interestingly, the expression of UGT1A1 has recently been demonstrated in human peripheral blood mononuclear cells [23].

Due to the strong linkage disequilibrium between the SNPs in the *UGT1* region (Figure 2) we cannot completely rule out the involvement of the other significant SNPs, such as those in the coding region of *UGT1A6* or in the promoter of *DNAJB3*, in the regulation of cf-DNA levels. Nevertheless, the main signal can be confined to the sub-block of the four top *UGT1A1* SNPs because the significance of the p-values of the other SNPs decreases along with the increase in distance from this sub-block (Table 2 and Figure 2).

One of the best characterised functions of UGT1A1 is the glucuronidation of bilirubin [21], and numerous recent GWA studies have identified *UGT1A1* alleles as the major regulators of serum bilirubin levels [24]. The four most significant SNPs associated with serum cf-DNA levels in this study (rs4148324, rs6742078, rs4148325 and rs887829) have also been identified as regulators of serum bilirubin concentrations [24]. The effect of these SNPs on bilirubin levels is, however, reported to be due to a functional TA-repeat polymorphism in the *UGT1A1* promoter TATA-box area; recent conditional linkage scans of this TA-repeat association demonstrated that it accounts for the linkage peaks observed for the other associated loci in the gene region [25,26]. Given that the ancestral alleles of the rs4148324, rs6742078, rs4148325 and rs887829 SNPs are in strong linkage with the wild-type form of the TA-repeat polymorphism (TA_6, rs8175347), and because all of these variants belong to the same haploblock in our population, we assume that the same regulation pattern holds true for both serum cf-DNA and bilirubin levels. The TA_6 form of the repeat polymorphism is associated with lower bilirubin levels due to higher promoter activity (via optimal transcription factor IID binding), resulting in higher UGT1A1 enzyme levels and enhanced bilirubin glucuronidation and

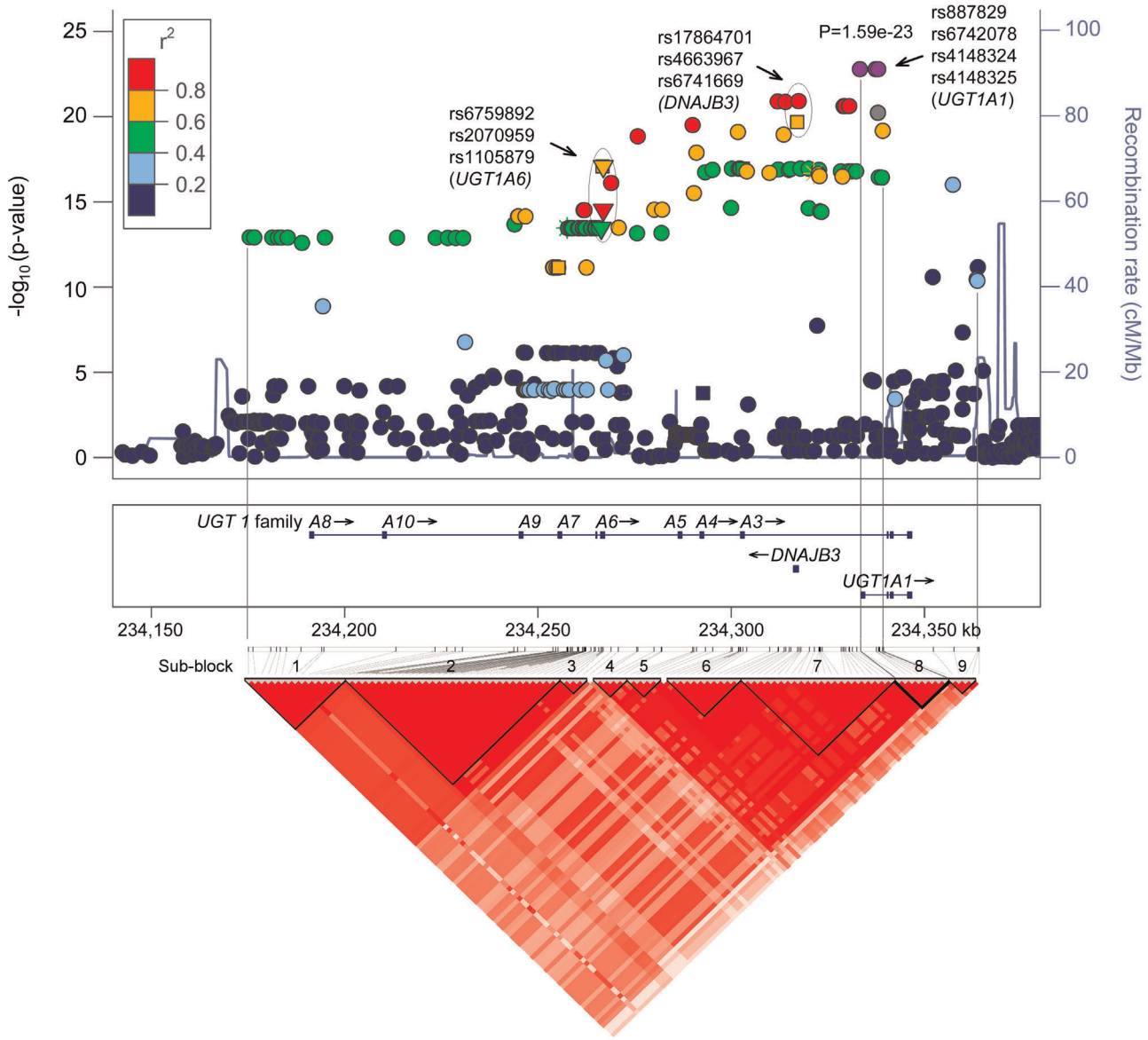

Figure 2. *UGT1* regional association plot and haploblock structure of the genome-wide analysis of SNPs associated with serum cf-DNA levels. The violet circles indicate the top SNPs in the *UGT1A1* region, the circled triangles indicate the non-synonymous SNPs in *UGT1A6* region, and the circled squares and circles indicate the SNPs in the *DNAJ3B* promoter region. The colour of the spots indicates LD (data from HapMap II CEU) with the index SNPs (violet spots). The blue line shows the recombination rate across the region (data from HapMap II CEU). The nine sub-blocks in the major haploblock are separated with black lines. The sub-block containing the four top SNPs is separated with a thick black line.
doi:10.1371/journal.pone.0035426.g002

clearance [27]. Because we observed lower cf-DNA levels in subjects bearing the wild type alleles (ß = −0.3358) of the four top SNPs in comparison with the minor allele carriers, we suggest that higher UGT1A1 production, or some cellular process associated with UGT1A1 induction, contributes to more effective turnover of serum cf-DNA.

Animal studies have demonstrated that the liver is the major site of cf-DNA clearance, although some portion of the circulating DNA also localises to other organs, such as the kidneys and spleen [14,16,17]. However, in humans, foetal cf-DNA has been shown to be cleared rapidly from the maternal circulation, and plasma nucleases have only a partial effect on cf-DNA disposal [20]. Moreover, relatively large DNA fragments (>150 base pairs) originating from non-renal or urinary tract tissues have been detected in the urine [28], indicating that the complete digestion of cf-DNA by nucleases is not required for its excretion. Beyond these observations, the metabolism and fates of different cf-DNA forms are unknown. Although our data does not provide a mechanistic basis of UGT1A1-mediated cf-DNA clearance, we propose that the metabolism of cf-DNA may be facilitated by UGT1A1, either directly by glucuronidation or indirectly via the interactions between UGT1A1 and the molecules related to DNA sensing and processing (Figure 3). It is also possible that a portion of the cf-DNA is endocytosed and/or co-processed with the traditional UGT1A1 substrates, such as the bilirubin-albumin complex [29].

Nevertheless, physiological interactions between UGT1A1 and nucleotides have been demonstrated [30] and the glucuronidation of a 2′,3′-dideoxynucleoside compound, the 3′-Azido-3′-deox-

Table 2. The top SNPs in each of the 9 sub-blocks in the *UGT1A* gene region (see Figure 2).

SNP†	Sub-block	Locus (bp)	Ancestral allele	Minor allele	MAF	β (S.E.)	p*	Imputed	Location
rs17864683	1	234243948	A	C	0.3221	0.3246 (0.0352)	2.06E−14	yes	intron, UGT1A10
rs10168416	2	234261826	G	C	0.4230	0.3236 (0.0325)	2.98E−15	no	intron, UGT1A8
rs1105879	3	234266941	C	A	0.4603	0.3127 (0.0324)	8.20E−18	no	missense, UGT1A6
rs17863787	4	234275833	G	T	0.3592	0.3092 (0.0338)	1.40E-19	no	intron, UGT1A6
rs6744284	5	234290036	T	C	0.3710	0.2818 (0.0335)	3.04E−20	no	intron, UGT1A6
rs6722076	6	234312056	A	G	0.3705	0.2824 (0.0334)	1.25E−21	yes	intron, UGT1A6
rs17864701	7	234317456	T	C	0.3722	0.2591 (0.0335)	1.19E−21	yes	intron, UGT1A6
rs887829	8	234333309	A	G	0.3983	0.3358 (0.0331)	1.59E−23	no	intron, UGT1A1
rs6742078		234337378	T	G	0.3983	0.3358 (0.0331)	1.59E−23	no	intron, UGT1A1
rs4148324		234337461	G	T	0.3983	0.3358 (0.0331)	1.59E−23	no	intron, UGT1A1
rs4148325		234338048	T	C	0.3983	0.3358 (0.0331)	1.59E−23	no	intron, UGT1A1
rs11690786	9	234357356	T	C	0.3839	0.2711 (0.0337)	9.67E−17	yes	intron, HEATR7B1

The four SNPs that are associated most significantly with serum cf-DNA levels are in sub-block 8.
†For each SNP, the ancestral allele was modelled, and the β coefficient represents the change in cf-DNA level (μg/ml) with one additional copy of the ancestral allele.
*Adjusted for age, sex, BMI and genetic East-West stratification in the Finnish population.
Abbreviations: SNP, single nucleotide polymorphism; Chr, chromosome; bp, base pairs; MAF, minor allele frequency; S.E., standard error.
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ythymidine (AZT), has been demonstrated, although this reaction is performed by the UGT2B7 enzyme [31]. However, at the present time, we can only speculate whether these observations are related to the glucuronidation of endogenous cf-DNA during the course of its cellular internalisation or (apoptotic) release. Neither can we exclude the possibility that the actual UGT1A1 substrate might be some lipophilic or proteinaceous structure associated with cf-DNA. Furthermore, the potential involvement of extrahe-

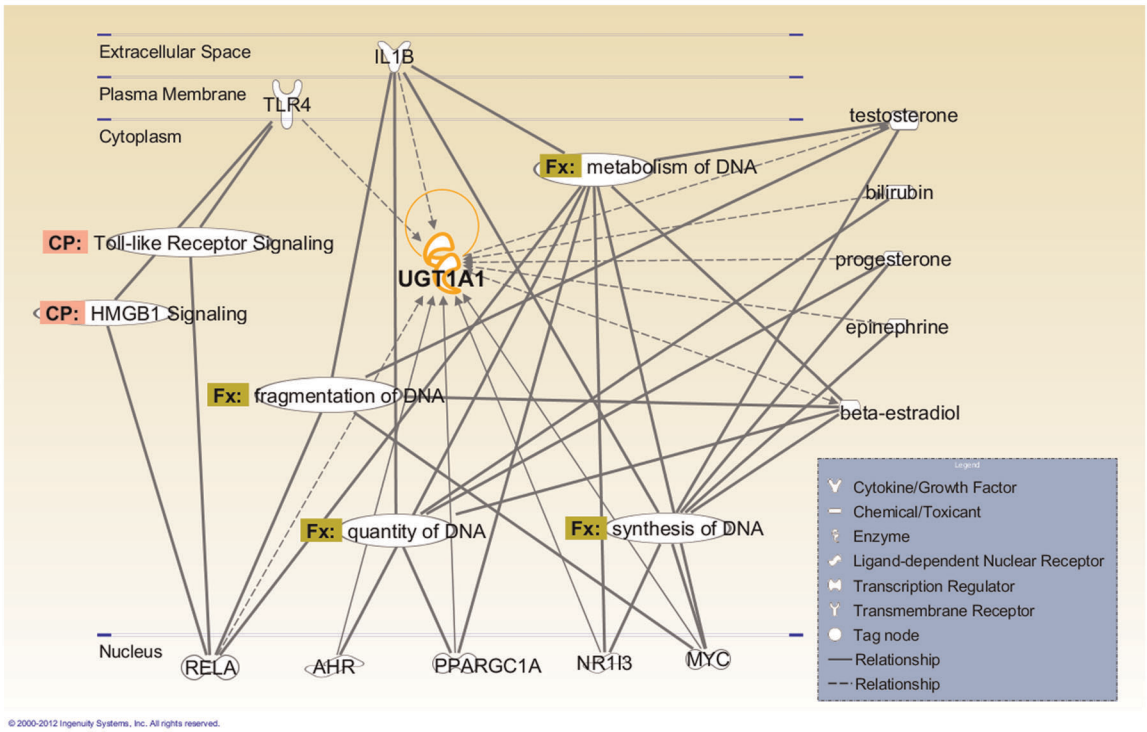

Figure 3. The UGT1A1 interaction network generated using the Ingenuity Pathway Analysis. The molecular relationships connecting UGT1A1 to DNA metabolism synthesis, fragmentation and quantity of DNA are indicated with Fx symbols, and the associated Canonical Pathways are indicated with CP symbols. Abbreviations: AHR, aryl hydrocarbon receptor; HMGB1, high-mobility group box protein 1; IL1B, interleukin-1 beta; MYC, v-myc myelocytomatosis viral oncogene homolog; NR1I3, nuclear receptor subfamily 1, group I, member 3; PPARGC1A, peroxisome proliferator – activated receptor gamma coactivator 1 alpha; RELA, v-rel reticuloendotheliosis viral oncogene homolog A (avian); TLR4, Toll-like receptor 4.
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patic UGT1A1-expression sites, such as leukocytes [23] or intestinal cells [32], in cf-DNA turnover cannot be addressed by our data.

Our statistical model for genetic variation in the UGTA1 explains only 5.3 % of the total variation in cf-DNA levels, although the association is highly statistically significant ($p = 1.6 \times 10^{-23}$). The somewhat modest coefficient of determination can be explained by the complex nature of the serum cf-DNA; different forms of cf-DNA might be assigned to different clearance pathways with dissimilar kinetics and saturability. It is also known that the serum contains higher cf-DNA levels than the plasma, allegedly due to the release of genomic DNA from leukocytes during blood clotting [33] Recently, however, this view has been challenged by the observation that leukocyte rupture is not the major factor causing increased serum cf-DNA levels [34]. Nevertheless, the strong association suggests a significant biological role for UGT1A1 in regulating cf-DNA levels, especially because no other polymorphisms outside the reported UGT1A1 loci emerged with genome-wide significance when the analysis was adjusted using the top SNP (rs4148324).

In conclusion, the results of this GWA study demonstrate that *UGT1A1* polymorphisms are associated with serum cf-DNA levels in young Finns. We propose that UGT1A1-associated processes are, either directly or indirectly, involved in the regulation of cf-DNA concentration. These results, however, are limited to one study cohort which is a major limitation of our study. Therefore the results should be replicated in an independent study cohort, and further investigation to establish the exact role of UGT1A1 in cf-DNA turnover is warranted.

Materials and Methods

Study population

The study population consisted of 1841participants in the Cardiovascular Risk in Young Finns study (aged 24–39 years; 1018 women and 823 men). The study was approved by the local ethics committees (the University Hospitals of Helsinki, Turku, Tampere, Kuopio and Oulu) and was conducted following the guidelines of the Declaration of Helsinki. All participants gave their written informed consent. For a detailed cohort description and the assessment of the variables listed in Table 1, see [35] and the references therein. The data in this study were from individuals with successful cf-DNA measurements and genotyping and who participated in the follow-up study in 2001.

Quantification of serum cf-DNA

Total circulating cf-DNA was determined directly in previously unthawed serum using the fluorescence-based Quant-iT^{TM} high-sensitivity DNA assay kit and a Qubit$^{®}$ fluorometer (Invitrogen, Carlsbad, CA, USA). All samples were analysed in duplicate, and the mean of the two measurements was used as the final value. At the mean serum cf-DNA levels of 0.650 μg/ml, the assessed intra- and inter-day variation coefficients were 2.2% and 4.7%, respectively. At the mean serum cf-DNA level of 1.02 μg/ml, the intra- and inter-day variation coefficients were 3.0% and 5.8%, respectively.

Genotyping

Genotyping was performed at the Welcome Trust Sanger Institute using a custom made Illumina Human 670 k BeadChips. Genotypes were determined using the Illumina clustering algorithm [36]. Fifty-sixsamples failed the Sanger genotyping pipeline QC criteria (i.e., duplicated samples, heterozygosity, low call rate, or Sequenom fingerprint discrepancies). Three samples were removed due to a low genotyping call rate (<0.95) and 54 samples were excluded for possible relatedness (pi.hat>0.2). A total of 11,766 single SNPs were excluded due to deviation from Hardy-Weinberg equilibrium (HWE) test (p≤1e-06), 7,746 SNPs failed the missingness test (call rate<0.95) and 34,596 SNPs failed the frequency test (MAF<0.01). After quality control, 546,677 genotyped SNPs remained available for further analysis. Genotype imputation was performed using MACH 1.0 [37,38] and HapMap II CEU (release 22) samples as reference. After imputation, 2,543,887 imputed SNPs were available. SNPs with squared correlations ≥0.3 between imputed and true genotypes were considered well imputed.

Statistical methods

The comparison of the basic study population variables presented in Table 1 was conducted with the Student's t-test, Mann-Whitney's test or chi-squared test. Prior to the GWA, all continuous variables were subjected to inverse normal transformation to minimise the incidence of type I errors and to reduce the impact of outliers [39]. A stepwise backward model (Akaike information criterion, AIC) was used to determine which covariates explained the most of the variation in the cf-DNA levels. In addition to the variables listed in Table 1, genetic main identity-by-descent (IBD) clustering components and interactions between age and gender as well as smoking status and the use of alcohol were included in the model. The variables that remained in the model and were thus used as the adjustment covariates in GWA were gender, age, systolic blood pressure, fasting glucose, triglycerides, CRP, homocysteine, daily smoking, use of alcohol, use of combined oral contraceptives, main IBD components and the gender*age and daily smoking*use of alcohol the interaction terms. These factors were observed to account for 30.5% of the variation in cf-DNA levels. To reveal other SNPs potentially associated with serum cf-DNA, the analysis was additionally adjusted with the top SNP, rs4148324. Standardised residuals were extracted from the model, and association analysis was performed using linear regression with an assumption of an additive genetic effect. PLINK [40] was used for the true genotyped SNPs, and ProbABEL [41] was used for imputed genotype dosages. If the same SNP was available in both genotyped and imputed form, the genotyped form was displayed and included in the results. A commonly accepted threshold for genome-wide statistical significance level ($p < 5 \times 10^{-8}$) was used to identify significant SNPs. Manhattan and Q-Q plots were drawn to confirm the validity of the analysis. The genomic inflation factor (lambda) was 0.9996475. The variance in serum cf-DNA level explained by each SNP (R^2) was calculated as follows: $R^2 = 1/[1+(\text{number of samples}-2)\times(\text{SE of SNP/beta estimate of SNP})^2]$. The individual genotype data for genome-wide significant SNPs in the same region were extracted from both the genotyped and imputed data; then the allele dosages of the imputed SNPs were rounded to best guess genotypes and transferred to Haploview [42] for haploblock analysis and tag SNP identification. Haploblocks were defined using the four gamete rule in Haploview, and the SNP with the lowest p-value in each haploblock was selected as the tag SNP. All tag SNPs were analysed with stepwise backward AIC to identify the potential independent signals in different haploblocks. Unless otherwise noted, the statistical analyses were performed the appropriate packages in R (MASS for stepwise AIC).

To identify the potential interactions via which UGT1A1 could be connected to cf-DNA regulation, a graph of the UGT1A1 molecular network and associated functions and Canonical Pathways was generated using IPA (Ingenuity® Systems, www.

ingenuity.com). In the IPA network analysis, the biological relationship between two molecules is represented as an edge (continuous or dashed line). All edges are supported by at least one reference from the literature, textbook or canonical information stored in the Ingenuity Knowledge Base.

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Author Contributions

Performed the experiments: JJ LPL. Analyzed the data: JJ LPL JK. Contributed reagents/materials/analysis tools: TL MH JV OTR MK NHK. Wrote the paper: JJ LPL.

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Characterization of the role of distinct plasma cell-free DNA (cf-DNA) species in age-associated inflammation and frailty

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Summary

Plasma cell-free DNA (cf-DNA) has recently emerged as a potential biomarker of aging, reflecting systemic inflammation and cell death. In addition, it has been suggested that cf-DNA could promote autoinflammation. Because the total cf-DNA pool comprises different cf-DNA species, we quantified the plasma levels of gene-coding cf-DNA, *Alu* repeat cf-DNA, mitochondrial DNA (mtDNA) copy number and the amounts of unmethylated and total cf-DNAs. We identified the relationships between these cf-DNA species and age-associated inflammation, immunosenescence and frailty. Additionally, we determined the cf-DNA species-specific transcriptomic signatures in blood mononuclear cells to elucidate the age-linked leukocyte responses to cf-DNA. The study population consisted of n=144 nonagenarian participants of the Vitality 90+ Study and n=30 young controls. In the nonagenarians, higher levels of total and unmethylated cf-DNAs were associated with systemic inflammation and increased frailty. The mtDNA copy number was also directly correlated with increased frailty but not with inflammation. None of the cf-DNA species were associated with immunosenescence. The transcriptomic pathway analysis revealed that higher levels of total and unmethylated cf-DNAs were associated with immunoinflammatory activation in the nonagenarians but not in the young controls. The plasma mtDNA appeared to be inert in terms of inflammatory activation in both the nonagenarians and young controls. These data demonstrate that the plasma levels of total and unmethylated cf-DNA and the mtDNA copy number could serve as biomarkers of frailty. In addition, we suggest that circulating self-DNA, assessed as total or unmethylated cf-DNA, might aggravate immunoinflammatory reactivity in very old individuals.

Introduction

Following tissue damage or conditions involving cellular stress and death, cells release cell-free DNA (cf-DNA) into the circulation. The concentration of plasma cf-DNA typically reflects the magnitude of damage and inflammation, and it has been shown that cf-DNA levels have predictive value in certain acute conditions (Butt and Swaminathan 2008). We (Jylhava *et al.* 2012) and others (Fournie *et al.* 1993) have introduced cf-DNA to the field of aging biomarkers suggesting that it serves as a novel biomarker of age-associated low-grade inflammation (inflammaging), and age-associated systemic decline. However, methods for assessing the levels of plasma cf-DNA have typically relied on the PCR-based quantification of a given coding sequence, even though the total cf-DNA pool is composed of different cf-DNA molecules in terms of quantity and quality. Specifically, the total cf-DNA pool comprises nuclear protein-coding, non-coding and repeat sequences as well as mitochondrial DNA (mtDNA). According to a high-throughput sequencing analysis performed in healthy individuals (Beck *et al.* 2009), the plasma cf-DNA sequence representation mirrors that of the genome; however, an overrepresentation of *Alu* repeat sequences in cf-DNA has been demonstrated, suggesting that repeat sequences are actively secreted in living cells (Stroun *et al.* 2001). In addition, exceptionally high concentrations of mtDNA have been detected in the plasma of trauma patients (Zhang *et al.* 2010), and variation in the cf-DNA methylation status is known to accompany a variety of conditions ranging from cancer to neurological and psychiatric diseases (Levenson and Melnikov 2012). However, all cf-DNA species can be captured using a single dsDNA-intercalacting dye-based measurement that quantifies the total cf-DNA irrespective of the sequence and fragment length. Therefore, in this study, we quantified the plasma levels of gene-coding cf-DNA (*RNAse P* gene), *Alu* repeat cf-DNA, and mtDNA copy number and determined the amount of unmethylated cf-DNA relative to the total cf-DNA, and analyzed these cf-DNA species separately to elucidate their role in age-associated inflammation and frailty.

The age-associated remodeling of the immunoinflammatory compartment typically manifests as a chronic inflammatory state, increased reactivity to self-antigens, including DNA (Agrawal *et al.* 2009), and a decline in the adaptive immune branch (Franceschi *et al.* 2000). Other body systems, such as those at the musculoskeletal and cognitive axes, also undergo a notable age-associated deterioration (Hunt *et al.* 2010). The generalized age-associated increased vulnerability to environmental and endogenous stressors can be defined as frailty, a condition in which the individual is at the limit of his/her physiological reserves in more than one homeostatic system

(Flicker 2008). Although inflammaging and frailty are often associated with chronic multimorbidity, they are not equivalent to it, but are indicative of systemic senescence and disability (Hubbard and Woodhouse 2010, Hunt *et al.* 2010). In keeping with this, chronic disability as well as the traditional markers of inflammaging, such as C-reactive protein (CRP) and interleukin-6 (IL-6), are stronger mortality predictors than multimorbidity (Marengoni *et al.* 2009). Longitudinal studies have also reported that elevated levels of CRP, IL-6 and tumor necrosis factor alpha (TNF-α) are independently associated with disability, frailty and muscle strength decline, suggesting that inflammation may be causally related to skeletal muscle catabolism and tissue homeostasis (Roubenoff 2003, Hubbard and Woodhouse 2010, Li *et al.* 2011). The potential value using cf-DNA as a biomarker can be attributed to the fact that virtually every tissue and cell type can release cf-DNA following death or injury. Consequently, any immune-competent cell capable of endocytosing cf-DNA can evoke a response to it, which depending on the motifs in the cf-DNA can be immunostimulatory, suppressive, or inert (Ishii and Akira 2005, Pisetsky 2007). In effect, accumulating evidence has demonstrated that endogenous DNA can serve as a danger-associated molecular pattern (DAMP) capable of triggering host immune responses (Ishii and Akira 2005). Potent immunostimulatory motifs in mammalian DNA include the unmethylated deoxycytidyldeoxyguanosine (CpG) stretches that resemble those found in bacterial DNA and in CpG oligodeoxynucleotide vaccine adjuvants (Ishii and Akira 2005, Pisetsky 2007). The role of unmethylated DNA acting as a key DAMP has been demonstrated recently *in vitro* by Agrawal et al. (2010) who observed that the level of global DNA methylation decreases with age, concomitantly with its increasing immunogenity (Agrawal *et al.* 2010).

The activity of endogenous DNA can also be determined by the components that are complexed with it. The factors known to render self-DNA immunostimulatory include the high mobility group box protein 1 (HMGB1) (Pisetsky 2007, Urbonaviciute *et al.* 2008), cathelicidin antimicrobial peptide (CAMP) (Lande *et al.* 2007) and the anti-chromatin antibodies (Leadbetter *et al.* 2002, Boule *et al.* 2004). The DNA originating from necrotic cells, undigested apoptotic cells or from macrophages that have digested the contents of apoptotic cells, is first encountered by the cell membrane and/or endosomal receptors that include the receptor for advanced glycation end products and the Toll-like receptor 9 (Ishii and Akira 2005, Pisetsky 2007). In addition, chromatin-immunocomplexes can also be internalized via the dendritic cell (DC) Fcγ receptors or the B cell receptor, and can be accompanied by immunostimulatory activity in the recipient cells (Leadbetter *et al.* 2002, Boule *et al.* 2004, Avalos *et al.* 2010).

In this study, we had two aims. First we determined the associations between the cf-DNA species (total cf-DNA, unmethylated cf-DNA, *RNAse P*-coding cf-DNA, *Alu* repeat cf-DNA

and mtDNA copy number) with immunoinflammatory parameters and frailty. Second, following our observations in the previous Vitality 90+ study (Jylhava *et al.* 2012) in which we demonstrated that the total cf-DNA level predicted a 4-year all-cause mortality and reflected the systemic low-grade inflammation, we investigated whether these associations could be linked with leukocyte immunoinflammatory responses, and if so, which of the cf-DNA species were responsible. To this end, we analyzed the cf-DNA species-specific genome-wide transcriptomic signatures in peripheral blood mononuclear cells (PBMCs), cells that are in constant contact with cf-DNA and involved in its turnover. The analyses were performed separately in the nonagenarians and young controls to delineate the age-associated characteristics of the cf-DNA and to elucidate its role a biomarker of aging.

Results

The characteristics and distribution of the study variables are presented in Table 1. In both the nonagenarians and young controls, a strong intercorrelation was observed between the total cf-DNA level and the unmethylated cf-DNA level and between and the levels of *RNase P*-coding cf-DNA and *Alu* repeat cf-DNA (Table 2). The mtDNA copy number did not correlate with any of the other cf-DNA species (Table 2) or the immunoinflammatory markers. A direct correlation between the levels of the proinflammatory mediators CRP and IL-6 and the total cf-DNA and unmethylated cf-DNA was observed only in the nonagenarians but not in the young controls (Table 2).

In the nonagenarians, the amounts of total cf-DNA, unmethylated cf-DNA and mitochondrial cf-DNA displayed a range of associations with the markers and indices of functional performance and frailty (Table 2) but not with the markers of immunosenescence (proportions of CD4+CD28- and CD8+CD28- cells) or the proportions of CD3+, CD4+ and CD8+ lymphocytes or monocytes (CD14+ cells) (Table 2). Similarly in the young controls, correlations were not observed between the plasma levels of the cf-DNA species and the leukocyte proportions (Table 2).

In the bioinformatics analysis, we assessed the transcriptomic signatures for the total cf-DNA level and the unmethylated cf-DNA level because of their correlation with the inflammatory markers. The transcriptomic signatures for the mtDNA copy number were assessed because of previous findings demonstrating an mtDNA-induced inflammatory response (Zhang *et al.* 2010). For the pathway analysis (IPA), we included the transcripts (assessed using the Chipster correlation tool) exhibiting expression levels in the PBMCs that correlated with the concentrations

of total cf-DNA, unmethylated cf-DNA and mitochondrial cf-DNA. The top 250 transcripts exhibiting expression levels that correlated with the plasma levels of total cf-DNA, unmethylated cf-DNA and mtDNA copy number in the nonagenarians and young controls are presented in Supporting Tables 1, 2 and 3 (S1, S2 and S3), respectively. The 10 most statistically significant canonical pathways harbored by the transcripts that exhibited expression levels correlating with the levels of total cf-DNA, unmethylated cf-DNA and mtDNA copy number are presented in Tables 3, 4, and 5, respectively. In addition, all the statistically significant canonical pathways harbored by these transcripts are presented in Supporting Tables 4, 5 and 6 (S4, S5 and S6). Although only seven significant canonical pathways were identified for the total cf-DNA level-correlated transcripts in the young controls and 10 significant canonical pathways were identified for the mtDNA copy number -correlated transcripts in the nonagenarians, to maintain uniformity, these pathways are presented as supporting Tables S4b and S6a.

In the bioinformatics analysis, notable differences were observed for the correlated transcripts and the resultant canonical pathways across the different cf-DNA species between the nonagenarians and the young controls (S1-S6). In the nonagenarians, a notable overlap was observed in the transcripts with expression levels that correlated with the levels of total cf-DNA and unmethylated cf-DNA (S1a and S2a). This overlap was also observed in the corresponding canonical pathways of which the majority was involved in immunoinflammatory responses or cytoskeleton/integrin-associated signaling (Tables 3a and 4a, S4a and S5a). In contrast, in the young controls, the correlated transcripts (S1b and S2b) and the resulting canonical pathways for the total cf-DNA level and unmethylated cf-DNA level were different; the pathways for the total cf-DNA level-correlated transcripts largely represented cellular turnover-related processes, whereas the pathways for the unmethylated cf-DNA level were largely involved in immune signaling. Interestingly, however, most of these immune-related transcript expression levels were inversely correlated with the amount of unmethylated cf-DNA (Tables 3b and 4b, S3b and S4b). In both the nonagenarians and young controls, the canonical pathways for the mtDNA copy number-correlated transcripts covered cellular signaling, metabolism and hormone signaling pathways (Tables 5a, 5b, S6a and S6b). In addition, three integrin/cytoskeleton-related pathways (*Paxillin Signaling*, *Integrin Signalling* and *ILK Signaling*) were identified in the nonagenarians (Tables 5a, 5b, S6a and S6b). However, pathways did not demonstrate overt immunoinflammatory activation associated with the mtDNA quantity (S6a and S6b).

Discussion

In this study, the concentrations of the total cf-DNA, unmethylated cf-DNA, *RNase P*-coding cf-DNA and *Alu* repeat cf-DNA, were significantly elevated in the nonagenarians compared with the young controls, suggesting that the age-accompanied increase in cellular senescence and death rate is manifested as elevated plasma cf-DNA. Moreover, the observation that higher concentrations of total cf-DNA and unmethylated cf-DNA were directly associated with inflammaging indicates that the plasma levels of these cf-DNA species are associated with central processes of aging, strengthening their role as aging biomarkers. However, we did not observe correlations between the cf-DNA levels and the markers of immunosenescence, indicating that the immune-related associations of cf-DNA are not extended to this niche of adaptive immunity. The finding that the plasma mtDNA copy numbers did not differ between the elderly and the young could reflect the aging-associated depletion of cellular mtDNA (Welle *et al.* 2003), manifesting as a decreased amount of mtDNA in relation to total cf-DNA in plasma. However, conflicting results have been reported with regard to the depletion of cellular mtDNA with age (Miller *et al.* 2003), which necessitates further research to elucidate the basis of our finding.

The observation that the levels of total cf-DNA, unmethylated cf-DNA and mtDNA copy number were directly correlated with the frailty score leads us to speculate that the tissues that are most prominently affected by frailty could be among the primary sources of these cf-DNA species. However, as the total and unmethylated cf-DNA levels were observed to better reflected the "generalized" frailty, including cognitive functions, while the mtDNA quantity was restricted to reflecting the physical aspect of frailty, we hypothesize that these cf-DNA species exhibit a degree of specificity with regard to their originating tissue. Specifically, catabolism-related cellular senescence and death in brain, skeletal muscle and nonmuscle lean tissue could be responsible for the elevation in total and unmethylated cf-DNA levels, whereas the plasma mtDNA level could increase following the depletion of mitochondria in sarcopenic skeletal muscle. Similar to our findings, Swarup et al. (2011) have suggested that the elevated cf-DNA levels observed in subjects with Friedreich's ataxia and spinocerebellar ataxia are due to muscular and neuronal degeneration in the patients (Swarup *et al.* 2011). However, it is also possible that frailty is associated with changes in cellular DNA methylation status in several other cell types as well, all of which can contribute to the pool of unmethylated plasma cf-DNA. For example, Bellizzi et al. (2012) have demonstrated that a lower global DNA methylation status in buffy coat cells is associated with frailty in 65-to 85-year-old individuals but not in ultranonagenarians (Bellizzi *et al.* 2012).

We next asked if the total cf-DNA, unmethylated cf-DNA and mtDNA plasma levels were associated with differential PBMC responses in the nonagenarians and young controls. In the nonagenarians, immunoinflammatory activity dominated the pathways of total cf-DNA and unmethylated cf-DNA levels. Extensive involvement of the integrin signaling and cytoskeleton/actin-remodeling pathways (i.e., *FAK Signaling, ILK Signaling, Signaling by Rho Family GTPases, RhoGDI Signaling, Actin Nucleation by ARP-WASP Complex, Regulation of Actin-based Motility by Rho, Paxillin Signaling and Rac Signaling*) was also observed in these data sets. Remodeling of the cytoskeleton through actin and integrin signaling pathways plays a key role in leukocyte effector functions, such as activation, migration and phagocytosis (Fenteany and Glogauer 2004). In addition, convergence of the immunoreceptor and integrin-ligation signaling pathways has been demonstrated downstream of the T-cell, B-cell and Fc receptors (Abram and Lowell 2007). As the *T and B cell Receptor Signaling* pathways and *Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes* were also identified in these data sets, we propose that conditions associated with high total and unmethylated cf-DNA levels could involve broad activation of the immune system. The identified *mTOR Signaling* pathway may also merge into this network because of its role in controlling antigen-presenting cell activation and T-cell and B-cell receptor pathways (Powell *et al.* 2012). However, the extent to which cf-DNA contributes to the immune system activation and autoinflammation warrants further research.

In the *EIF2 Signaling* pathway, the downregulated expression of several ribosomal proteins was associated with increased levels of total and unmethylated cf-DNA in the nonagenarians. Various stress conditions are known to downmodulate EIF2-regulated global translation accompanied by the selective translation of proteins required for coping with the stressors, mitigating cellular injury or inducing apoptosis (Wek *et al.* 2006). Because our data sets also identified several stress-related pathways (*p53 Signaling, HMGB1 Signaling, mTOR Signaling*, *NRF2-mediated Oxidative Stress Response* and *Production of Nitric Oxide and Reactive Oxygen Species in Macrophages*) and an apoptotic pathway (*Myc Mediated Apoptosis Signaling*), it appears that fluctuation in the plasma levels of total and unmethylated cf-DNA is inherently linked with various cellular stress conditions in the old individuals. Therefore, we hypothesize that high levels of total and unmethylated cf-DNA or the processes associated with elevation of these cf-DNA species could be among the stressors that contribute to attenuated EIF2-regulated global translation.

In the young controls, the pathways identified for the total cf-DNA level were mainly involved in cellular metabolism and cell cycle regulation. Because the *EIF2 Signaling* pathway emerged without any stress-related pathways in this data set, it is likely that the total cf-DNA level in young individuals fluctuates with cellular turnover and is not associated with stress or immune

responses. In contrast, the pathways for the unmethylated cf-DNA level included various immunoinflammatory pathways that exhibited a tendency toward downregulation with increasing concentrations of unmethylated cf-DNA. Thus, it appears that a somewhat reversed situation prevails in the PBMC responses associated with the unmethylated cf-DNA level in the nonagenarians and the young individuals. Tentatively, these observations suggest that cf-DNA might acquire its proinflammatory properties with aging or in the "inflammation-primed" milieu. Indeed, Agrawal et al. (2009) reported that DCs from aged subjects exhibited increased reactivity to human DNA compared to DCs from young subjects. The same group also demonstrated that the age-associated decrease in DNA methylation status led to the enhanced immunogenicity of the DNA when delivered into DCs (Agrawal *et al.* 2010). Furthermore, Atamaniuk et al. (2012) reported that cf-DNA or plasma from end-stage renal disease patients exhibiting continuous innate immunity activation is capable of inducing IL-6 production in monocytes (Atamaniuk *et al.* 2012). Other inherent properties of self-DNA may also contribute to its immunomodulatory capacity. Antagonizing effects of suppressive elements in mammalian DNA have been demonstrated against CpG-driven immune activation; the telomeric sequences (TTAGGG) and their ability to form G-tetrads have been identified as the key suppressive motifs in self-DNA (Gursel *et al.* 2003). Telomere shortening occurs with aging; however, the relevance of this phenomenon in the potential cf-DNA-related immunomodulation cannot be addressed using our data.

In both the nonagenarians and young controls, the pathways identified for the plasma mtDNA copy number included *Protein Ubiquitination Pathway* and the *Estrogen Receptor Signaling* pathway, which have been identified as regulators of mitochondrial homeostasis (Neutzner *et al.* 2008) and biogenesis (Klinge 2008), respectively. Likewise, the pathways *Melatonin Signaling*, *Glucocorticoid Receptor Signaling* and *Androgen Signaling* pathways identified in the young controls might reflect a more global external control of these signaling pathways on mitochondrial turnover, which could manifest as fluctuation in the plasma mtDNA quantity. Nevertheless, the lack of immunoinflammatory engagement in the pathways suggests that mtDNA is not immunostimulatory under physiological concentrations. Therefore, the potential mtDNA-mediated inflammatory activation may involve other cell types or conditions, such as neutrophils, which have recently been demonstrated to be activated by mtDNA released following trauma (Zhang *et al.* 2010).

Overall, the results of the transcriptomic analysis provide information that higher levels of total and unmethylated cf-DNA are associated with different PBMC activation states and responses *in vivo* in very old and young individuals – a finding that could be of relevance with regard to biomarker discovery and the mechanisms of immune aging. However, a weakness of this

study is that is does not reveal the cell type-specific responses associated with the cf-DNA levels. Therefore, for example, potential DC-restricted DNA-sensing pathways might have been masked due to the responses of more abundant cell types. In addition, a clear limitation of the study is that the median IL-6 level appeared unusually high in the healthy young controls. Possible explanations include the possibility that some individuals were recently engaged in strenuous physical exercise before the blood collection or were about to develop an infection. The strengths of this study include a well-characterized and relatively large cohort of very elderly individuals and a multi-directed approach to characterize the role of cf-DNA in immune aging and frailty.

In conclusion, we suggest that the total circulating cf-DNA and its unmethylated content serve as indicators of inflammaging and frailty, whereas the plasma mtDNA concentration serves as a marker of the physical aspect of frailty. Our results also suggest that increasing concentrations of total and unmethylated cf-DNAs might potentiate autoinflammation in very old individuals. However, further investigations will be required to elucidate the basis of these findings.

Experimental Procedures

Study population

The study population consisted of n=144 nonagenarians (101 women and 43 men) participating in the Vitality 90+ study, which is an ongoing prospective study involving individuals aged 90 years and older, living in the city of Tampere, Finland. The individuals in the current study were born in 1920 and were recruited and characterized as in the previous Vitality 90+ study cohorts (Goebeler *et al.* 2003). A home-visiting trained medical student performed the blood tests, physiological measurements, interviews and performance tests. The data concerning the recent infections in the subjects were elicited using a questionnaire, and if the subject reported suffering from any infectious disease during the last two weeks, the subject was excluded from the analyses in this study. Written informed consent was obtained from each participant and the study protocol followed the guidelines of the Declaration of Helsinki. The Ethics Committee of the Pirkanmaa Hospital District and the Ethics Committee of the Tampere Health Center approved the study protocol. The control subjects (n=30, 21 women and 9 men aged between 19 and 30 years) consisted of healthy laboratory personnel who had no diagnosed chronic diseases and had not suffered from any infectious diseases within the previous two weeks.

Biochemical measurements

The EDTA-blood was collected and the plasma was separated by centrifugation for 15 min at 700 g followed by transfer to new tubes and centrifugation for 15 min at 1,000 g. The plasma was stored at -70°C. The total cf-DNA was measured directly in plasma using a Quant-iT^{TM} DNA High-Sensitivity Assay kit and a Qubit® fluorometer (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The samples were analyzed in duplicate, and the mean of the two measurements was used as the final value. For the mean cf-DNA level of 0.593 µg/ml, the intra- and inter-day variation coefficients for the Quant-iT^{TM} DNA High-Sensitivity Assay were 1.9% and 4.7%, respectively, and were 2.3% and 5.2%, respectively, for the mean cf-DNA level of 1.007 µg/ml. The amount of unmethylated cf-DNA was determined by quantifying the 5-methyl-2-deoxy cytidine using the DNA Methylation EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA, Cat. no. 589324) and subtracting the resulting value from the total cf-DNA concentration.

For the quantitative PCR (qPCR)-based assessment of the genomic equivalents (GEs) of the *RNase P*-coding cf-DNA and the *Alu* repeat cf-DNA and the mtDNA copy number, the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract the plasma cf-DNA from 200 µl of plasma. The amount of input cf-DNA for each of the qPCR assays was 2 µl which was determined by assessing the linear range for each of the qPCR assays and testing the reactions for inhibition. The GE quantity of the single-copy gene, *RNase P*, was determined using the *RNase P* detection reagents (Applied Biosystems, Foster City, CA, USA, part no. 4316831). The TaqMan qPCR protocol to quantify the *Alu* repeat cf-DNA was adapted from a previously described protocol (Stroun *et al.* 2001). The primer sequences used were *Alu* F 5′-GGAGGCTGAGGCAGGAGAA-3′ and *Alu* R 5′-ATCTCGGCTCACTGCAACCT-3′, and the probe sequence was 5′-(FAM)CGCCTCCCGGGTTCAAGCG-3′. The standard curve for the *RNase P* and *Alu* repeat detection assays was constructed using Human Genomic control DNA (Applied Biosystems, Foster City, CA, USA, part no. 4312660). The mtDNA copy number was determined with TaqMan qPCR using the following primers: hmitoF 5′-CTTCTGGCCACAGCACTTAAAC-3′, and hmitoR 5′-GCTGGTGTTAGGGTTCTTTGTTTT-3′, and a probe 5′-(FAM)ATCTCTGCCAAACCCC-3′ (described in (Malik *et al.* 2011)). The standard curve for the assessment of the mtDNA copy number was constructed using a purified mitochondrial genome (Standard Reference Material no 2392-I, National Institute of Standards and Technology, Gaithersburg, MD, USA). The qPCR assays were run with the ABI PRISM® 7900 HT Sequence Detection System with 40 cycles of amplification under standard cycling conditions (2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C.

The plasma CRP concentration was measured using the Human CRP Immunoassay (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA). The plasma IL-6 concentration was measured using the PeliKine human IL-6 ELISA kit (Sanquin Reagents, Amsterdam, The Netherlands). The plasma IL-10 concentration was determined with the PeliKine Compact™ human IL-10 ELISA kit (Sanquin Reagents, Amsterdam, The Netherlands).

RNA extraction and whole-genome transcriptomic analysis

The blood samples were subjected directly to leucocyte separation using Ficoll-Paque density gradients (Ficoll-Paque™ Premium, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The PBMC layer was collected, and the cells were suspended in 150 µl of RNAlater solution (Ambion Inc., Austin, TX, USA) and stored at -70 °C until analyzed. The RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase digestion (AppliChem GmbH, Darmstadt, Germany). The concentration and quality of the RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The Illumina TotalPrep RNA amplification Kit (Ambion Inc., Austin, TX, USA) was used to amplify 330 ng RNA for hybridization on the HumanHT-12 v4 Expression BeadChip (Cat no. BD-103-0204; Illumina, San Diego, CA, USA) at the Core Facility of the Department of Biotechnology, University of Tartu, Estonia. The quality of the biotinylated complementary RNA (cRNA) products was determined with Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). The chips were scanned using Beadscan (Illumina Inc., CA, USA).

The bioinformatic microarray data analysis was performed using the Chipster v2.0 software (http://chipster.csc.fi/) (Kallio *et al.* 2011). The quality of the data was confirmed using the box blot, density blot and principal component analyses. For the data preprocessing and normalization, the lumi pipeline was used, and the Array_Address_ID was used as the probe identifier. The background correction was performed using the bgAdjust.affy package, and the data were log2-transformed to normality. To filter out the bad-quality data, the preprocessed data were filtered by expression (fluorescence intensity), i.e., the probes with an expression value of <5 or >100 were removed from the analysis. To identify the transcripts exhibiting expression levels correlating with the concentrations of total cf-DNA, unmethylated cf-DNA and plasma mtDNA copy number, the concentrations of the cf-DNA species were log-transformed and correlated with Pearson's rho using Chipster's correlate with phenodata -tool which assigns each transcript a correlation coefficient (from -1 to +1, without a p-value). The microarray expression correlation analyses were performed out separately for the nonagenarians and the control subjects, and from each of the analysis, the 250 transcripts that were best correlated were transferred to the Ingenuity

Pathway Analysis (IPA, http://www.ingenuity.com). The IPA Canonical Pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to each of the data set containing the 250 correlated transcripts. The significance of the association between the data set and the canonical pathway was measured in 2 ways. 1) The ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules in the given pathway. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the transcripts in the dataset and the canonical pathway is explained by chance alone. A significance level of $p<0.05$ was considered statistically significant. The correlation coefficients of the transcripts were identified in the results as follows: the transcripts with expression levels correlated directly with the levels of total cf-DNA, unmethylated cf-DNA or mtDNA copy number are identified in red, and the transcripts with expression levels correlated indirectly with levels of total cf-DNA, unmethylated cf-DNA or mtDNA copy number are identified in green. The resulting canonical pathways were filtered to exclude disease-specific pathways and pathways not relevant to PBMCs.

Flow cytometry

The flow cytometric analysis of the different leukocyte subtype distributions has been described in detail (Marttila *et al.* 2011). Briefly, the PBMCs were separated by Ficoll-Paque density gradient (Ficoll-Paque™ Premium, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and stored in liquid nitrogen until analysis using flow cytometry (BD FACSCanto II, the results were analyzed using the BD FACS Diva, version 6.1.3, BD Biosciences, Franklin Lakes, NJ, USA). The antibodies used to label the cells were FITC-CD14 (cat. no. 11-0149), PerCP-Cy5.5-CD3 (45-0037), APC-CD28 (17-0289) (eBioscience, San Diego, CA, USA), PE-Cy™7-CD4 (cat. no. 557852) and APC-Cy™7-CD8 (557834) (BD Biosciences).

Physiological measurements and the assessment of functional performance and frailty

The frailty score for the nonagenarians was assessed based on the criteria outlined by Fried et al. (Fried *et al.* 2001). The points for calculating the frailty score (min 0, max 5) for each individual were assessed as follows: if the individual met the criteria/threshold value in any of the five assessment steps, he/she was awarded one point in each criterion, and the points were summed to yield the frailty score. The criteria yielding the frailty points were as follows: 1) Mini-Mental Score Examination (MMSE) score ≤ 22: 2) weight loss of $\geq 10\%$ of body weight in the previous two years or BMI < 18.5 kg/m^2: 3) self-reported fatigue (the individual reported that he/she felt fatigued 'often' in a questionnaire in which the options were feeling tired 'often', 'sometimes' or 'never'): 4)

low hand grip strength (the maximum hand grip strength of the hand primarily used was in the lowest sex- and BMI-specific quartile, in accordance with Fried et al. (Fried *et al.* 2001)), and 5) low moving capability (the individual was not able to walk independently on a level surface or on stairs, as assessed by the Barthel index points for "mobility" and "stairs", i.e., the summed score of these two values was < 25).

The methods for the assessment of MMSE, chair-stand test, handgrip strength and Barthel index have been previously described (Jylha *et al.* 2007, Tiainen *et al.* 2010).

Statistical analyses

The differences in the distributions of the study variables between the nonagenarians and young controls were analyzed using the Mann-Whitney U-test or student's t-test where appropriate. The correlations between the cf-DNA species and the biochemical and physiological variables were analyzed using Spearman's rho. The statistical analyses were performed using the IBM SPSS Statistics version 19 (IBM Corp., Sommers, New York, USA).

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Conflicts of interest: none declared

Author Contributions

J.J. collected the samples, designed the experiments and performed most of the experiments, analyzed the data and wrote the manuscript

T.N. performed some of the experiments

S.M. collected the samples and performed some of the experiments

M.J. was responsible for the Vitality 90+ study design and cohort recruitment

A.H. was responsible for the Vitality 90+ study design and cohort recruitment

M.H. designed the experiments and provided the facilities and reagents

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Supporting Information listing

Supporting Experimental procedures

Supporting Tables

The microarray data are available in the GEO database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE40366.

Tables

Table 1. Characteristics of the study population. Statistically significant differences in the study variables between the nonagenarians and young controls are shown in bold.

	Nonagenarians (n=144)		**Young controls (n=30)**		
	Median	IQR	Median	IQR	p for difference*
Total cf-DNA (µg/ml)	0.884	0.788-986	0.820	0.662-0.901	**0.002**
Unmethylated cf-DNA (µg/ml)	0.701	0.618-0.811	0.643	0.503-0.732	**0.004**
mtDNA (copy number/ml)	$3.79E^8$	$2.73E^8$-$5.07E^8$	$4.05E^8$	$2.63E^8$-$5.41E^8$	0.818
RNase P cf-DNA (GE)	13.38	9.02-19.98	7.25	4.56-13.70	**<0.001**
Alu repeat cf-DNA (GE)	71.05	51.01-101.09	41.85	29.25-62.04	**<0.001**
CRP level (ng/ml)	2.15	0.82-4.27	1.50	0.58-2.82	**0.041**
IL-6 level (pg/ml)	3.97	2.41-6.19	4.11	1.78-6.34	0.546
IL-10 level (pg/ml)	1.51	0.98-2.53	2.18	1.08-3.14	0.154
CD4+ cells (%)[a]	63.50	51.95-73.23	59.75	55.95-62.03	0.055
CD8+ cells (%)[a]	28.00	20.18-39.93	31.85	27.18-35.15	0.218
CD4+CD28- cells (%)[b]	9.60	4.18-18.25	0.25	0.10-1.58	**<0.001**

CD8+CD28- cells (%)[c]	68.40	51.58-77.03	16.95	12.65-29.10	**<0.001**
CD14+ cells (%)[d]	8.30	5.68-11.93	2.65	1.80-3.23	**<0.001**
BMI (kg m^{-2})	26.12	22.81-29.49	NA		
Handgrip (kg)	20	16-24	NA		
Chair stand test time (s)	15	12-23	NA		
MMSE score	25	22-27	NA		
Barthel index	95	90-100	NA		
Frailty score (percentage of individuals with each score, min 0, max 5)	0 = 25.0 1 = 32.6 2 = 19.4 3 = 18.8 4 = 3.5 5 = 0.7		NA		

Abbreviations: BMI, body mass index; CD, cluster of differentiation; CRP, C-reactive protein; GE, genomic equivalent; IL, interleukin; IQR, interquartile range; kyn, kynurenine; MMSE, Mini-Mental State Examination; NA, not available; trp, tryptophan

[a] percentage of total lymphocytes

[b] percentage of CD4+ cells

[c] percentage of CD8+ cells

[d] percentage of live-gated cells

* Mann-Whitney *U* test

Table 2. Spearman correlations between the different cf-DNA species and study variables in the nonagenarians and young controls. Statistically significant correlations are shown in bold.

		Nonagenarians (n=144)					**Young controls (n=30)**				
		total cf-DNA	unmethylated cf-DNA	mtDNA copy number	*RNase P* cf-DNA	*Alu* cf-DNA	total cf-DNA	unmethylated cf-DNA	mtDNA copy number	*RNase P* cf-DNA	*Alu* cf-DNA
unmethylated cf-DNA	r	**0.953**	.	.	.	.	**0.954**	.	.	.	.
	p	**<0.001**	.	.	.	.	**<0.001**	.	.	.	.
mtDNA copy number	r	0.126	0.131	.	.	.	-0.023	-0.053	.	.	.
	p	0.132	0.125	.	.	.	0.904	0.782	.	.	.
RNase P cf-DNA	r	**0.173**	**0.172**	0.096	.	.	**0.739**	**0.731**	0.013	.	.
	p	**0.038**	**0.043**	0.252	.	.	**<0.001**	**<0.001**	0.945	.	.
Alu cf-DNA	r	0.117	0.113	0.083	**0.821**	.	**0.640**	**0.652**	0.082	**0.078**	.
	p	0.164	0.186	0.323	**<0.001**	.	**<0.001**	**<0.001**	0.665	**<0.001**	.
CRP level	r	**0.311**	**0.325**	0.059	0.113	0.025	0.057	-0.016	0.304	0.061	-0.002
	p	**<0.001**	**<0.001**	0.482	0.176	0.767	0.763	0.931	0.103	0.748	0.993
IL-6 level	r	**0.237**	**0.192**	0.145	-0.060	-0.042	0.093	-0.031	0.150	0.039	0.029
	p	**0.004**	**0.024**	0.084	0.476	0.619	0.626	0.869	0.430	0.836	0.878
IL-10 level	r	0.126	0.043	0.061	0.110	0.104	0.007	-0.118	0.245	-0.101	-0.008
	p	0.132	0.619	0.471	0.188	0.216	0.971	0.536	0.192	0.595	0.965
CD4+ cells[a]	r	-0.054	-0.023	-0.028	0.037	0.065	-0.161	-0.176	0.240	-0.002	0.057
	p	0.544	0.795	0.748	0.674	0.464	0.395	0.354	0.201	0.990	0.764

CD8+ cells[a]	r	0.012	0.001	0.005	-0.049	-0.079	0.291	0.356	-0.192	0.327	0.304
	p	0.894	0.991	0.954	0.581	0.376	0.118	0.054	0.310	0.078	0.103
CD4+CD28- cells[b]	r	0.072	0.013	-0.022	-0.082	-0.104	0.088	0.134	-0.304	-0.147	-0.183
	p	0.414	0.886	0.802	0.355	0.239	0.664	0.481	0.101	0.438	0.332
CD8+CD28- cells[c]	r	-0.082	-0.115	-0.109	-0.084	-0.048	-0.076	-0.011	-0.215	0.174	0.174
	p	0.355	0.199	0.215	0.340	0.589	0.689	0.953	0.255	0.358	0.357
CD14+ cells[d]	r	0.065	0.047	0.064	0.077	0.118	0.133	0.128	-0.224	-0.030	-0.024
	p	0.464	0.599	0.472	0.384	0.183	0.482	0.499	0.234	0.875	0.898
BMI	r	0.026	-0.019	**-0.209**	0.011	-0.005	NA	NA	NA	NA	NA
	p	0.764	0.829	**0.013**	0.896	0.954					
Handgrip strength	r	-0.123	**-0.185**	**-0.188**	-0.059	-0.072	NA	NA	NA	NA	NA
	p	0.153	**0.034**	**0.029**	0.479	0.409					
Chair stand test time	r	**0.219**	0.165	**0.262**	0.115	0.046	NA	NA	NA	NA	NA
	p	**0.022**	0.091	**0.006**	0.235	0.636					
MMSE score	r	**-0.201**	**-0.233**	-0.109	-0.095	0.022	NA	NA	NA	NA	NA
	p	**0.016**	**0.006**	0.194	0.259	0.798					
Barthel index	r	**-0.230**	**-0.222**	-0.036	-0.033	-0.002	NA	NA	NA	NA	NA
	p	**0.006**	**0.009**	0.671	0.698	0.981					
Frailty score	r	**0.258**	**0.290**	**0.182**	0.098	0.047	NA	NA	NA	NA	NA
	p	**0.002**	**0.001**	**0.029**	0.242	0.574					

Abbreviations: BMI, body mass index; CD, cluster of differentiation; CRP, C-reactive protein; IL, interleukin; MMSE, Mini-Mental State Examination; NA, not available

[a]percentage of total lymphocytes, [b]percentage of CD4+ cells, [c]percentage of CD8+ cells, [d]percentage of live-gated cells

Table 3. The top 10 IPA Canonical Pathways harbored by the 250 transcripts that exhibited expression levels correlating with the plasma total cf-DNA concentration in the nonagenarians (a) and young controls (b).

3a.

Ingenuity Canonical Pathways	-log(p)	Ratio	Molecules
EIF2 Signaling	6.65	0.068	RPL24↓, RPS17↓/RPS17L↓, RPL21↓, RPL31↓, RPL26↓, RPL15↓, RPL39↓, PIK3CG↑, PIK3C3↑, EIF5↓, EIF3A↑, RPL8↓, RPS27A↓, RPSA↓
Role of JAK1 and JAK3 in γc Cytokine Signaling	4.76	0.104	JAK1↓, PIK3CG↑, SYK↑, PIK3C3↑, STAT3↑, STAT1↑, IL2RB↓
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.59	0.052	JAK1↓, RHOQ↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, CYBB↑, IFNGR2↑, PRKCH↓, NCF4↑, STAT1↑
mTOR Signaling	4.31	0.052	RHOQ↑, RHOT1↑, RPS17↓/RPS17L↓, RHOC↓, PIK3CG↑, PIK3C3↑, EIF3A↑, PRKCH↓, HIF1A↑, RPS27A↓, RPSA↓
IL-9 Signaling	4.10	0.125	JAK1↓, PIK3CG↑, PIK3C3↑, STAT3↑, STAT1↑
IL-8 Signaling	3.98	0.051	GNAS↓, CCND2↓, RHOQ↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, CYBB↑, DEFA1↑, PRKCH↓
IL-15 Signaling	3.82	0.090	JAK1↓, PIK3CG↑, SYK↑, PIK3C3↑, STAT3↑, IL2RB↓
FAK Signaling	3.81	0.069	ITGB1↓, FYN↓, ASAP1↑, PIK3CG↑, PIK3C3↑, ACTG1↓, PTEN↑
ILK Signaling	3.74	0.052	ITGB1↓, RHOQ↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, HIF1A↑, CREB5↑, ACTG1↓, PTEN↑
p53 Signaling	3.63	0.073	CCND2↓, STAG1↑, PIK3CG↑, PIK3C3↑, APAF1↑, GNL3↓, PTEN↑

3b.

Ingenuity Canonical Pathways	-log(p)	Ratio	Molecules
B Cell Development	2.06	0.083	CD19↓, HLA-DOA↓, CD79A↓
Regulation of eIF4 and p70S6K Signaling	2.01	0.034	RPS26↑, PPP2R3A↑, EIF2B1↓, EIF4G3↑, EIF2A↓, EIF3L↓
Cdc42 Signaling	1.86	0.034	IQGAP2↓, HLA-DOA↓, WASL↑, PAK2↓, MYL5↓, HLA-DPB1↓
Cyclins and Cell Cycle Regulation	1.82	0.046	PPP2R3A↑, SUV39H1↓, CDKN2D↑, CDKN1B↓
EIF2 Signaling	1.55	0.029	RPS26↑, EIF5↓, EIF2B1↓, EIF4G3↑, EIF2A↓, EIF3L↓
Glycosaminoglycan Degradation	1.37	0.035	FGFRL1↑, GUSB↑
Pyruvate Metabolism	1.33	0.022	PDHA1↓, AKR7A3↓, GLO1↓

Table 4. The top 10 IPA Canonical Pathways harbored by the 250 transcripts that exhibited expression levels correlating with the plasma unmethylated cf-DNA concentration in the nonagenarians (a) and young controls (b).

4a.

Ingenuity Canonical Pathways	-log(p)	Ratio	Molecules
EIF2 Signaling	5.26	0.059	RPL24↓, RPS17↓/RPS17L↓, RPL39↓, PIK3CG↑, PIK3C3↑, EIF5↓, RPL21↓, EIF3A↑, RPL31↓, RPL26↓, RPLP0↓, RPSA↓
Integrin Signaling	5.03	0.057	ITGB1↓, FYN↓, PAK2↑, RHOQ↑, ASAP1↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, ARPC4↓, ACTG1↓, PTEN↑
IL-8 Signaling	4.87	0.057	GNAS↓, CCND2↓, PAK2↑, RHOQ↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, CYBB↑, DEFA1↑, MAP4K4↓
FAK Signaling	4.83	0.078	ITGB1↓, FYN↓, PAK2↑, ASAP1↑, PIK3CG↑, PIK3C3↑, ACTG1↓, PTEN↑
Signaling by Rho Family GTPases	4.40	0.047	ITGB1↓, GNAS↓, PAK2↑, RHOQ↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, ARPC4↓, CYBB↑, PIP4K2A↓, ACTG1↓
p53 Signaling	3.74	0.073	CCND2↓, STAG1↑, PIK3CG↑, PIK3C3↑, APAF1↑, GNL3↓, PTEN↑
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	3.74	0.069	MYO5A↑, FYN↓, PIK3CG↑, ARPC4↓, ACTG1↓, LCP2↓, PTEN↑
HMGB1 Signaling	3.71	0.070	RHOQ↑, HMGB1↑, RHOT1↑, RHOC↓, PIK3CG↑, PIK3C3↑, IFNGR2↑
Rac Signaling	3.46	0.057	ITGB1↓, PAK2↑, PIK3CG↑, PIK3C3↑, ARPC4↓, CYBB↑, PIP4K2A↓
RhoGDI Signaling	3.40	0.045	ITGB1↓, GNAS↓, PAK2↑, RHOQ↑, RHOT1↑, RHOC↓, ARPC4↓, PIP4K2A↓, ACTG1↓

4b.

Ingenuity Canonical Pathways	-log(p)	Ratio	Molecules
iCOS-iCOSL Signaling in T Helper Cells	2.73	0.049	LAT↓, TRAT1↓, IKBKE↓, HLA-DMB↑, PLEKHA2↑, ITK↓
PPARα/RXRα Activation	2.32	0.037	HSP90AB1↓, ACVR1↓, IKBKE↓, STAT5B↓, MED12↓, MED24↓, AIP↓
IL-9 Signaling	2.09	0.075	CISH↓, SOCS2↓, STAT5B↓
Mitotic Roles of Polo-Like Kinase	2.07	0.058	CDC25B↓, HSP90AB1↓, PPP2CA↑, CDC7↓
Antigen Presentation Pathway	1.99	0.075	NLRC5↓, CANX↑, HLA-DMB↑
CD28 Signaling in T Helper Cells	1.86	0.038	LAT↓, ARPC5↑, IKBKE↓, HLA-DMB↑, ITK↓
PI3K/AKT Signaling	1.83	0.036	HSP90AB1↓, PPP2CA↑, IKBKE↓, PRKCZ↓, BCL2↓
Role of NFAT in Regulation of the Immune Response	1.74	0.030	LAT↓, GNB5↓, IKBKE↓, HLA-DMB↑, ORAI1↓, ITK↓
TNFR1 Signaling	1.69	0.057	TRADD↓, MADD↑, IKBKE↓
Phospholipase C Signaling	1.64	0.027	HDAC6↓, MPRIP↓, PLD3↑, LAT↓, GNB5↓, PRKCZ↓, ITK↓

Table 5. The top 10 IPA Canonical Pathways harbored by the 250 transcripts that exhibited expression levels correlating with the plasma mtDNA copy number in the nonagenarians (a) and young controls (b).

5a.

Ingenuity Canonical Pathways	-log(p)	Ratio	Molecules
Protein Ubiquitination Pathway	2.81	0.037	USP21↑, PSME1↓, UBE4B↑, UBE2N↓, DNAJB11↓, USP9X↓, PSMB1↓, PSME2↓, PSMB8↓, USP48↓
Estrogen Receptor Signaling	2.26	0.044	TAF6L↑, CCNH↓, POLR2C↓, MAP2K2↑, HNRNPD↓, H3F3C↓
Paxillin Signaling	2.04	0.045	NCK2↑, TLN1↑, PTPN12↑, ITGB7↓, ACTN1↑
Integrin Signaling	1.88	0.033	NCK2↑, MAP2K2↑, ILK↑, TLN1↑, ITGB7↓, ACTN1↑, CAPN3↑
PI3K/AKT Signaling	1.77	0.036	MAP2K2↑, YWHAH↑, ILK↑, CTNNB1↓, MCL1↓
Assembly of RNA Polymerase II Complex	1.60	0.054	TAF6L↑, CCNH↓, POLR2C↓
ERK/MAPK Signaling	1.53	0.029	MAP2K2↑, YWHAH↑, TLN1↑, STAT1↓, H3F3C↓, ELF1↓
ILK Signaling	1.52	0.031	NCK2↑, ILK↑, RSU1↑, CTNNB1↓, ITGB7↓, ACTN1↑
Death Receptor Signaling	1.42	0.046	TANK↓, DAXX↑, CFLAR↑
Glycosphingolipid Biosynthesis - Ganglioseries	1.41	0.036	DBT↑, ST3GAL5↓

5b.

Ingenuity Canonical Pathways	-log(p)	Ratio	Molecules
Protein Ubiquitination Pathway	5.05	0.048	USP24↑, UCHL3↓, ANAPC10↓, USP39↑, TCEB1↓, ANAPC4↑, HSCB↓, PSMD12↓, HSP90AA1↓, PSMD14↓, PSMD1↓, USP34↑, AMFR↑
Purine Metabolism	2.41	0.023	NUDT5↓, ATP5H↓, ATP1B1↓, SMARCA5↓, HSP90AA1↓, CLPX↓, DGUOK↑, ATP5S↓, POLB↑
Assembly of RNA Polymerase I Complex	2.34	0.154	TAF1C↑, TBP↑
Cleavage and Polyadenylation of Pre-mRNA	2.09	0.167	WDR33↑, CSTF2↓
Assembly of RNA Polymerase III Complex	2.02	0.125	TBP↑, GTF3A↓
Melatonin Signaling	2.01	0.050	GNAI3↓, CALM1↓, GNRH1↑, MAP2K1↓
Glucocorticoid Receptor Signaling	1.95	0.027	MAP3K14↑, SRA1↓, YWHAH↓, IL10↑, PRKAA1↑, TBP↑, HSP90AA1↓, MAP2K1↓
Estrogen Receptor Signaling	1.79	0.037	SRA1↓, TBP↑, H3F3A/H3F3B↓, MAP2K1↓, MED27↓
Fructose and Mannose Metabolism	1.72	0.022	AKR7A2↑, NUDT5↓, MDP1↓
Pantothenate and CoA Biosynthesis	1.70	0.033	PPCS↓, COASY↑