CLINICAL EVALUATION CRITERIA FOR AGING AND AGING-RELATED MULTIMORBIDITY

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CLINICAL EVALUATION CRITERIA FOR AGING AND AGING-RELATED MULTIMORBIDITY

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EDITORIAL
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Editorial: Clinical Evaluation Criteria for Aging and Aging-Related Multimorbidity

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Editorial on the Research Topic

Clinical Evaluation Criteria for Aging and Aging-related Multimorbidity

It is becoming increasingly clear that population aging brings a train of degenerative, malignant and other chronic diseases, such as cancer, type 2 diabetes, chronic obstructive pulmonary disease, neurodegenerative diseases, heart disease, aggravation of infectious diseases. This is also accompanied by other diverse functional, physical and mental impairments. These conditions do not emerge separately from each other, but have related aetiologies and mutually exacerbate each other. This multitude of morbid conditions has been often termed as "multimorbidity" or "co-morbidity." Moreover, it has been suggested that a promising approach to address the entire host of old-age-related morbidities would be by treating their underlying determinative factors–namely fundamental degenerative processes of aging.

Yet, there is currently no agreed method to estimate the direct effects of therapy on tackling the aging process as such, for which there is presently no agreed formal or clinical definition or criteria. Moreover, essentially, there is no agreed formal or clinical definition and criteria for old-age multimorbidity either. Correspondingly, there are no agreed scientifically grounded criteria to select interventions against degenerative aging and old-age multimorbidity or to evaluate their effectiveness. There are clinical methods to diagnose individual age-related diseases and dysfunctions, and assess interventions against those individual diseases and dysfunctions. Yet their integrated evaluation as "aging-related ill health" or "multimorbidity," as well as the selection and evaluation of effective interventions against these conditions, remain as unresolved methodological challenges. As a result, there is no agreed formal conceptual basis for incentivizing industrial development, nor regulatory adoption, of diagnostics and therapies against degenerative aging and aging-related multimorbidity (Moskalev et al., 2016; Stambler, 2017).

The main aim of the current Research Topic was to contribute to establishing the methodological basis for developing, and regulatory adoption of, diagnostic criteria for aging and aging-related multimorbidity. The articles published in this research topic provided a broad and diverse exploration of clinical criteria for aging and old-age multimorbidity that utilized diverse physiological, functional, genetic, epigenetic and other biomarkers and methods of bioinformatics. Of special interest were the selecting of the most informative and economic diagnostic parameters (biomarkers and functional essays) for aging and old-age multimorbidity and developing guidelines and analytical methodologies for clinical testing of interventions against degenerative aging and old-age multimorbidity.

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The articles published in this research topic reconfirmed the multiplicity of approaches, and demonstrated once again how far we are yet from any kind of general or "consensus" agreement about the metrics of aging and aging-related multimorbidity. The combinations of examined parameters were quite distinct for all the researchers, according to their diverse methodological backgrounds, the measurement techniques and the data that were convenient, available and affordable. Practically, there were as many methodological focuses of evaluation as there were studies. This diversity is a salient characteristic of the broad and complex aging field, and is likely unavoidable, but it showcased once again that in the clinical evaluation of aging and multimorbidity, there may be a common language, but not many common rules. Yet, the articles of this research topic outlined some of the potential directions for more integration, harmonization and standardization of the aging evaluation field.

First of all, they emphasized the need for a systemic composite approach that would combine multiple evaluation criteria and parameters from multiple organ systems (the nervous system and cognition, respiratory and cardiovascular system, muscular system, etc.) and from different levels of biological organization. Thus, in this topic, Vaiserman and Krasnienkov, in their review of telomere length as a marker of biological age, emphasized the importance of combined assessment of biomarkers of aging. Though telomere length has been widely used as a purported biomarker of aging, the authors argued that as a stand alone measure, it may have limited predictive value and clinical importance, yet in combination with other parameters (such as certain immune parameters, indices of epigenetic age, indices of homeostatic dysregulation, frailty index, etc.) it can improve the risk evaluation for aging-related ill health (Vaiserman and Krasnienkov). Krut'ko et al. reporting their method and computer system for dialog optimization of aging biomarker panels for biological age assessment, reconciled with a possibly unlimited multiplicity of approaches to aging evaluation, and argued for the need to select and optimize particular panels of biomarkers for particular tasks, using pre-defined optimization criteria (Krut'ko et al.). In this topic, Li et al., in their study of hamartin as an endogenous neuroprotective molecule induced by hypoxic preconditioning, they showcased that a fruitful approach to developing and selecting aging evaluation criteria may be by actual trials of potential geroprotecitive interventions (Li et al.).

Considering aging evaluation in diverse organ systems, Strasser stressed the clinical significance of assessing muscular fitness in secondary care, striving to improve practical guidelines for such assessment, with specific reference to older persons (Strasser). Gustafsson and Ulfhake provided an in depth review of the loss of muscle function and mass (sarcopenia) in the framework of human aging, healthspan and lifespan, with a special consideration of a potential neurogenic origin of sarcopenia, and argued for enhancing physical activity with appropriate predictive clinical monitoring (Gustafsson and Ulfhake).

Further emphasizing the interrelatedness of aging evaluation criteria, in this topic, the studies of Papathanasiou et al. and Chadjikyprianou et al. exemplified the types of methodological instruments commonly found in the toolkits of geriatric assessment and geriatric treatment. These chiefly relied on functional frailty assessments, especially cognitive assessments and self reports, with the aim to evaluate the relationships between multiple aspects of functional aging impairment. Thus, Papathanasiou et al. related between multimorbidity, trauma exposure, and frailty of older adults in the community (Papathanasiou et al.); while Chadjikyprianou et al. in their longitudinal neurocognitive study of aging, considered the relation of sex, age, education and APOE-4 with cognitive performance, utilizing such measures as executive functions and verbal episodic memory (Chadjikyprianou et al.).

The two last studies of this research topic provided further general directions toward harmonization of discourse on aging evaluation. Thus, Kim et al. suggested a compendium of age-related diseases and traits within a framework of genome-wide association studies (GWAS) and phenome-wide association studies (PheWAS). Even though the terms "age-related diseases (ARDs)" and "age-related traits (ARTs)" are commonly used, there are currently no accepted criteria for their definition, selection and registration. The authors make a step toward establishing an evidence-based list of such age-related diseases and traits, based on their prevalence with increasing age, suggesting a basis for further discussion and consensus building (Kim et al.). Finally, Hartmann et al. in their ranking of biomarkers of aging by citation profiling and effort scoring, provided an overview of different biomarker types often considered for aging assessment (routine and research laboratory biomarkers, physical capability and organ function parameters) and piloted their ranking system based on the biomarkers' citation profile (the review count score) and estimated effort of use (effort score) (Hartmann et al.). Clearly, given the vast multitude of potential aging biomarkers and evaluation parameters, it is important to establish some sort of scoring or prioritization criteria, to facilitate their clinical use.

Altogether, this research topic showcased a wide variety of approaches and directions toward clinical evaluation criteria for aging and aging-related multimorbidity, and we hope it will contribute to stimulating the discussion and involvement for the further development of such evaluation criteria, which we believe are vitally important for healthy longevity research, development, application and education.

AUTHOR CONTRIBUTIONS

IS and AM contributed to conceptualization and writing of the article and approved the submitted version.

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REVIEW
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Hamartin: An Endogenous Neuroprotective Molecule Induced by Hypoxic Preconditioning

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Hypoxic/ischemic preconditioning (HPC/IPC) is an innate neuroprotective mechanism in which a number of endogenous molecules are known to be involved. Tuberous sclerosis complex 1 (TSC1), also known as hamartin, is thought to be one such molecule. It is also known that hamartin is involved as a target in the rapamycin (mTOR) signaling pathway, which functions to integrate a variety of environmental triggers in order to exert control over cellular metabolism and homeostasis. Understanding the role of hamartin in ischemic/hypoxic neuroprotection will provide a novel target for the treatment of hypoxic-ischemic disease. Therefore, the proposed molecular mechanisms of this neuroprotective role and its preconditions are reviewed in this paper, with emphases on the mTOR pathway and the relationship between the expression of hamartin and DNA methylation.

Keywords: hamartin, ischemia, hypoxia, neuroprotection, TSC1

INTRODUCTION

Most aging of the brain is associated with some degree of ischemia/hypoxia for decline in cerebral blood flow (Rosenberg, 2019). Hypoxia/ischemia is a common pathophysiological process seen clinically that can, if left uninterrupted, lead to cell death culminating in serious brain damage (Jovandaric and Milenkovic, 2014; Jiang et al., 2018). Brain ischemia/hypoxia(ischemic stroke) is one of the most common causes of disability and mortality worldwide and is a prominent age-related diseases (Lucke-Wold et al., 2014). Aging is a strong risk factor for poor post-stroke outcome (Roy-O'Reilly et al., 2020). Organisms can forestall this procces, however, using endogenous protective mechanisms that insulate brain cells from the hypoxic/ischemic environment. One of these mechanisms is described by the term hypoxic/ischemic preconditioning (H/IPC), a complex process that appears to function through upregulation of several endogenous molecules that have been shown to exert neuroprotective effects under hypoxic conditions, including VEGF,

EPO, and HSP70 (Gora-Kupilas and Josko, 2005; Montero et al., 2007; Bespalov et al., 2014). Hypoxic/ischemic preconditioning is described in detail below.

Tuberous sclerosis complex 1 (TSC1), or hamartin, has recently been proposed as new addition to the list of endogenous neuroprotective molecules (Hadley et al., 2013; Papadakis et al., 2013; Xia et al., 2013). In a study by Papadakis et al. (2013), while hamartin expression was unaffected by hypoxic conditions alone, it was upregulated when preconditioning was performed prior to ischemia, and conferred protection from ischemic injury on the otherwise vulnerable hippocampal CA1 neurons. Hamartin is also known for its capacity to bind with the TSC2 product tuberin, to form a hamartin-tuberin complex that plays a crucial role in the rapamycin (mTOR) signaling pathway (Plank et al., 1998). The mTOR pathway is known to govern cellular responses to hypoxia (Srivastava et al., 2015); therefore, it is not surprising that the neuroprotective function of hamartin was found to proceed through mTOR pathway signaling (Hadley et al., 2019).

The mechanism whereby hamartin expression is unaffected by ischemia alone, and yet is upregulated by preconditioning prior to ischemia, remains unclear. One possible explanation for this phenomenon evokes the role of epigenetics. Studies have shown that a change in DNA methylation at the Tsc1 promoter can affect mRNA and protein expression of mTOR (Zhang et al., 2015), suggesting that epigenetic changes may produce a downstream effect on the neuroprotective function of mTOR. In this article, we review the neuroprotective role of hamartin, provide insight into the role of mTOR pathway signaling in its mechanism, and provide clarity regarding the epigenetic role of DNA methylation in the regulation of hamartin expression.

HYPOXIC/ISCHEMIC PRECONDITIONING (HPC/IPC) AND ENDOGENOUS NEUROPROTECTION

Hypoxic/ischemic preconditioning (HPC/IPC) refers to a process capable, through prior exposure to a state of moderate hypoxia/ichemia in organisms, organ systems, individual organs, tissues, or cells, of conferring increased resistance to subsequent severe hypoxia/ischemia in these biological units (Shao and Lu, 2012; Altintas et al., 2016). Murry et al. (1986) first described IPC in 1986 after finding that dogs subjected to repeated sublethal ischemia exhibited protection against subsequent sustained cardiac ischemia and reperfusion injury. Although neurons are very sensitive to hypoxia/ischemia, previous research has demonstrated that even tolerance to cerebral ischemia can be induced by IPC (Kitagawa et al., 1990). The underlying mechanisms have not been fully deciphered yet. The process by which this tolerance develops is highly complex, involving a profusion of signaling pathways and their mediators [for example, the Janus-activated kinase (JAK) and PKC], as well as gene expression, together responsible for sensing, transducing, modulating, and effecting preconditioned resistence; these include adenosine, excitatory and inhibitory amino acids (for example, glutamate and γ-amino-butyric acid), reactive oxygen species (for example, O_2, H_2O_2, and OH), transcription factors (for example, NF-kappaB and HIF-1), membrane channels (for example, calcium ions and ATP-sensitive K + channels), heat shock proteins (for example, Hsp-70 and Hsp-27), cytokines (for example, IL-6, IL-1βand TNF-α), and mitochondrial biogenesis (Hagberg et al., 2004; Lu et al., 2005; Long et al., 2006; Marini et al., 2007; Dornbos and Ding, 2012; Thompson et al., 2012; Cai et al., 2014; Chen et al., 2016; Mukandala et al., 2016; Basheer et al., 2018; Jackson et al., 2018). In general, the neuroprotective effect of HPC/IPC appears to depend on both the downregulation of detrimental cellular mediators and biomolecules, and the upregulation of their beneficial counterparts (Lu et al., 2005).

Upregulation of hypoxia inducible factor-1 (HIF-1) by HPC/IPC, for instance, plays a pivotal role in preconditioning-mediated neuroprotection. HIF-1 is a transcription factor responsible for regulating the expression of genes that contribute to hypoxic/ischemic tolerance by modulation, in turn, of several downstream mediators known to be involved in ischemic neuroprotection (Taie et al., 2009). Erythropoietin (EPO) and vascular endothelial growth factor (VEGF) are two of the molecules upregulated by HIF-1, and are known to be stimulants of cell survival and neurogenesis in animal models (Sun et al., 2003; Gu et al., 2008; Chen et al., 2010). EPO can exert neuroprotective effects against hypoxic injury reducing apoptosis by affecting ERK pathways, JAK2/STAT5/Bcl-xL signaling, and others signal transduction pathway (Bartesaghi et al., 2005; Ma et al., 2014; Jeong et al., 2017). VEGF reduced hypoxic lesions in the brain through activation of VEGF signaling, such as VEGF/VEGFR2/Flk1 pathway, MEK/ERK1/2 pathway and so on, to protect neuronal cell from injury (Gomes et al., 2007; Laudenbach et al., 2007). Another mechanism by which HPC/IPC may perform its neuroprotective function is by reducing oxidative damage to tissues and cells. Ischemia/reperfusion injury generates free radicals at concentrations that can damage cellular structures, including proteins, lipids, and DNA (Hatwalne, 2012); HPC/IPC, by contrast, appears to produce these free radicals at a low level that is sufficient to initiate endogenous neuroprotective pathways (Thompson et al., 2012).

Another process that has demonstrated neuroprotective effects in the context of ischemia and may mediate the results of HPC/IPC is DNA methylation, a type of epigenetic modification that regulates gene expression (Hwang et al., 2017). During HPC/IPC, DNA methylation of certain genes is thought to regulate transcriptomic responses to moderate ischemia that ultimately result in the production of ischemic tolerance (Meller et al., 2015). Support for this contention is derived from the finding that DNA methyltransferases (DNMTs), enzymes responsible for DNA methylation, are found to be altered after HPC/IPC. DNMTs can establish specific DNA methylation patterns to protect the brain from damage by modifying gene expression to promote neuroprotection (Zhang et al., 2014; Felling and Song, 2015).

HPC/IPC may also exert its effects on a smaller scale. Modification of protein subunits or amino acids through processes such as phosphorylation alter the activity of the proteins they form and have been shown to be involved in the regulation of several cellular responses in the brain

(Takagi, 2014). In support of the importance of phosphorylation to HPC/IPC, Shamloo and Wieloch (1999) found that the level of tyrosine-phosphorylated proteins were increased in the brain after IPC. Similarly, protein phosphatase levels, which regulate dephosphorylation of serine/threonine residues in proteins, were also found to be changed after HPC/IPC treatment (Cid et al., 2007; Zhang et al., 2014). The protein activity regulated by phosphorylation may produce a variety of cellular consequences, including, among others, alteration in the levels of phosphorylated extracellular signal-regulated kinases 1/2 (ERK1/2), change in the location of a protein kinase, and modification of ion influx through the N-methyl-D-aspartate receptor (Shamloo and Wieloch, 1999; Li et al., 2005; Niu et al., 2005; Long et al., 2006; Qi et al., 2007). These changes create a buffer for neurons against hypoxic/ischemic injury caused by autophagy, necroptosis, apoptosis and other mechanisms (Tregub et al., 2016; Ren et al., 2017; Wang et al., 2018).

THE STRUCTURE AND ACTIVITY OF HAMARTIN

Tuberous sclerosis is a disorder involving the formation of hamartomas in multiple organ systems, particularly in the brain, skin, heart, lungs, and kidney (Nguefack et al., 2012; Resende et al., 2013). Studies have identified the TSC1 gene, located on 9q34 (Fryer et al., 1987), as the etiological culprit in this disease. Structurally, the TSC1 gene has 23 exons and produces an 8.6 kb mRNA transcript, the transcriptional product of which is hamartin. Hamartin is a 1,164-amino-acid/130 KDa tumor suppressor protein expressed in most human tissues (Plank et al., 1999; Johnson et al., 2001). It is hydrophilic and has transmembrane domains at amino acids 127–144 and within its coiled-coil region at residues 719–998 (Nellist et al., 1999). Amino acid residues 145–510 contain the functional unit for activation of Rho GTPase, and amino acid residues 881–1,084 interact with the N-terminal domains of the ezrin, radixin, and moesin (ERM) family of actin-binding proteins (**Figure 1**; van Slegtenhorst et al., 1997; Jacks and Kissil, 2009), which are responsible for motility and neuro-polarization.

The typical molecular activity of hamartin is predicated on the formation of a functional protein complex through binding with tuberin (Slegtenhorst et al., 1998). Although both hamartin and tuberin may have distinct functions outside of their combined complex, hamartin binding to tuberin stablilizes the latter (Chong-Kopera et al., 2006; Huang and Manning, 2008), allowing the complex to proceed to function as the GTPase activating protein (GAP) for the ras homolog RheB, which is highly expressed in the brain (Li et al., 2004). RheB-GTP can interact with the target of rapamycin (TOR) complex 1 (TORC1) to precipitate phosphorylation of TORC1 targets, including p70 S6 kinase and elongation factor 4E binding proteins (Guertin and Sabatini, 2007); thus, formation of the hamartin-tuberin complex is a crucial means by which to inhibit the mTOR pathway.

Differential phosphorylation sites on the hamartin protein may serve as the basis for a "molecular switch" that regulates the formation of its functional complex with tuberin. Astrinidis et al. (2003) demonstrated that endogenous hamartin was threonine-phosphorylated at three sites (Thr 417, Ser 584, and Thr1047) in a reaction catalyzed by cyclin-dependent kinase 1 (CDK1), one of which (Thr417) is located in the hamartin-tuberin interaction domain (**Figure 1**); the authors proceed to conclude that hamartin phosphorylation controls the activity of the complex during the cell cycle at the G2/M phase. Phosphorylation may also act to negatively regulate the activity of the hamartin-tuberin complex. A study by Lee et al. (2007) suggested that the IKKβ kinase phosphorylated hamartin at both Ser487 (a non-traditional phosphorylation site) and Ser511 (an orthodox phosphorylation site), and found that phosphorylation at these sites enhances dissociation of the complex, which in turn induces mTOR activation.

THE NEUROPROTECTIVE ROLE OF THE HAMARTIN/mTOR PATHWAY

The mTOR pathway is critically involved in intracellular signaling events during I/R injury and increases the phosphorylation of the mTOR confers neuroprotection against I/R (Arabian et al., 2019). mTOR has been proposed as a novel target for neuroprotective treatment of hypoxia/ischemia brain injury (Chen et al., 2012). The mTOR pathway modulated autophagy, inducible nitric oxide synthase (iNOS), oxidative state, the mitochondrial and non-mitochondrial oxygen consumption rate, and so on to prevent neurons form hypoxia/ischemia injury (Dutta et al., 2015; Arabian et al., 2019; Zhang et al., 2019).

Despite the fact that the full scope of hamartin-tuberin complex function has not been revealed, its role in inhibition of mTOR activity is well-established (**Figure 2**; Chen et al., 2012). Inactivating variant in either hamartin or tuberin resulted in the hyperactivation of the mechanistic target of mTOR pathway and dysregulated mTOR signaling resulted in increased cell growth and proliferation (Salussolia et al., 2019). It is also clear that important neuroprotective role of TSC in the context of hypoxic/ischemic conditions may depend on mTOR pathway (Liu et al., 2019).

Controversy remains regarding the nature of the alterations to mTOR produced by hypoxic/ischemic conditions, however. Zare Mehrjerdi et al. (2013) found that remote ischemic preconditioning (RIPC) decreased apoptosis, an effect that was associated with increased p-mTOR, while mTOR remained unaltered; mechanistic confirmation was obtained in this study when rapamycin abolished all protective effects of RIPC. On the contrary, Yang et al. (2015) demonstrated essentially the opposite findings: cerebral ischemia in rats resulted in an increase of mTOR transcripts and protein concurrent with apoptotic and necrotic neuronal death, while inhibition of mTOR by rapamycin markedly reduced ischemia-induced damage. Clinical findings have mimicked this latter pattern, with patients treated using rapamycin showing a decrease in the number of stroke or transient ischemic attacks compared with the non-rapamycin control group (Beek et al., 2009). As in the treatment of ischemia, there is also controversy over mTOR activation-mediated modulation of neuroprotection

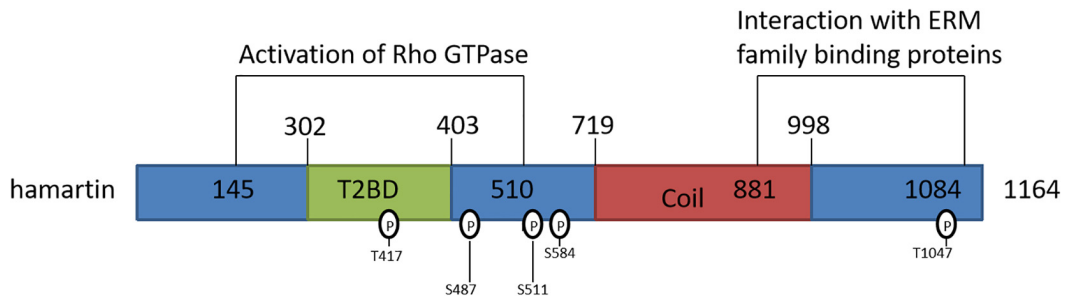

FIGURE 1 | Schematic representation of the hamartin protein and its functional domains. T2BD: TSC2-binding domain; Coil: predicted coiled-coil domain.

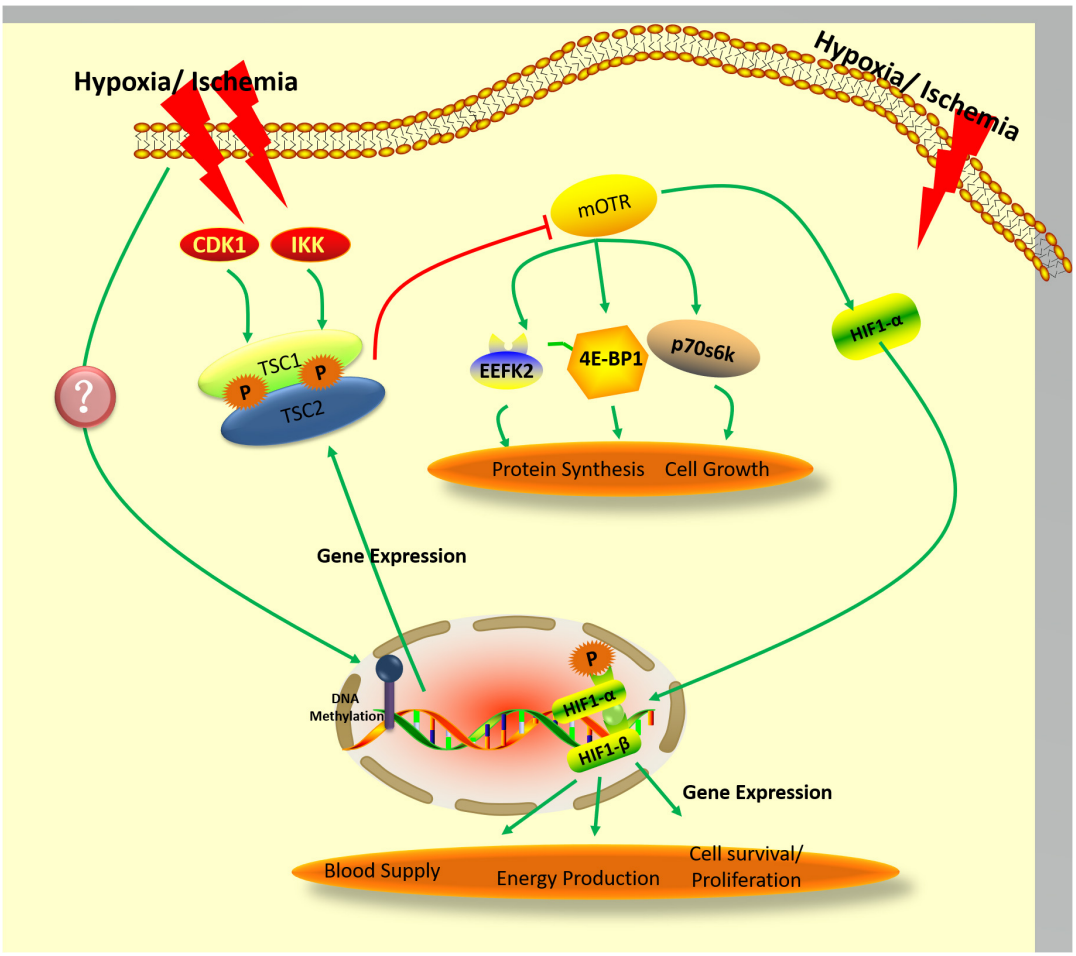

FIGURE 2 | Proposed pathway governing the expression and activity of hamartin under hypoxic/ischemic conditions.

during hypoxia treatment (Bilali et al., 2008; Liu et al., 2015). Chen et al. (2012) address this with their proposal that multiple processes underwritten by mTOR signaling, such as anti-apoptosis, regeneration of neurons, removal of neurotoxins, and angiogenesis, may support neuronal survival in the wake of hypoxic/ischemic brain injury. While, it is worth noting that the clinical use of temsirolimus (CCI-779), an mTOR inhibitor, in oncology (Galanis et al., 2005). It would be of great clinical value to explore positive or negative effects of mTOR inhibitor on neuroprotection in patients with cancer and stroke.

Despite these uncertainties surrounding the pathway's details, mTOR signaling during ischemia/hypoxia is known to depend on hamartin-tuberin complex formation (Brugarolas et al., 2004; **Figure 2**). It is also well-established that phosphorylation of tuberin is a crucial step in oxygen-sensing pathways relevant to the cellular response to energy depletion (Inoki et al., 2003;

Leontieva and Blagosklonny, 2012). In addition to precipitating cellular energy depletion, hypoxia results in activation of the AMPK/TSC2/RheB pathway, which culminates in mTOR inhibition (Liu et al., 2006). The hamartin-tuberin complex inhibits mTORC1 by acting on RheB when the cell is subjected to hypoxic or energy-poor conditions, and thereby enacts downstream control over protein synthesis and cell growth through regulation of p70S6K, 4E-BP1, and EEF2K (Browne and Proud, 2004). As mentioned above, phosphorylation of hamartin appears to be an important component of the control of hamartin-tuberin complex formation (Astrinidis et al., 2003; Lee et al., 2007), but it is still unclear whether the phosphorylation of hamartin is also involved in oxygen-sensing pathways. Recently, it was reported that protein kinase B (also known as AKT) can regulate IKKβ kinase under ischemic/hypoxic conditions (Chong et al., 2005; Song et al., 2005), and that inhibition of cyclin-dependent kinases (CDK) improves the survival of hippocampal CA1 neurons. AKT regulates the dissociation, while CDK1 regulates the formation, of the hamartin-tuberin complex – either indirectly, or directly through phosphorylation of hamartin (Astrinidis et al., 2003; Lee et al., 2007), as was also mentioned above. It has further been shown that hypoxia can affect the activity of AKT and CDK activity (Kook et al., 2008; Song et al., 2017). Therefore, other signals such as hamartin phosphorylation may also transudce hypoxia, resulting in mTOR inhibition-mediated neuroprotection.

HAMARTIN-MEDIATED ENDOGENOUS NEUROPROTECTION

One component of brain physiology that has been particularly useful to efforts to elucidate the role of hamartin in endogenous neuroprotection is the differential resistance to hypoxia exhibited between hippocampal regions. It has been long-established that CA1 hippocampal neurons are highly vulnerable to hypoxic conditions, while CA3 cells are relatively resistant to ischemic injury. This contrast has spurred research interest in determining the molecular foundations of CA3 resistance (Chen et al., 1996; Ouyang et al., 2007; Sun et al., 2009). Hadley et al. (2013), for instance, have investigated hamartin in this context, reporting that, while hamartin levels in CA1 neurons are unaffected during ischemia alone, they are upregulated when antecedent ischemic preconditioning is instituted; by contrast, hamartin can be induced by ischemia in CA3 neurons. This led them to propose that hamartin is a critical mediator both of the resistance of CA3 neurons to global ischemia, and of the tolerance conferred by IPC on CA1 neurons. In addition, knockdown and overexpression studies of hamartin have demonstrated increased and decreased vulnerability of neurons, respectively, to cell death following oxygen-glucose deprivation (Johnson et al., 2001). These findings are consistent with the classification of hamartin as an endogenous neuroprotective molecule in the brain.

The mechanism by which hamartin fulfills its neuroprotective function may involve modulation of metabolic programming on the molecular level:

(1) Hamartin highly related with ATP product and biosynthesis. Wang et al. found increases in mitochondrial respiration, glycolysis, and lipid synthesis in hamartin-deficient dendritic cells (Wang et al., 2013). *TSC1/2*$^{-/-}$ cells are hypersensitive to glucose deprivation and this has been linked to increased p53 translation and activation of apoptosis (Choo et al., 2011). These observations appear to translate to larger units of organization, as it was found in another study both that cells containing mutated hamartin were enlarged by a factor of 2–3, and that the size of organs that contained the most hamartin mutant cells were increased (Potter et al., 2001). On the other hand, it has been shown that energy efficiency promotes a reduction in cell size (Sengupta et al., 2013), as well as protection of neurons from ischemic/hypoxic injury (Shao and Lu, 2012). Thus, the upregulation of hamartin induced by ischemic preconditioning may produce the opposite outcome seen with its inhibition, reducing cellular energy demand and thereby conferring protection on the neurons that express it against ischemic insults.

(2) Hamartin modulated autophagy, a critical regulator of cellular metabolism and homeostasis. Autophagy is well known as a physiological which prolongs cell survival though the recycling of cellular macromolecule to generate energy (Rabinowitz and White, 2010). This process replenishes pools of cellular precursors in response to pressure (Ryter et al., 2013). Autophagy, which is a mechanism for the degradation of cellular components that has come to prominence for its involvement in a number of important diseases (such as obesity, cancer, and neurodegenerative disorders), has been revealed to be critical to the regulation of energy balance in the brain (Choo et al., 2010; Coupe and Bouret, 2012). Autophagy might also participate directly in the degradation of glycogen, lipid and protein to produce ATP to meet celluar demand (Mizushima, 2007; Kovsan et al., 2009; Kim and Lee, 2014). Sheng et al. (2010) showed that activation of autophagy occurred during IPC, provided protection against subsequent permanent focal ischemia, and that induction of autophagy with the mTOR inhibitor rapamycin reproduced the neuroprotective effect seen with IPC. Since hamartin is also induced by IPC and functions through mTOR signaling, the endogenous neuroprotective effect of hamartin may depend on autophagy; indeed, hamartin has been shown to promote autophagy through its inhibitory effect on mTORC1 (Hadley et al., 2013; Papadakis et al., 2013; Xia et al., 2013). In addition to its energy-conserving effect secondary to mTOR inhibition, autophagy also appears to exert its neuroprotective effect through an anti-apoptotic mechanism (Jing et al., 2012).

Thus, taken together, the current data suggest that the endogenous neuroprotection conferred by hamartin may arise both from the energy conservation and anti-apoptosis it promotes, in a manner that can proceed either through autophagy, or independently.

MODULATION OF HAMARTIN EXPRESSION BY DNA METHYLATION

One mechanism that may account for the expression pattern exhibited by hamartin is epigenetic induction through changes in DNA methylation. The relationship between epigenetics and hamartin has been demonstrated experimentally, with higher methylation rates seen in the hypothalamic neurons of Sprague Dawley (SD) rats that received high-fat ketogenic diets found to correspond to decreased expression of hamartin (Zhang et al., 2015). Similarly, Wang et al. (2017) revealed reduced expression of hamartin in fibrotic mouse lungs concurrently with an increase in hamartin promoter methylation.

As mentioned earlier, DNA methylation is a type of epigenetic modification that involves potentially stable, heritable genetic modifications that control gene expression, typically without altering DNA sequences (Petronis, 2010). Developmental, environmental, or pathogenic stimuli can cause epigenetic changes, which can affect gene expression and thus the regulation of many cellular processes (Shetty et al., 2018). DNA methylation is the best-studied epigenetic event, and has been found to take place at the 5-C position of the cytosine residues of CpG dinucleotides in a reaction that is catalyzed by DNA methyltransferase (DNMT) (Zhang et al., 2014; Schubeler, 2015). Higher methylation rates of CpG dinucleotides in promoters represses gene expression, while lower methylation rates promote gene expression by facilitating transcription factor binding and the attraction of methyl-binding proteins (Fazzari and Greally, 2004). It has been found that gene expression and DNA methylation changes in aneurysmal subarachnoid hemorrhage patients undergoing remote ischemic preconditioning are involved in coordinated cell cycle and inflammatory responses (Nikkola et al., 2015). IPC induction of Arid5a and Nptx2, modulators of neuronal cell death, were shown to be demethylated in regulatory regions, suggesting the involvement of DNA methylation in IPC-induced neuroprotection (Cai et al., 2019). Thus, it follows that lower methylation rates in its promoter region could result in increased hamartin expression.

In a recent study by our group, we found that hypoxic preconditioning may change expression and activity of the methyltransferase enzymes DNMT3A and DNMT3B (Sheng et al., 2010). It has been reported that decrease of Dnmt1 expression at 4 days post-ischemia may be related to ischemia-induced delayed neuronal death (Lee et al., 2013). In a study involving methyltransferase-mutated mice, $Dnmt^{S/+}$ heterozygotes were shown to be resistant to mild ischemic damage, suggesting that DNMTs adversely impact neuroprotection after ischemia (Endres et al., 2000). Other work has used the nucleotide analog 5-Aza-2'-deoxycytidine (5-aza-cdR) as a DNMT inhibitor to observe the effect of DNA methylation on gene expression (Desjobert et al., 2015; Zhang et al., 2016). Wang et al. (2017) used this strategy to investigate hamartin, demonstrating that 5-aza-cdR significantly upregulated hamartin levels in lung fibroblast cells. Comparable results were achieved on an oral squamous cell line treated with 5-aza-cdR, which in this case produced a significant increase in expression of TSC genes (Chakraborty et al., 2008). 5-Aza-CdR has been approved by FDA for disease treatment through affecting genes directly or indirectly (Yang et al., 2010; Dastjerdi et al., 2014) 0.5-aza-cdR have been used in a clinical setting in myelodysplastic syndrome (Abou Zahr et al., 2015), Therefore, it implied that 5-Aza-CdR may be used as a potential clinical treatment medicine for ischemia/hypoxia brain damage through up-regulation TSC/down-regulation mTOR. Thus, it is conceivable in light of our work on the modulation of DNMTs by hypoxic preconditioning that ischemic/hypoxic conditions may induce DNMTs to alter DNA methylation rates at the hamartin gene, modulating its expression to promote the neuroprotective effect called for under these circumstances (**Figure 2**).

CONCLUSION

Endogenous neuroprotective molecules such as VEGF and HIF-1 are induced by IPC/HPC, whereupon they act to increase neuronal tolerance to hypoxia/ischemia. Therefore, upregulation of these molecules through either chemical or physical (i.e., IPC/HPC) means may prove beneficial in conferring protection against hypoxic/ischemic insults. Hamartin appears to be one such endogenous neuroprotective molecule, which is also well-known for its role in regulating activity of the mTOR pathway that is responsible for controlling cell metabolism and survival. Hamartin regulates formation of the hamartin/tuberin complex that mediates its activity on the mTOR pathway; complex formation may be modulated by differential phosphorylation. Finally, evidence is emerging that epigenetics may play a role in neuroprotection by impacting the expression of hamartin; specifically, DNA methyltransferase changes may result in upregulation of the expression of hamartin in response to hypoxic conditions.

AUTHOR CONTRIBUTIONS

SL, XJ, and GS: review conception and design. CR, JX, NL, and CH: literature review. CS, AC, and YD: language modification. All authors contributed to the article and approved the submitted version.

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Importance of Assessing Muscular Fitness in Secondary Care

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INTRODUCTION

Muscle atrophy is an unfortunate effect of aging and many diseases and can compromise physical function and impair vital metabolic processes (Argilés et al., 2016; Strasser et al., 2018). One of the many threats to independent life is the age-related loss of muscle mass and strength (by ∼1 % per year from the age of around 30 years) referred to as sarcopenia (Goodpaster et al., 2006). These structural and functional changes in skeletal muscle contribute significantly to adverse health outcomes such as falls, fractures, functional impairments, and mobility limitations accompanied by elevated risk for hospitalization, morbidity and mortality in older persons (Bianchi et al., 2016). Furthermore, malnutrition is common among older people and often poorly recognized and underdiagnosed (Roberts et al., 2019). Insufficient dietary intake is not only related to the development of sarcopenia (Beaudart et al., 2019), but is also a major risk factor for cognitive or functional impairments and mortality in older patients (Sánchez-Rodríguez et al., 2019; Zanetti et al., 2019).

In a recent systematic review with data of a total of 34,955 participants older than 60 years, the prevalence of sarcopenia in community-dwelling individuals was 11% in men and 9% in women, whereas in nursing-home individuals the prevalence was 51% in men and 31% in women and in hospitalized individuals 23 and 24% for men and women, respectively (Papadopoulou et al., 2020). Nevertheless, health care practitioners often inadequately address the multifactorial issues that contribute to age-related and disease-related skeletal muscle changes, such as the following key factors: reduced physical activity and/or energy intake, anabolic resistance, changes in hormones (mainly sexual hormones, growth hormone, insulin-like growth factor 1, and insulin), and low-grade systemic inflammation (Cruz-Jentoft et al., 2010). Sex-specific hormonal changes that occur with aging, with reduced amounts of testosterone and estrogen in men and women, respectively, are an important factor related to sex differences in skeletal muscle structure, function, and metabolism (Gheller et al., 2016), and furthermore, a major contributing factor to the development of sarcopenic obesity with age, associated with accelerated functional decline and increased risks of cardiometabolic diseases, compared to sarcopenia or obesity alone (Roh and Choi, 2020).

The purpose of this opinion article is to reinforce the necessity of easy-to-use clinical measures for identifying patients at risk of developing sarcopenia or related disorders and to further provide practical guidelines that should be considered in the implementation of an exercise program in secondary care.

MEASUREMENT OF MUSCULAR FITNESS IN CLINICAL SETTINGS

As an objective measure, the assessment of muscular strength has achieved considerable clinical value and is considered as a key characteristic of sarcopenia with low handgrip strength (<27 kg for men and <16 kg for women) as the first defining characteristic (Cruz-Jentoft et al., 2018). A

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recent meta-analysis evaluating the relationship between muscular strength measures and mortality in outpatient populations with chronic diseases and in critically ill hospitalized patients found evidence of associations between all measures of muscular strength investigated (handgrip strength, knee extension strength, and other) and all-cause mortality (Jochem et al., 2019). This growing body of evidence should be a stimulus for physicians to incorporate muscular strength improvement as a high priority in the overall clinical treatment approach to patients with multiple chronic conditions to improve patient management and patient health, though it may be even better to use regular exercise as a preventative strategy. Being physically active is one of the most important approaches that people of all ages can take to maintain their health and normal functioning. Future trials should be conducted to develop validated cut-points for diagnosing low muscular fitness and their predictive value for hard end-points, such as survival.

The implementation of simple physical capability tests that may reliably assess the muscular strength state of the patient has value in any phase of aging, but particularly in the elderly routine because of the bidirectional interplay between multimorbidity and functional impairment (Calderón-Larrañaga et al., 2019). Recent findings of a prospective study from UK Biobank suggested that from all physical capability markers used to define sarcopenia, slow gait speed ($\leq$0.8 m/s) and low handgrip strength seemed to be the main drivers of the noticed association with health outcomes—more than low muscle mass—and should be considered in clinical practice (Petermann-Rocha et al., 2020). According to the concept of "coordinated deadaptation," functional capacities (and structure) of the cardiovascular and respiratory systems will decline when skeletal muscle function due to aging and disease, but also as a result of physical inactivity, decreases (Burtscher, 2013). The Longevity Check-up 7+ project recently showed that pulmonary function was positively associated with muscular function, assessed by chair stand and handgrip strength tests (Landi et al., 2020). Thus, timely detection of lower muscular strength and function may be helpful in evaluating potential pulmonary function impairment. This loss of function is often the result of changes in muscle quality independent of muscle mass (Correa-de-Araujo et al., 2017). Potential mechanisms include changes in muscle tissue composition and muscle cell metabolism based on high levels of inter- and intra-muscular adipose tissue and intramyocellular lipids. For future clinical practice, measures of muscular strength can be combined with measures of muscle quality such as phase angle (Uemura et al., 2020) or ultrasound-measured thigh muscle echogenicity, also known as echo intensity, to create a score that would better predict functional strength and clinical outcomes across the adult age span (Bourgeois et al., 2019). Although more expensive and time-consuming, the use of magnetic resonance imaging (MRI) allows additional evaluation of fat/connective tissue infiltration of the muscles (Prado and Heymsfield, 2014). This enables a more tailored approach for treatment, which may help in improving the effectiveness and acceptability of therapies currently available.

RECOMMENDATIONS FOR THE MANAGEMENT OF SARCOPENIA

Initiating early treatments to maintain proper muscle mass and function are crucial for optimal patient outcomes across the healthcare continuum (Prado et al., 2018). Interventions to support muscularity include resistance exercise and nutrition because both have a positive impact on protein anabolism. An initial resistance training program should be performed on 2 non-consecutive days per week and may progress to a regimen of 3 days per week. The training load should be systematically increased to keep the maximum possible repetitions per set between 10 and 15, corresponding to 60–80% of one- repetition maximum (Fragala et al., 2019). A minimum of two sets per muscle group per week should be performed at the beginning of the program and be increased progressively (every 4 weeks by one set) to a maximum of six sets (in rehabilitation) and 10 sets (in health promotion) per muscle group per week (Strasser and Schobersberger, 2011). Generally, resistance training should consist of exercises for all major muscle groups. However, training of the small muscle groups of the lower limbs (e.g., single leg knee extensions) is a powerful approach to combat exercise intolerance in patients with chronic obstructive pulmonary disease and heart failure (Burtscher, 2013). Although traditional slow-velocity resistance training is primarily associated with improvements in muscular strength level, there is convincing evidence that muscle power training with higher-velocity and lower-intensity (30–60% of one repetition maximum or the use of own body as resistance) would be a more effective strategy to improve both muscular strength and power output, as well as functional abilities (i.e., sit-to-stand, walking ability, stairs climbing) in elderly populations, including the frail oldest old (Cadore and Izquierdo, 2018).

However, poor exercise compliance and adherence to exercise training programs is a common problem in older multimorbid patients. In these cases, health care professionals should focus on patients' strengths rather than their weaknesses. Motivating patients to be active can help alleviate the loss in muscular function associated with aging and disease. The clinical environment can be easily used for health promotion activities, in particular advocating for reduced sedentary time and increased physical activity to promote muscle conditioning and thereby supporting patients' self-management (Murayama et al., 2020). Nevertheless, many of the barriers to exercise and some of the reasons for poor adherence come from outside the clinical environment, such as lack of time or lack of skills, costs of sports programs or equipment. With time economy as the primary concern, the 2018 US Physical Activity Guidelines for Americans revealed new opportunities for promoting physical activity by recognizing that even short and sporadic bouts of high relative intensity incidental physical activity count for health (Piercy et al., 2018), which may be an attractive option for people living a sedentary lifestyle to be more active from earlier in life to stop them having problems later on.

For patients who are not able to perform active exercise, such as in the ICU setting, the use of neuromuscular electrical

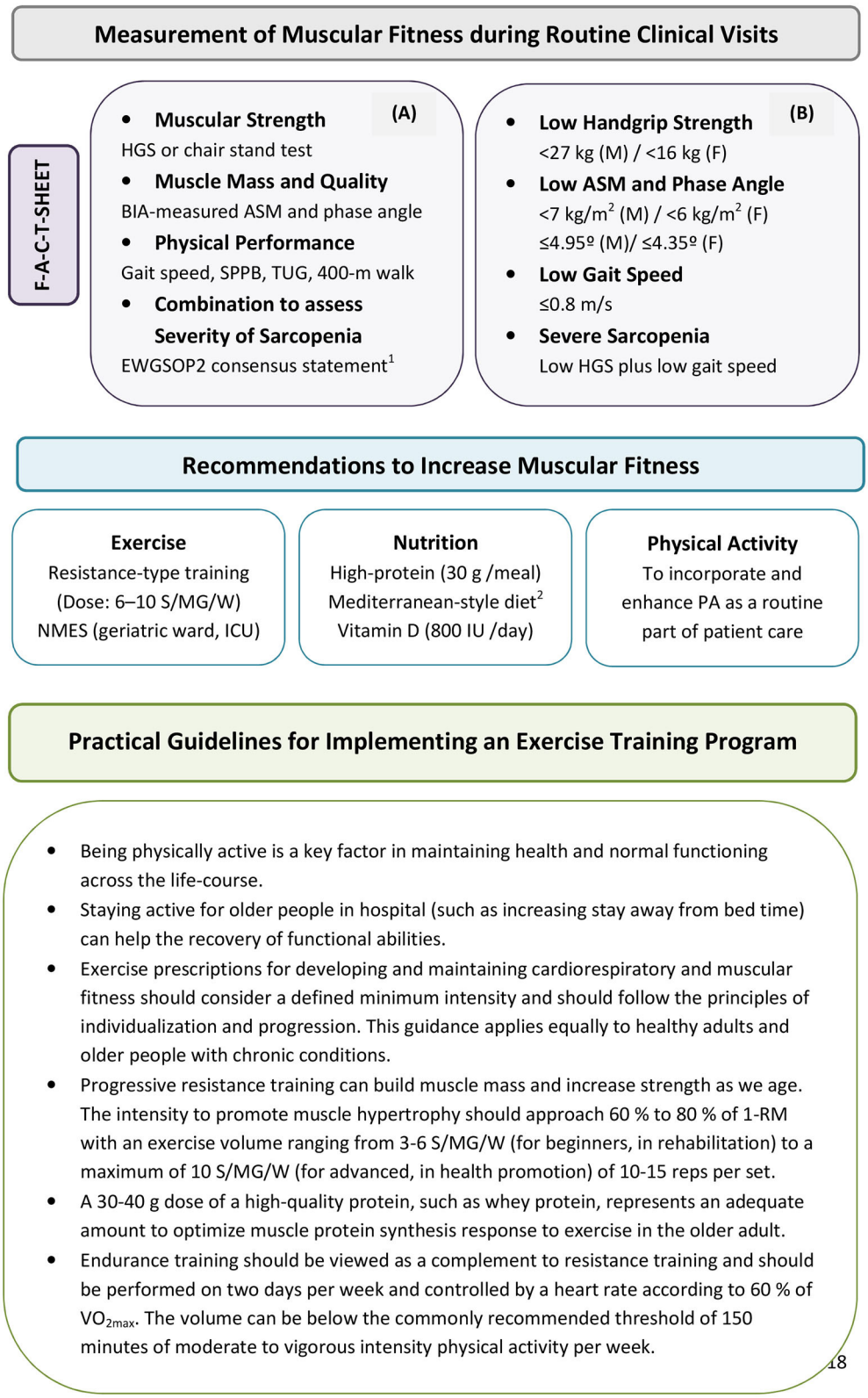

FIGURE 1 | Procedures for incorporating muscular strength and function estimations into routine clinical assessments in a pragmatic manner **(A)** for identifying patients at risk for low muscular fitness in secondary care. **(B)** Recommendations listed in figure provide practical guidelines that should be considered in the

(Continued)

FIGURE 1 | implementation of an exercise and physical activity program. The steps of the pathway are represented as Find-Assess-Combine-Treat or F-A-C-T [modified by Cruz-Jentoft et al. (2018)]. EWGSOP2 recommends for case-finding the use of the SARC-F questionnaire as a screen for sarcopenia risk (Malmstrom et al., 2016). [1]Cruz-Jentoft et al. (2018); [2]Capurso et al. (2019). ASM, appendicular skeletal muscle mass; BIA, bioelectrical impedance analysis; EWGSOP2, European Working Group on Sarcopenia in Older People 2; F, females; HGS, handgrip strength; ICU, intensive care unit; M, males; MD, mediterranean diet; NMES, neuromuscular electrical stimulation; PA, physical activity; S/MG/W, sets per muscle group per week; SPPB, Short physical performance battery; TUG, Timed-up-and-go test; 1-RM, one-repetition maximum; VO_{2max}, peak oxygen uptake.

stimulation (NMES) appears to be a potential adjunct to prevent muscle atrophy and loss of muscle strength (Dirks et al., 2015). Moreover, this technique is a useful clinical tool to preserve leg lean mass during hospitalization in geriatric patients (Karlsen et al., 2020). At the molecular level, NMES stimulates the regenerative capacity of satellite cells and induces downregulation of genes (e.g., myostatin, MuRF1 and MAFbx) involved in muscle atrophy (Karlsen et al., 2020). NMES intensity should be as high as individually tolerated, and a minimum of three sessions per week with large pulses (between 300 and 450 μs) and high frequency (50–100 Hz in young and around 30 Hz in older adults) should be performed (Adams, 2018).

A recent RCT in elderly men found that whey protein supplementation following resistance exercise induced changes in muscle microRNAs (miR-208a and-499a and-206) similar to what is reported in young men (D'Souza et al., 2019). These findings confirm a potential involvement of specific microRNAs in the regulation of hypertrophic signaling pathways following an acute resistance training stimulus. Thus, circulating microRNAs may serve as a predictive marker of the physiological state of skeletal muscle and may have important significance for the screening of early sarcopenia and related conditions (He et al., 2020), but also provide an understanding into mechanisms involved in the aging process such as anabolic resistance (Margolis et al., 2017).

In addition, the supplementation of essential amino acids and vitamin D can further augment protein anabolism and has been shown to improve muscle composition in mobility-limited older adults (Englund et al., 2017). The current recommended dietary allowance (RDA) for protein of 0.8 grams protein per kilogram of body weight per day might not be adequate for maintaining muscle health in old age. Therefore, experts have proposed an increase in dietary protein recommendations for older age groups to 1.0–1.2 g/kg body weight per day, and an even higher protein intake (1.2–1.5 g/kg body weight/day) is advised for those who are exercising or for older people with disease or injury (Bauer et al., 2013). Nevertheless, the training component *per se* is of primary importance when it comes to improving muscle mass and strength, as well as functional capacity, as a substantial part of the older population does benefit from a resistance-type exercise training intervention (Churchward-Venne et al., 2015).

Although testosterone supplementation in older men has been shown to improve body composition, yet the effects on muscular strength or physical function are still conflicting (Endo et al., 2020). It has been shown that testosterone replacement therapy does not offer any benefit beyond resistance exercise alone in elderly patients with low to normal serum testosterone, but may be an effective short-term intervention to overcome age-related deficits in adaptive responses to resistance training (Gharahdaghi et al., 2019). However, because of potential adverse events, the clinical meaningfulness of testosterone in the management of sarcopenia should be carefully evaluated.

It is essential to maintain good muscular fitness over the long-term to improve health outcomes. In this regard, it is important to incorporate physical performance assessment and promotion into healthcare in a manner that engages both clinicians and patients (Brannan et al., 2019). Accordingly, understanding and addressing muscular strength, mass, and function in older persons and to communicate the health-promoting effects of regular exercise and physical activity combined with nutritional advice affords clinicians with a vitally important opportunity to identify patients at risk of developing sarcopenia or related disorders and, more importantly, to preserve muscle mass and function. Procedures for incorporating diagnostic tools into routine clinical practice in a pragmatic manner and recommendations that should be considered in the implementation of an exercise training program to increase muscular fitness are provided in **Figure 1**. Such a multimodal approach improves the efficiency of therapy and ameliorates the functional capacity even in advanced disease stages; these are crucial to avoid long-term care, thereby promoting quality of life for older people.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Telomere Length as a Marker of Biological Age: State-of-the-Art, Open Issues, and Future Perspectives

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Telomere shortening is a well-known hallmark of both cellular senescence and organismal aging. An accelerated rate of telomere attrition is also a common feature of age-related diseases. Therefore, telomere length (TL) has been recognized for a long time as one of the best biomarkers of aging. Recent research findings, however, indicate that TL *per se* can only allow a rough estimate of aging rate and can hardly be regarded as a clinically important risk marker for age-related pathologies and mortality. Evidence is obtained that other indicators such as certain immune parameters, indices of epigenetic age, etc., could be stronger predictors of the health status and the risk of chronic disease. However, despite these issues and limitations, TL remains to be very informative marker in accessing the biological age when used along with other markers such as indices of homeostatic dysregulation, frailty index, epigenetic clock, etc. This review article is aimed at describing the current state of the art in the field and at discussing recent research findings and divergent viewpoints regarding the usefulness of leukocyte TL for estimating the human biological age.

Keywords: leukocyte telomere length, age-related telomere shortening, telomerase, biomarker of biological age, aging-related disease, mortality

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INTRODUCTION

The last century has been characterized by an unprecedented rise in human life expectancy across the globe. This demographic trend, however, was generally not accompanied by the same extension in healthspan and productivity (Lee et al., 2020). This is because aging *per se* is the major risk factor for most pathological conditions that limit healthspan and promote chronic disorders affecting the elderly, including immuno-senescence, cardio-metabolic disorders, osteoporosis, sarcopenia, arthritis, cataracts, neurodegenerative diseases and most cancers (Franceschi et al., 2018). Therefore, development of effective tools for slowing down the aging rate is currently a priority agenda for research organizations and for health policy makers across countries (Yabluchanskiy et al., 2018). In this context, an important task is the development of effective tools to evaluate the rate of aging both in individuals and in groups of patients participating in clinical trials specifically targeting aging and related multimorbidity (Ferrucci et al., 2020; Moskalev, 2020). Historically, functional tests such as grip strength, walking speed, chair rising, and standing balance times were most commonly used in such studies (Ferrucci et al., 2016; Justice et al., 2016). These tests, however, are too sensitive to transient changes in health status caused by non-chronic diseases such as colds and indigestion, so they can hardly provide a reliable estimate

of the aging rate *per se*. Therefore, considerable efforts were made over the past few decades to develop measures of biological aging which are reliable, precise, and robust. Among them, there are parameters characterizing aging-related processes such as genomic instability, cellular senescence, DNA methylation, proteostasis, and mitochondrial function (Jylhävä et al., 2017; Ferrucci et al., 2020). None of the above measures, however, represent an exhaustive measure of biological age.

According to the American Federation for Aging Research (2016), a true biomarker of aging should meet the following criteria:

1) predict remaining life expectancy better than chronological age,
2) monitor mechanisms underlying the aging process but not a specific disease,
3) be subject to repeated tests without harming the individual,
4) be testable in both laboratory animals and humans.

Telomere length (TL) possesses many properties that make it suitable to be used as a biomarker of aging. Telomeres of vertebrates represent repetitive (TTAGGG)n sequences located at the ends of linear chromosomes. These chromosomal structures are known to be crucially involved in the process of cellular senescence (Victorelli and Passos, 2017) and their age-associated shortening is commonly regarded as an important contributor to organismal aging (McHugh and Gil, 2018). Therefore, TL is currently recognized by many authors as powerful biomarker of aging and aging-associated pathological conditions (Fasching, 2018). The advantages of TL in order to be a biomarker of aging include a correlation with chronological age throughout the entire life course, predictive power for the disease condition and mortality and also large responsiveness to either adverse or beneficial exposures (Hastings et al., 2017). Moreover, it is recognized as an attractive biomarker candidate to track changes in the human aging rate over the course of intervention trials designed to delay the onset or retard the progression of aging-associated pathological conditions. However, while TL satisfactorily meets the criteria 3 and 4 of the American Federation for Aging Research due to an opportunity to a minimally invasive, repeated testing without harm to human subjects and a testability in both laboratory animals and humans, its compliance with the criteria 1 and 2 is rather questionable (Sanders and Newman, 2013).

Presently, peripheral blood leukocyte TL (LTL) is often identified in cross-sectional and longitudinal analyses when certain patient cohorts are compared to age- and sex-matched individuals. However, even though convincing evidence has been obtained from *in vitro* and *in vivo* models that TL reflects levels of both the cellular senescence and chronic disease-related oxidative stress, epidemiological and clinical findings are far less conclusive (Sanders and Newman, 2013). Indeed, a large inconsistency was observed among studies. Such a discrepancy could be, at least partly, attributable to differences in the methods of TL measurement and statistical modeling used, variations among investigated populations, etc. This inconsistency was, however, so great that some authors have even questioned whether the link between TL and aging-associated processes really exists and, consequently, it has led to hot debates about whether TL is really a reliable and valid tool to evaluate the rate of aging in human populations (Sanders and Newman, 2013). Moreover, the phenomenon of the age-related telomere shortening is extremely complex and biological mechanisms underlying this process are not yet definitively established. In particular, it remains still unclear whether telomeric aging reflects a mitotic clock-like process or it is rather a biomarker of stress or a biological mechanism that transfers stress-associated signals to the cell (Koliada et al., 2015; Notterman and Schneper, 2020). If the latter, telomere shortening can be rather a proxy for life-course stress exposures than a genuine cause of aging (von Zglinicki and Martin-Ruiz, 2005). Nevertheless, despite this uncertainty, TL currently remains one of the most widely used biomarkers of aging in epidemiological and clinical studies. In recent years, TL is also being increasingly used as a potential biomarker in personalized medicine (Gorenjak et al., 2018).

The present review is aimed at discussing diverging views and research findings regarding the usefulness of TL for estimating the human biological age.

BASIC PRINCIPLES OF TELOMERE BIOLOGY

Telomeres play vital roles in multiple cellular processes because they protect chromosomes from end-to-end fusions and chromosomal instability (Aksenova and Mirkin, 2019). The repetitive TTAGGG sequences that constitute telomeric DNA are bound by the protective protein complex, Shelterin. This complex, together with proteins involved in chromatin remodeling, shapes the telomere structure thereby protecting chromosome ends (Tomita, 2018). Two key telomere features are the formation of DNA loops at chromosome ends (T-loops) and the transcription of telomeres producing G-rich RNA (TERRA). In the t-loop structure, the 3′ end of the G-rich strand protrudes as a single stranded overhang, known as the G-overhang (Turner et al., 2019). This G-strand overhang loops back to form a t-loop and invades the 5′ double stranded telomeric duplex, thereby forming so-called D-loop. This structure ensures that loose DNA ends are housed internally within the nucleoprotein structure (Turner et al., 2019). The formation of such looped structures is an important mechanism that protects telomeres from premature degradation. Despite their heterochromatic state, telomeres are able to be actively transcribed resulting in a production of long non-coding RNAs called TERRA (telomeric repeat-containing RNA). TERRA molecules play crucial role in telomere biology, including regulation of telomerase activity and formation of heterochromatin at chromosome ends (Bettin et al., 2019; Lalonde and Chartrand, 2020).

At each somatic cell division cycle, telomeres shorten by 50–200 bp through incomplete synthesis of the lagging strand during the DNA replication (Srinivas et al., 2020). This is due to inability of DNA polymerase to completely replicate the 3′ end of the DNA strand (a phenomenon commonly referred to

as "the end-replication problem") (Watson, 1972; Olovnikov, 1973). Moreover, since the G-rich telomere repeat sequence is known to be highly susceptible to oxidative damage (Oikawa and Kawanishi, 1999), telomeres may be directly damaged by oxidative stress, thereby driving the cell into senescence (Barnes et al., 2019). Considering this, it has been recently suggested that telomere-induced senescence of post-mitotic cells might be a key driver of aging (von Zglinicki et al., 2020).

In culture, somatic cells have limited replication potential reaching a time point at which cell division ceases. This time point is characterized by shortening ("attrition") of particular telomeres to a critical size incompatible with their functioning, thereby resulting in cell cycle arrest and cellular senescence. Therefore, TL is suggested to limit the cell division number and acts as a "mitotic clock" in the cell (Olovnikov, 1996), and telomere shortening may cause decreasing of proliferative potential and be a marker for cellular senescence (Liu et al., 2019a). In a multicellular organism, TLs are highly heterogeneous across different tissues and cell types depending, at least partly, on the tissue-specific proliferation rate, but they generally tend to decrease with age in all proliferating tissues (Demanelis et al., 2020).

The size of critically short ("uncapped") telomeres may be stabilized by telomerase, a reverse transcriptase enzyme that can elongate chromosome ends *de novo*. Two main components of human telomerase are telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) serving as a template for the telomere elongation (Rubtsova and Dontsova, 2020). In humans, this enzyme is known to be expressed during early *in-utero* development, is inactivated in most adult cells except for the germ line and embryonic stem cells and immune cells, and reactivated in the majority of cancers (Shay and Wright, 2019). Telomerase was shown to be insufficient to maintain normal TL even in proliferating stem cells that may express it; therefore, gradual shortening of telomeres also occurs in these cells (Lai et al., 2018; Celtikci et al., 2020) (**Figure 1**). Since most human somatic cells have low or no telomerase activity, it results in age-related telomere erosion and associated pathological processes. Therefore, activation of telomerase is regarded by several authors as promising therapeutic modality in the treatment of degenerative aging disorders (Bernardes de Jesus and Blasco, 2011; Prieto-Oliveira, 2020). However, even although telomerase indeed has potential in anti-aging medicine, the fact that it is over-expressed in about 90% of human cancers raises doubts about the applicability of telomerase activators in clinical practice (Smith-Sonneborn, 2020).

GENETIC AND ENVIRONMENTAL DETERMINANTS OF TL

TL and the rate of age-related telomere shortening depend on both genetic and environmental factors. In twin studies, high heritability of TL was demonstrated and many specific loci related to TL were identified. For example, in the study by Hjelmborg et al. (2015), the heritability of LTL at baseline was estimated at 64%; the heritability of age-dependent LTL attrition rate was less (28%) but also significant. Genetic mutations related to short TL have been found to lead to diseases such as pulmonary fibrosis, dyskeratosis congenita, and also some other clinical conditions commonly grouped under the term "telomere syndrome" (El-Chemaly et al., 2018). A relative contribution of genetically determined TL to the risk of various neoplastic and non-neoplastic disorders was repeatedly evaluated by a Mendelian Randomization approach (a research strategy aimed at evaluating causality in observational epidemiological studies). Mendelian Randomization studies have indicated that long genetically predicted TLs (gTLs) are commonly associated with greater risks for several cancers, whereas short gTLs are associated with increased risks of some age-related degenerative diseases, including cardiovascular disorders (CVDs) and Alzheimer's disease (Telomeres Mendelian Randomization Collaboration et al., 2017; Protsenko et al., 2020). Recenely, a potential causal link between short gTLs and accelerated facial aging was demonstrated using Mendelian randomization analysis in participants of the UK Biobank data (Zhan and Hägg, 2020).

Two potential sources of TL heritability include inherited variation in non-telomeric regions (e.g., single nucleotide polymorphisms affecting telomere maintenance) and TL variability in gametes which produce zygotes (the "direct" inheritance). Strong evidence has been provided that TLs in parental germ cells may impact TL in offspring cells thereby contributing to heritability of TL (Delgado et al., 2019). Indeed, TL was shown to increase with age of father in sperm (correspondingly, descendants of older fathers inherit longer telomeres), while higher maternal age at conception appears to be associated with shorter offspring TL (Eisenberg and Kuzawa, 2018; Eisenberg et al., 2019). Remarkably, the impact of parental germ cells on TL remains significant even despite the telomere "reprogramming" throughout embryonic development, initially by the recombination-based mechanisms and then through the activity of telomerase at the blastocyst stage and later (Kalmbach et al., 2014, see also **Figure 2** for schematic illustration).

Multiple findings indicate that TL may be also significantly modulated by environmental and life-style factors throughout the life course. Among important factors affecting life-course TL dynamics, there are developmental experiences such as intrauterine unfavorable events (Entringer et al., 2018; Habibi et al., 2020), TL at birth (Gorenjak et al., 2020), as well as early life adversity (family's low socioeconomic status, childhood neglect or abuse, etc.) (Belsky and Shalev, 2016; Ridout et al., 2018; Beijers et al., 2020). This is all the more important that early-life exposures can likely affect health status and longevity not only in directly exposed individuals but also in their offspring (Vaiserman et al., 2017). Recently, evidence was obtained that TL may be substantially involved in such trans-generational effects. According to a hypothesis recently proposed by Epel (2020), TL can be inherited not just through classical genetic mechanisms but also through direct (epigenetic-like) transmission of the parental germ-line TL. More specifically, if parental gametes are vulnerable to stress, then chromosomes, together with their telomere sequences which were shortened in consequence of stress exposure, can be directly transmitted to offspring thereby affecting health outcomes and longevity in

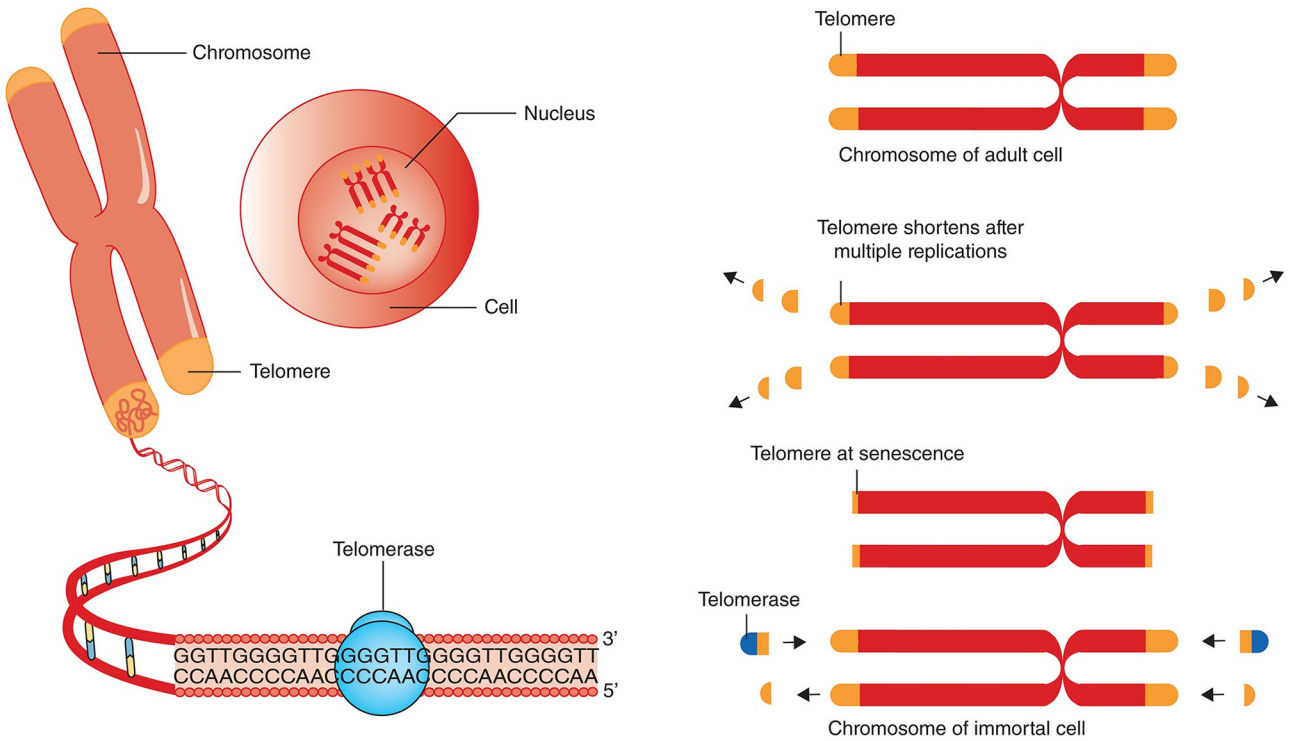

FIGURE 1 | Telomere attrition, telomere length and telomerase. Chromosomes have repeated base segments called telomeres that shorten with each replication cycle (cell division). The enzyme telomerase has the capability to extend the telomere ends, thus prolonging cell life and potentially inducing immortality (which is a cancer cell hallmark). The figure and its legend are reproduced from the article by Aunan et al. (2016) with permission from John Wiley and Sons (License Number 4938770663443). Copyright © 2016 BJS Society Ltd Published by John Wiley & Sons Ltd.

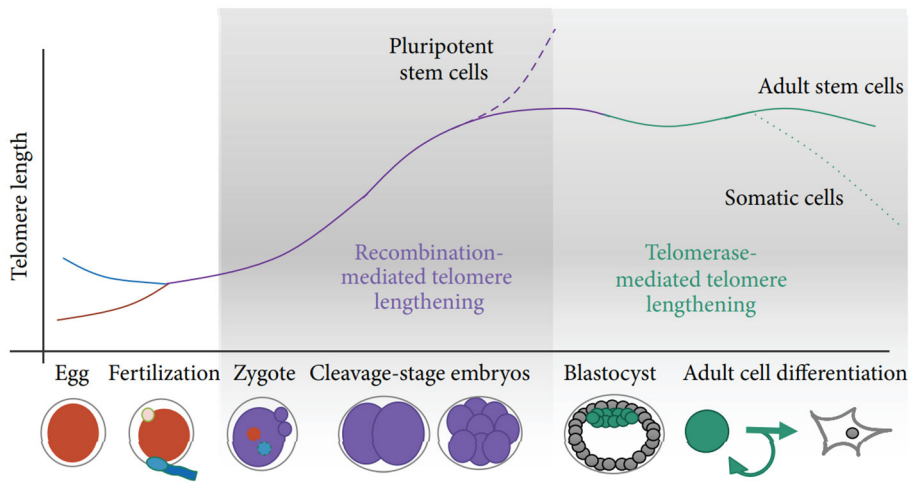

FIGURE 2 | Schematic of telomere length reprogramming in mammalian embryonic development. The greatest telomere lengthening takes place during the earliest stages of preimplantation development, the cleavage-stage embryo, which may coincide with zygote genome activation. Recombination-mediated telomere lengthening (ALT) is also purportedly responsible for reprogramming in pluripotent stem cells, including ESCs, ntESCs, and iPSCs. Later, in development and adult life, telomerase becomes the dominant telomere maintenance mechanism for the inner cell mass and in tissue-specific telomere replenishment in stem cell niches. The figure and its legend are reproduced from the article by Kalmbach et al. (2014) distributed under the terms of the Creative Commons Attribution License. Copyright: © 2014 Keri Kalmbach et al.

subsequent generations. Adult-life exposures to environmental factors such as infection (Ilmonen et al., 2008), psycho-emotional stress (Epel et al., 2004; Boccardi and Boccardi, 2019; Mayer et al., 2019), nutrition (Canudas et al., 2020; Galié et al., 2020), physical activity (Semeraro et al., 2020), smoking (Astuti et al., 2017), alcohol consumption (Dixit et al., 2019), marital status (Chen et al., 2020), therapeutic interventions (Srinivas et al., 2020), etc. were also shown to have long-term impacts on TL.

In conclusion, better understanding of these processes can likely lead to developing novel treatment strategies specifically targeted at reversing damaging effect of stress on telomeres (Shalev and Hastings, 2019; Epel, 2020) and thereby to promote human healthspan.

LIFE-COURSE DYNAMICS OF TL

An inverse correlation between TL and human chronological age is well-documented in the literature. For instance, in a systematic review of such a relationship in adults, a significant negative correlation of about −0.3 between mean chronological age and mean LTL was found across 124 cross-sectional studies (Müezzinler et al., 2013). This relationship, however, is not linear and depends on the stage of the human life cycle. Following fertilization, TL was shown to decrease up to the stage of embryo cleavage and, thereafter, to increase up to the blastocyst stage (Turner et al., 2010). These changes are in line with changes in telomerase activity during this time (Wright et al., 2001). At the stage of blastocyst, both telomerase activity and TLs are again substantially increased. The gestational dynamics of TL has been determined in few studies only, and results of these studies are contradictory. A decrease in TL and telomerase activity throughout 6–11 weeks of human gestation was observed by Cheng et al. (2013); after that, they both remained rather constant until birth. In the study by Sorochynska et al. (2018), however, both TL and telomerase activity were shown to increase between 5 and 10 weeks of gestation. After that, they reached a plateau and remained constant up to gestational week 12.

In newborns, TLs appear to be associated with parental TLs, though controversy exists regarding the relative maternal and paternal contributions (Eisenberg, 2014; Turner et al., 2019). After birth, telomeres are steadily shortened with age. The rate of telomere attrition was shown to vary throughout a human lifetime (Turner et al., 2019). It is much more pronounced during first two years of life (throughout the period of rapid somatic growth) than during later life (Frenck et al., 1998; Zeichner et al., 1999). It has been also found that those individuals who have either shorter or longer TL in their childhood, compared to the average population value, tend to maintain this rank throughout the rest of the life course (Benetos et al., 2013). In other words, those individuals who enter their adult life with either short or long telomeres are likely to have short or long telomeres, respectively, during the remaining lifetime. This indicates that inter-individual variation in TL is established mainly early in life and that early life is an important stage in determining TL and may have a lasting effect on TL throughout the entire life course (Factor-Litvak and Susser, 2015). Thereby, at any age point, the resulting TL is a joint function of the newborn TL setting and of the TL attrition rate over the lifetime (Entringer et al., 2018). Interestingly, findings from some studies indicated that rate of age-dependent telomere shortening during adulthood was more pronounced in those persons who had longer telomeres at baseline (Turner et al., 2019). Several authors suggest that the effect of the dependency of age-related LTL attrition on the baseline LTL can be explained by a well-recognized statistical phenomenon such as a "regression to the mean" (Verhulst et al., 2013). Such an effect occurs when individuals who are measured with an extreme error (no matter negative or positive) at baseline, will on average tend to be measured with a much smaller error at follow-up (Barnett et al., 2005). In analyzing the potential role of this statistical artifact in age-dependent TL dynamics, Verhulst and co-authors revealed modest effect which, however, remained statistically significant even when correcting for the "regression to the mean" effect, indicating that high baseline TL is really associated with higher rate of age-related TL attrition (Verhulst et al., 2013).

In the blood from healthy newborns, median leukocyte TLs (LTLs) ranged from 8.5 to 13.5 kb in the Okuda et al. (2002) study and from 7.0 to 11.6 kb in the Factor-Litvak et al. (2016) study. With age, LTL was found to shorten with an average annual rate of 30–35 bp (Herrmann et al., 2018), reaching about 5–6 kb in people over 60 years old (Calado and Dumitriu, 2013). This appears to be an important point in the context of current debates on whether the human longevity has a maximal natural limit. Steenstrup et al. (2017) suggested that LTL of 5 kb is the "telomeric brink," which denotes a high risk of impending death. Such a telomeric brink can be likely reached, according to the authors, within the present-day life expectancy and, a fortiori, within the 100-years life expectancy (see **Figure 3** for illustration). If so, then current upward trend in life expectancy may confront a biological limit because of crossing the telomeric brink. Since longer TLs are beneficial for a healthy aging (Boccardi and Boccardi, 2019), this feature of telomere biology is apparently vital for the normal functioning of aging organism.

It should be taken into account, however, that findings from some studies contradict the prediction of Steenstrup and co-authors. For example, in the study by Arai et al. (2015), the average TL values dropped to about 3.5 kb in centenarians and then remained approximately unchanged or, paradoxically, even enlarged in supercentenarians compared to younger age groups. More specifically, TLs of unrelated persons were found to shorten by ∼21 ± 8 (males) and 29 ± 4 (females) bp/year, while after 100 years of age, TLs were found to increase by 59 ± 25 (males) and 48 ± 11 (females) bp/year (**Figure 4**). Evidence was obtained that centenarians can maintain TL and telomerase activity better than non-centenarians, and that healthy centenarians have significantly longer telomeres than unhealthy centenarians (Terry et al., 2008; Tedone et al., 2014, 2019). This could be likely explained by preserved telomerase activity in several cell lines. For example, stimulated T cells from centenarians expressed higher telomerase activity levels and they proliferated better than T cells from younger unrelated 67- to 83-year-old individuals (Tedone et al., 2019). The paradoxical ≪elongation≫ of TL in centenarians may likely be explained by

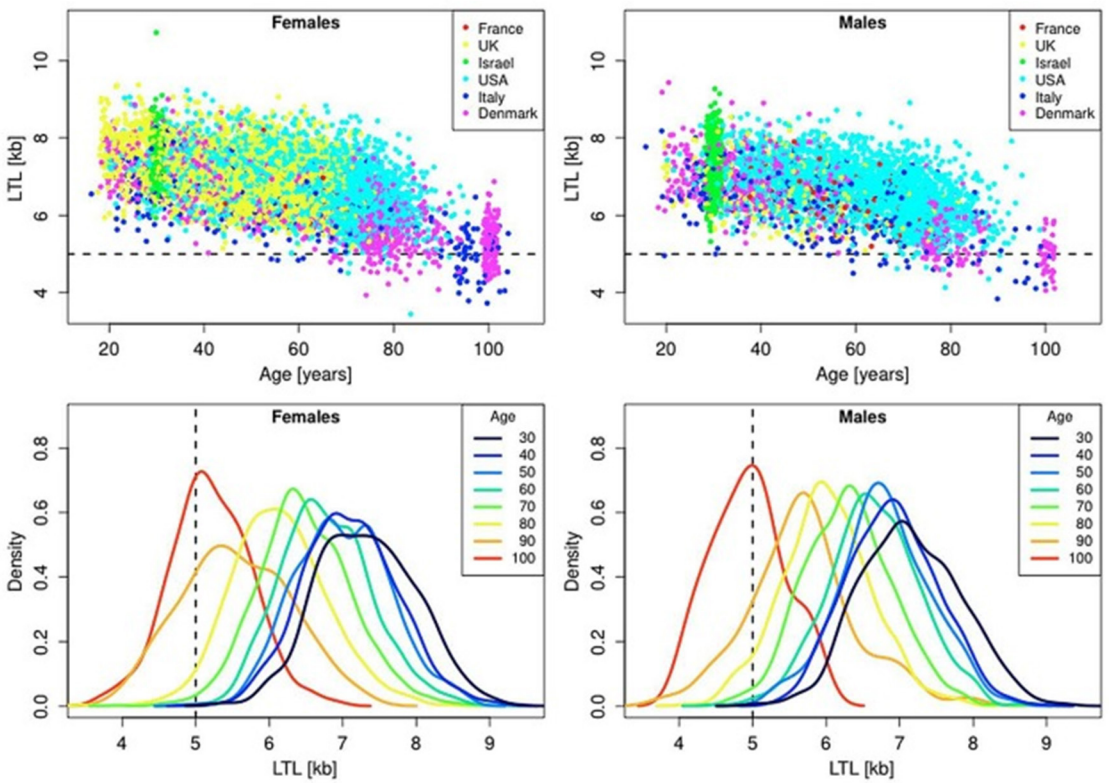

FIGURE 3 | Scatter plots and density plots of LTL as a function of age for males and females residing in different countries. Measurements of LTL were performed in the same laboratory on DNA donated by participants in different studies in different countries. The horizontal dashed lines in the top panels and vertical dashed lines in the bottom panels indicate LTL values of 5 kb. The bottom plots are smoothed histograms obtained by kernel density estimation. The figure and its legend are reproduced from the article by Steenstrup et al. (2017) distributed under the terms of the Creative Commons Attribution License (CC BY 3.0). Copyright © 2017 Steenstrup et al.

a survivor effect (Mather et al., 2011). Indeed, if individuals with shorter TLs are more susceptible to age-associated pathologies and, consequently, are at higher risk of mortality, then these individuals will tend to die earlier. Therefore, in cross-sectional studies of oldest-old participants, only survivors with relatively long TLs may be involved, and, thereby, the variability in TL can be reduced (Mather et al., 2011). The variability of TL was indeed found to be reduced in lymphocytes, CD45RA-positive T-cells and memory T-cells in healthy oldest-old persons compared to mid-life controls (Halaschek-Wiener et al., 2008).

TL AND DISEASE

Recently, a hypothesis was proposed that aging trajectory and longevity potential may be "programmed" early in development (Vaiserman et al., 2018). In particular, it is assumed that intrauterine growth restriction (IUGR) can result in a low birth weight and a high risk for metabolic disorders in adulthood, whereas fetal macrosomia and resulting high birth weight can predict an enhanced risk for cancers in adulthood (Vaiserman, 2018). Telomere biology is suggested to play an important role in mediating these programming effects. According to the "fetal programming of telomere biology" hypothesis proposed by Entringer et al. (2018), both the initial TL and the life-course rate of telomere shortening are highly plastic and sensitive to early-life conditions. As a consequence, stress exposures (maternal-placental-fetal endocrine or metabolic disturbances, oxidative and immune/inflammatory stresses, etc.) acting during prenatal and early postnatal development can program the telomere biology in a way that promote cellular senescence and, as a result, lead to accelerated rate of organismal aging. Possibility of developmental programming of initial TL and rate of age-related telomere erosion could be particularly important in the context of aging and aging-associated pathology. According to a two-hit developmental model of accelerated aging (Belsky and Shalev, 2016; Shalev and Belsky, 2016), shorter TL in early life may be associated with poor health status later in life. Building on these ideas, Aviv and Shay (2018) further hypothesized that lengthened telomeres may enhance the risk for disorders linked to increased proliferation rate, including cancer, while shortened telomeres can enhance the risk for pathologies related to a restricted cellular proliferation and tissue degeneration, such as atherosclerosis-associated cardiovascular disorders.

In epidemiological studies, TL was repeatedly found to reflect the risk for aging-related chronic pathological conditions.

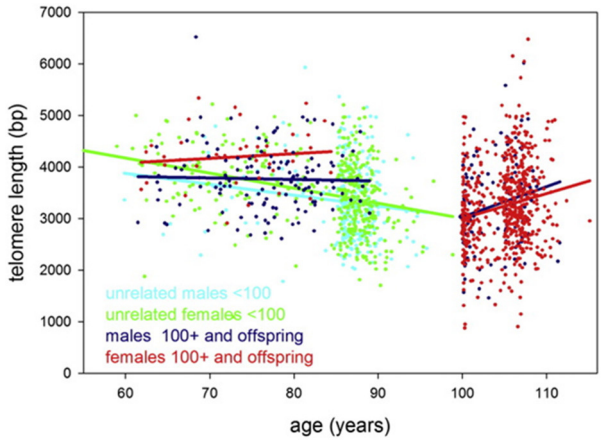

FIGURE 4 | Telomere length in study participants up to 115 years of age. Leukocyte telomere length vs. age is shown for males (blue or cyan) and females (green or red). Centenarians, (semi)supercentenarians, and centenarian offspring are shown in blue (males) or red (females), respectively. Unrelated participants younger than 100 years are indicated in cyan (males) or green (females). Regression lines belonging to these groups are indicated by the same color. The figure and its legend are reproduced from the article by Arai et al. (2015) distributed under the terms of the Creative Commons CC-BY license. Copyright © 2015, Elsevier.

In patients with coronary heart disease, TLs were found to be significantly shorter than that in controls; moreover, TL was inversely correlated with the severity of this disease (Xu et al., 2019). The convincing evidence was obtained that short telomeres are indicative of higher risk for atherosclerosis and related vascular complications (De Meyer et al., 2018; Herrmann and Herrmann, 2020). Low telomerase activity and short LTLs were shown to be associated with atherosclerotic plaque instability and higher risk for coronary heart disease, myocardial infarction or stroke (Yeh and Wang, 2016; Tian et al., 2019; Zhan and Hägg, 2019). Significant associations were observed between the high rate of age-related LTL attrition and hypertension (Liu et al., 2019b). In a systematic review comprising 119,439 individuals in total, a weak to moderate association between obesity and TL was observed in 39 of 63 included studies; a significant heterogeneity, however, was observed between studies (Mundstock et al., 2015). An association of short LTL with higher risk for developing type 2 diabetes has been also demonstrated; this association, however, was significantly influenced by body mass index (BMI), diabetes type, age, region, and sex (Zhao et al., 2013; Willeit et al., 2014; Wang et al., 2016; Krasnienkov et al., 2018). Short LTL was also shown to be associated with different aspects of metabolic syndrome (Khalangot et al., 2017, 2019, 2020). Accelerated telomere shortening and low telomerase activity were shown to be associated with skeletal pathologies mediated by the age-related abnormal reconstruction of the subchondral bone, such as osteoporosis and osteoarthritis (Fragkiadaki et al., 2020).

Available data on TL in aging-associated neurodegenerative disorders are rather inconsistent. No evidence for shorter TLs in patients with Parkinson's disease was obtained in meta-analysis by Forero et al. (2016a). An evidence for shorter TL in patients with Alzheimer's disease has been provided in another meta-analysis by Forero et al. (2016b). One potential explanation for this could be the enhanced level of oxidative stress in those patients (Levstek et al., 2020). In a more recent study, however, a U-shaped association between the TL and the risk for Alzheimer's disease was demonstrated, with both shorter and longer telomeres associated with an increased risk of this disease in the general population (Fani et al., 2020). Given the uncertainty of findings, the contribution of telomere attrition in the pathogenesis of neurodegenerative disorders is yet to be fully elucidated (Levstek et al., 2020).

Epidemiological associations between TL and risk for cancers are also ambiguous. The tumor cells are known to be able to maintain TL for unlimited growth either by reactivation of telomerase or by a specific recombination-based mechanism (Okamoto and Seimiya, 2019). From this, it is commonly thought that the risk of diseases mediated by increased proliferative activity including most cancers have to be correlated with long telomeres (Celtikci et al., 2020). In fact, however, cancerous cells frequently have paradoxically shorter telomeres relative to those observed in normal tissues (Okamoto and Seimiya, 2019). The evidence that cancers are associated with short telomeres in surrogate tissues (e.g., in blood cells) was provided in a meta-analysis by Wentzensen et al. (2011). The strongest evidence has been obtained for gastric, esophageal, renal and bladder cancers. A significant association between short TL and poor cancer survival has been also demonstrated (Zhang et al., 2015). The association between short telomeres and the risk for cancer was consequently confirmed in other meta-analyzes. In particular, convincing evidence has been obtained for the association of short telomeres with elevated risk of gastrointestinal, head and neck cancers (Zhu et al., 2016). However, in a meta-analysis by Zhang et al. (2017), an association between the TL and the risk of total cancers was found to be marginally positive. Subgroup analyses performed within this meta-analysis showed that such a positive association was stronger for a lung cancer, for men and for studies with more precise methods for DNA extraction and TL measurement. In conclusion, the inconsistency in the effects of TL on cancer outcomes can be likely explained by the variable measurement methods. Therefore, the standardization of measurement and reporting of TL is required in cancer epidemiology before the prognostic value of TL might be accurately evaluated (Adam et al., 2017).

From the research findings above, it can be concluded that epidemiological associations between TL and age-dependent pathological conditions are often inconsistent and a mechanistic understanding of these associations is still lacking. Remains uncertain whether age-related telomere shortening is a cause or merely a consequence of aging-associated diseases (De Meyer et al., 2018). Moreover, despite convincing evidence has been provided for links between TL and specific age-related diseases, no significant relationship was found between the TL and syndrome of aging-related physiological decline (frailty), a geriatric clinical syndrome characterized by marked vulnerability to adverse health outcomes (Zhou et al., 2018; Araújo Carvalho et al., 2019).

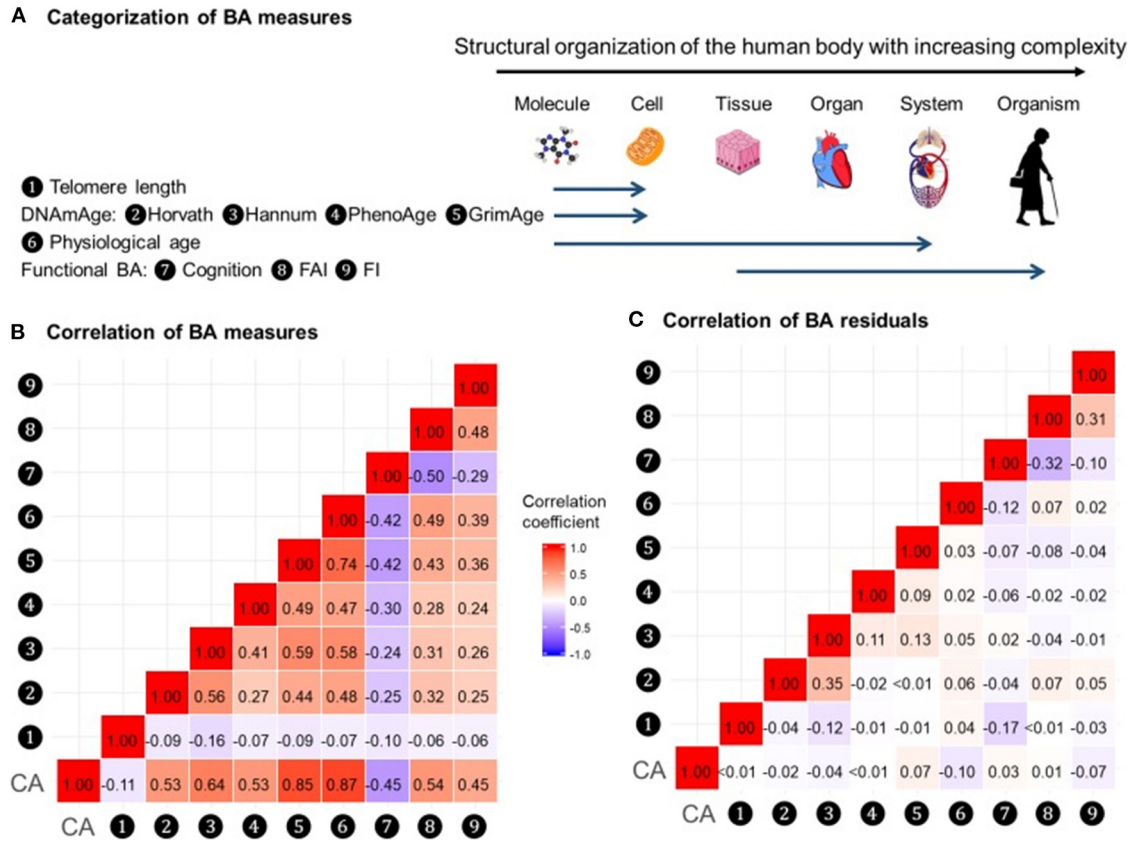

FIGURE 5 | Correlations of BAs in 288 individuals (612 complete measurements). A total of 612 complete measurements assessed from 288 individuals were included to estimate the correlations of BAs. BAs were broadly categorized into four groups according to the main biological structural levels where the BA measurements were implemented **(A)**. We estimated the repeated-measure correlation coefficients between BAs and between BA residuals and illustrated the correlation coefficients in heat maps **(B,C)**. Red and blue tiles represented positive and negative correlations, respectively; color density indicated the magnitude of correlation coefficients. All BAs were correlated to varying degrees **(B)**. After regressing out CA from BAs, most of the original correlations were attenuated **(C)**. BA, biological age; DNAmAge, DNA methylation age estimator; FAI, functional aging index; FI, frailty index; CA, chronological age. The figure and its legend are reproduced from the article by Li et al. (2020) distributed under the terms of the Creative Commons Attribution License. Copyright © 2020, Li et al.

TL AND MORTALITY

Numerous epidemiological findings indicate that short telomeres are associated with higher morbidity and all-cause mortality (Wang et al., 2018) and TL appears to be a better predictor for survival than chronological age (Adwan Shekhidem et al., 2019). For example, in the Swedish Twin Registry study, individuals with the shortest LTL quartile had 44% higher risk of all-cause mortality than those with the longest quartile (Wang et al., 2018). Moreover, the meta-analysis of all eligible studies performed by the authors (121,749 persons with 21,763 deaths in total) indicated that individuals with the shortest LTL quartile had 26% higher risk of all-cause mortality compared to those with the longest quartile, although substantial between-study heterogeneity has been revealed. In an individual-participant-data meta-analysis of 2 large prospective cohort studies from Europe and the U.S. conducted by Mons et al. (2017), age-adjusted hazard ratios for the shortest LTL quintile compared to the longest were found to be higher by 23, 29, and 10% for all-cause mortality, cardiovascular mortality, and cancer mortality, respectively. Likewise, in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, patients in 2-4 LTL quartiles had 18% lower hazard ratio for all-cause mortality and 17% lower hazard ratio for CVD-mortality when compared to those in the 1st quartile (Pusceddu et al., 2018). The associations between TL and mortality were, however, not consistent across studies. For example, in a systematic review by Mather et al. (2011), a significant correlation between shortened TL and increased risk of mortality was observed in 5 of 10 studies included in the analysis, while it was absent in 5 others. All these studies, however, were performed with a cross-sectional design, which do not allow an inference of causality. Given this, and also because of a very high inter-individual variability of TL in same-age individuals, longitudinal research of intra-individual rates of age-related decline in TL is likely a more useful design than the cross-sectional one in studying the relationship between TL and mortality. However, this link was investigated with a longitudinal design in few studies only. In the study by Epel et al. (2008),

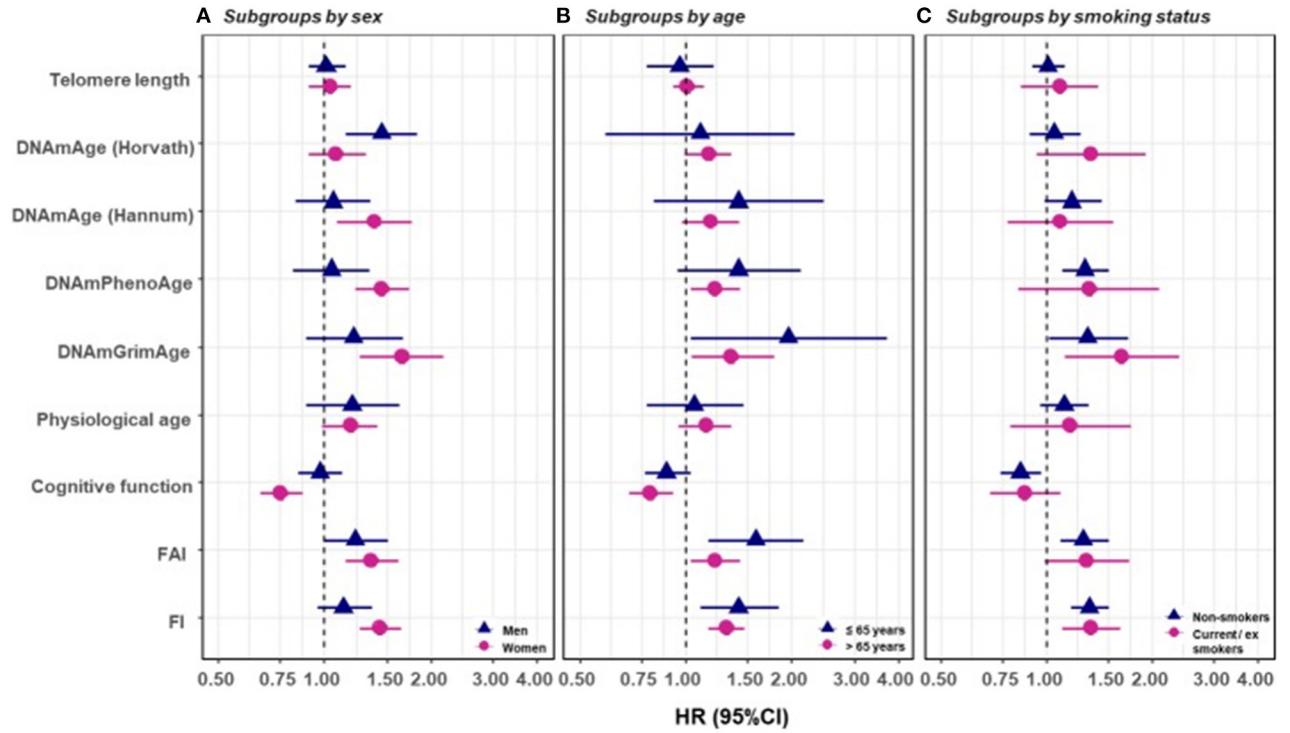

FIGURE 6 | Survival analyses of baseline BAs with the risk of all-cause mortality in subgroups classified by sex, baseline smoking status, and baseline age (one-BA models). A total of 845 individuals were included to estimate the mortality associations of BAs in subgroups. We used Cox regression models to estimate the change in mortality risk associated with a one-SD increment of the respective BA at baseline assessment (one-BA models). All models controlled for sex, educational attainment, smoking status, and BMI, stratified by participants' birth year, and accounted for left truncation and right censoring. Attained age was used as the time-scale and thus age was inherently adjusted for. BA-mortality associations were illustrated in the forest plot **(A–C)**, in which points and horizontal lines denoted HRs (95% CIs) and point shapes and colors represented subgroups. The associations of BAs with mortality risk were generally stronger in women (except for Horvath DNAmAge and physiological age), more pronounced in the younger individuals (except for Horvath DNAmAge, physiological age and cognitive function), and a bit stronger in current or ex-smokers (for Horvath DNAmAge and DNAmGrimAge). BA, biological age; DNAmAge, DNA methylation age estimator; FAI, functional aging index; FI, frailty index; CA, chronological age; HRs (95%CIs), hazard Ratio (95% Confidence Interval). The figure and its legend are reproduced from the article by Li et al. (2020) distributed under the terms of the Creative Commons Attribution License. Copyright © 2020, Li et al.

short baseline LTL was associated with 2.3-fold higher risk for mortality from cardiovascular disease in females; in male, age-related LTL shortening, but not baseline LTL, was associated with 3-fold higher risk for cardiovascular mortality. In studying 3259 adults of European ancestry residing in the U.S., short telomeres were associated with an increased risk of non-cancer mortality as individuals approached the upper boundary of their longevity (Arbeev et al., 2020). The longitudinal changes in LTL in relation to all-cause, cardiovascular, and cancer mortality were observed in 247 elderly Swedish males (Yuan et al., 2018). Short LTLs at baseline were strongly associated with mortality risks, with a 40–70% enhanced risk of all-cause mortality, and a 2-fold enhanced risk of cancer mortality. The association between TL and mortality, however, has not been confirmed in several other studies. For example, in the large population-based follow-up research by Martin-Ruiz et al. (2005), TL in blood monocytes had no predictive power for age-related morbidity and mortality in the oldest-old aged 85 years and over. In addition, whereas no association was observed between LTL and overall survival or death from any specific cause, a significant association between LTL and self-reported health status was found in both cross-sectional and longitudinal data (Njajou et al., 2009), indicating that LTL may not be a strong predictive biomarker for prognosis of survival in older individuals, but it may be a potentially useful biomarker of healthy aging.

TL AND OTHER BIOMARKERS OF AGING: WHETHER THEY MEASURE THE SAME PROCESSES?

When discussing the applicability of LTL as a reliable biomarker of aging, the comparison of LTL and other biomarkers of aging from the same individuals may certainly provide valuable information. In this context, the comparison of LTL with biological age measure such as DNA methylation-based epigenetic clock [DNA methylation (DNAm) age], which is currently regarded as the most precise estimation of biological

age (Bell et al., 2019; Levine, 2020; Salameh et al., 2020), seems to be particularly valuable. It is yet questionable whether LTL and DNA methylation change in tandem or contribute independently to the prediction of biological age. To answer this question, several studies have been recently conducted. Evidence that TL and epigenetic clock estimates are independent predictors of chronological age and mortality risk was obtained in the study by Marioni et al. (2018) performed in two Scottish cohorts aged from 70 to 90 years. In both cohorts studied, combined whole-blood TL and DNAm age explained more variance in age than each of them individually. In a combined cohort analysis, TL and DNAm age explained 2.8 and 28.5% of the variance in age, respectively, and jointly they explained 29.5%. Also in a combined cohort, one standard deviation increase in a baseline DNAm age was associated with a 25% increased mortality risk ($p < 0.001$) while in the same model, one standard deviation increase in a baseline TL was independently associated with an 11% reduced mortality risk only ($p = 0.05$). In addition, weak, non-significant correlations have been observed between the estimates of epigenetic clock and TL. In older adults, the whole-blood DNAm-based mortality risk scores were shown to be strongly associated with TL; they, however, demonstrated much stronger associations with all-cause mortality than the TL-based ones (Gao et al., 2018). More recently, a significant correlation between DNAm age and chronological age was found in the study by Banszerus et al. (2019). However, no evidence of either association between the relative LTL and DNAm age or between LTL and the DNAm age acceleration was observed in the studied cohort, suggesting that LTL and DNAm age measure different aspects of biological age. In the subsequent study, a weak but significant inverse relationship between DNAm age acceleration and relative LTL was shown (Vetter et al., 2019). The authors concluded that DNAm age is a biomarker of aging phenotypes, which are not (only) linked to pathways associated with mitotic age as measured by relative LTL. In this context, it should be noted, however, that TL is not the only unique indicator of mitotic aging. Indeed, as it was shown by Lowe and co-authors, epigenetic clocks also can be indicative of mitotic age, as demonstrated by advanced ticking in cultured cells that were forced into replicative senescence but no change in those cells forced into senescence via DNA damage (Lowe et al., 2016). Recently, a novel DNAm-based TL estimator was introduced by Lu et al. (2019). This epigenetic biomarker was developed by regressing measured LTLs on the blood methylation data (140 CpGs) for 2,256 individuals. This estimator correlated negatively with age in different tissues and in various cell types and outperformed LTL measurements generated using classical Southern Blot-based approach when predicting morbidity and mortality.

In the Belsky et al. (2018) study, eleven different methods were used to quantify biological aging in the same individuals. These methods included TL and the rate of TL shortening, and also three epigenetic-clocks and their ticking rates, and three biomarker-composites. The associations between these measures and aging-related outcomes such as cognitive decline, physical functioning, and also subjective signs of aging, including aged facial appearance were determined. The 71–cytosine-phosphate-guanine epigenetic clock and composite biomarkers were found to be associated with aging-related outcomes studied, however, the effect sizes were modest only, and TL-based measure was not associated with any of outcomes. Moreover, unexpectedly, poor agreement was observed among all applied measures of biological aging. The authors assumed that different methods of quantifying biological aging could not measure the same aspects of the aging process.

In a recent study by Li et al. (2020), nine measurements of biological age (**Figure 5A**) were assessed in a 20-year follow-up in 845 individuals from a Swedish population-based cohort. All measurements of biological age were more or less correlated with one another. Interestingly, the correlations among LTL and other biological age measurements were lower than among all other measurements (**Figure 5B**). The correlation became greater if the biological age residuals constructed by regressing out the chronological age-related part from respective biological age (commonly referred to as indices of age acceleration in the DNAm age-related studies) were used (**Figure 5C**). The largest effects were shown for estimators of methylation age (GrimAge) and the frailty index. In predicting the mortality of the study participants, two estimators of methylation age (Horvath and GrimAge) and the frailty index remained predictive in joint models, indicative of the complementarity between them, and all biological age measurements except for LTL have been related to mortality risk independently of chronological age (**Figure 6**).

Basing on the findings above, it can be concluded that measurements of biological aging conducted either with TL-based approaches or with other approaches, such as the DNA methylation-based epigenetic clock, could measure different aspects of the aging process. Therefore, it seems reasonable to use them together rather than separately.

USING TL IN HUMAN STUDIES: ANALYTICAL AND METHODOLOGICAL CHALLENGES

Analytical Aspects

The implementation of LTL as routine marker into clinical practice is currently hampered by several unresolved pre-analytical and analytical issues (Semeraro et al., 2020). One serious problem is considerable inter-individual variability of LTLs which was repeatedly observed in molecular epidemiological studies across multiple populations and which may substantially complicate the interpretation of individual data (Bodelon et al., 2014). For example, in the Factor-Litvak et al. (2016) study, LTL range was 7.01–11.6 kb in the newborns, 6.19–9.81 kb in their mothers (age range, 17–42 years), and 5.83–9.88 kb in fathers (age range, 17–56 years). Another important issue is that TL may substantially vary across various tissue types. There is also evidence that TL may vary even within the same organ depending on the site of sampling (Semeraro et al., 2020). However, in some cases, TLs may be correlated in different organs even despite these differences. Correlation between TLs in leukocytes and skeletal muscles was

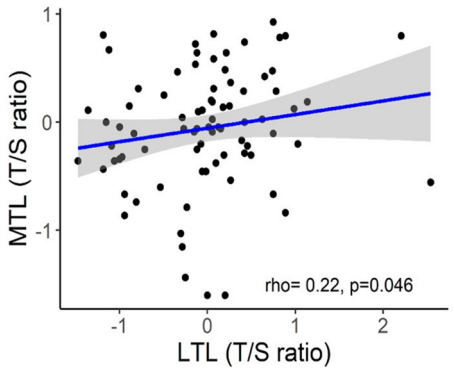

FIGURE 7 | Correlation between skeletal muscle TL (MTL) and LTL. The figure and its legend are reproduced from the article by Hiam et al. (2020) distributed under the terms of the Creative Commons Attribution License (CC BY 3.0). Copyright © 2020 Hiam et al.

demonstrated in recent study by Hiam et al. (2020) conducted in healthy individuals across a wide age range (18–87 years). Those subjects who had long (or short) TL in one tissue also tended to have long (or short) TL in another (**Figure 7**). This correlation between TLs in leukocytes and other somatic tissues within an individual may be likely explained by high inter-individual TL variation, which is already evident at birth (Factor-Litvak et al., 2016). This variation was found to be about three times larger than that in TL within the individual's somatic tissues (Aviv and Levy, 2019). There are, however, differences in TLs amongst somatic tissues primarily related to the replicative history of stem/progenitor cells in these tissues.

The most reliable information in this respect would be certainly provided from studies performed with cadaver specimens. Variability of TL in different cadaver tissues from the same dead human donors has been, however, investigated in few studies to date. In the study by Dlouha et al. (2014), TL was studied in tissues obtained from deceased donors aged from 29 weeks to 88 years. The relative TL (rTL, telomere repeat abundance in a DNA sample relative to a standard sample) was significantly higher in blood compared to liver, brain, heart, muscles and skin and moderately higher compared to spleen and mucosa, while no differences were obtained with adipose and renal tissues. The longest rTL was found in leukocytes (1.34 times longer than the reference TL) and kidney (1.15), and the shortest one was observed in the skin (0.48), liver (0.57), and brain (0.58). These inter-tissue differences were likely due to differences in the proliferation rate and different life spans of the various cell types, and also in oxidative stress levels in these tissues. These results, however, could be confounded by the small sample size and also by the variable health status of donors. Convincing evidence supporting differences in TL amongst human tissues has been provided in a more recent research by Demanelis et al. (2020). In this large-scale study, rTL has been measured during the Genotype-Tissue Expression (GTEx) project in more than 25 tissue types from 952 deceased donors (age from 20 to 70). Generally, rTLs were shown to be positively correlated between tissues. The whole-blood rTL was found to be a proxy for rTLs in most of the tissues studied. rTL varied across tissues and was shown to be shortest in the whole blood and longest in testicular tissue. In most of the tissues studied, except for the testis and cerebellum, rTLs were inversely associated with age; this association was found to be strongest in tissues with shorter average rTL. Moreover, importantly, evidence was provided that TL-associated genetic variants can affect expression of nearby genes and that TL may mediate effects of age on gene expression in various tissues. In addition, components of telomerase have been found to be more highly expressed in testis than in any other tissue. For schematic representation of the main findings from the study by Demanelis et al. (2020), see **Figure 8**.

Another analytical issue in human studies is that TL is most commonly determined in peripheral blood leukocyte samples. This cell source was chosen for TL analysis because it allowed avoid invasive procedures. However, it still remains questionable whether findings in circulating leukocytes can be generalized to other tissues. There is evidence from several studies that TL in leukocytes reflects systemic TL in other tissues. For example, TLs were found to be strongly correlated in adult tissues such as leukocytes, skin, skeletal muscle and subcutaneous fat, and rates of telomere shortening were similar in these tissues (Daniali et al., 2013). It indicates that TL in circulating leukocytes may be a good surrogate marker for TL in other somatic cell types. There is, however, some evidence that this could be not true for certain tissues (Thomas et al., 2008). It remains far from clear whether correlations exist between TLs in leukocytes and in tissues with low proliferative activity, such as neurons (Mather et al., 2011).

One more important methodological problem is that blood leukocytes are highly heterogeneous cell population which includes various cell types, such as lymphocytes, monocytes, granulocytes, etc. The composition of this cellular population is known to be highly variable, even in healthy persons, depending on various stress exposures (Semeraro et al., 2020). Different stressors may trigger a redistribution of leukocytes from immune reservoirs to the circulation and certain peripheral tissues (Dhabhar et al., 2012). This is an important issue given the fact that TLs differ in leukocyte subtypes isolated from the same donors. For example, in the Lin et al. (2010) study, the highest level of the telomerase activity and longest TLs were observed in B cells while CD4+ T cells had slightly higher telomerase activity and similar TLs than CD8+CD28+ T cells. Moreover, transient expression of telomerase can occur as a result of the antigen-induced lymphocyte stimulation. Although the telomerase activity is low enough in resting human T lymphocytes, it was shown to be transiently upregulated upon antigen presentation (Huang et al., 2017). In addition, pronounced differences exist in the rate of age-related telomere attrition in different tissues and cell types. For example, the decline in TL with age was found to be far more pronounced in lymphocytes than in granulocytes (Aubert et al., 2012a) (see **Figure 9** for illustration).

Another important point is that, for each particular individual, LTL is a very dynamic parameter reflecting transient changes in the immune system which have nothing to do with aging. Indeed, inflammatory conditions may induce proliferation of leukocytes

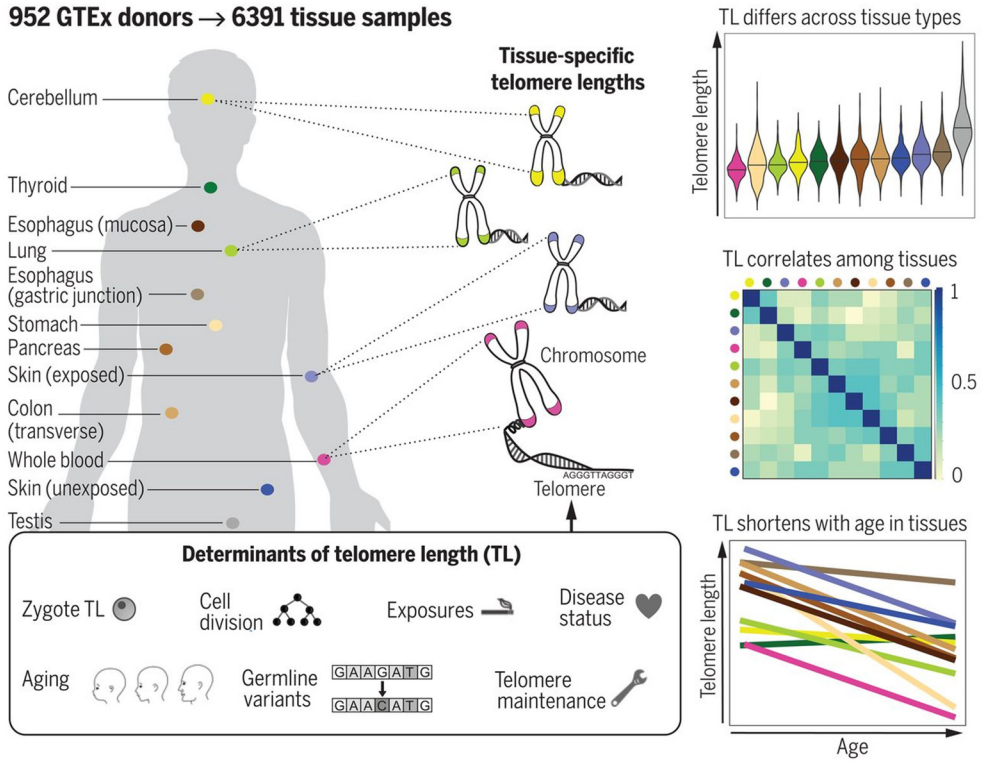

FIGURE 8 | TL in human tissues. Using a Luminex-based assay, TL was measured in DNA samples from >25 different human tissue types from 952 deceased donors in the GTEx project. TL within tissue types is determined by numerous factors, including zygotic TL, age, and exposures. TL differs across tissues and correlates among tissue types. TL in most tissues declines with age. The figure and its legend are reproduced from the article by Demanelis et al. (2020) with permission from AAAS (License Number 4910920603987). Copyright © 2020 The Authors.

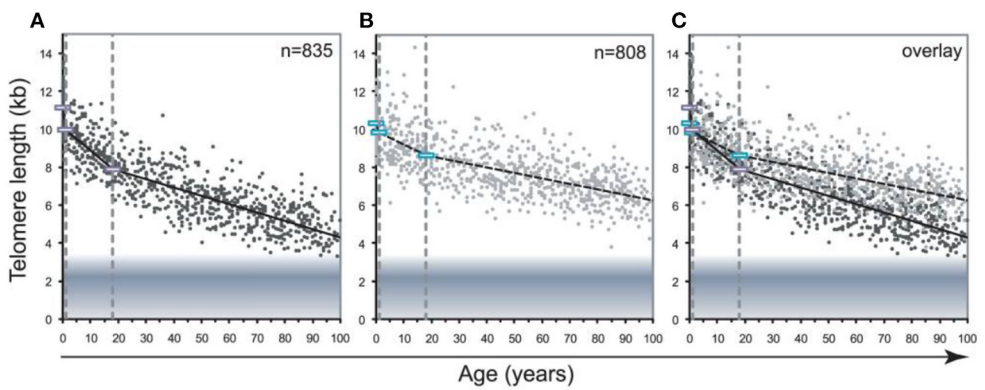

FIGURE 9 | Decline in telomere length with age differs between lymphocytes and granulocytes. The median telomere length in nucleated blood cells from 835 healthy individuals ranging from birth (umbilical cord blood) to 102 years of age were measured by flow FISH. The results were used to calculate the telomere attrition over time using linear regression in three age segments. **(A)** Median telomere length in lymphocytes (black dots). **(B)** Median telomere length in granulocytes (gray dots). Breakpoints in the piece-wise linear regression lines are marked by rectangles and the three age groups are marked by dotted vertical gray lines at 1 and 18 years. On average 8 individuals were tested per age-year. **(C)** At any given age, a wide range of telomere length was observed and the decline in telomere length with age in lymphocytes was more pronounced than in granulocytes. The shaded area represents the estimated length of subtelomeric DNA. Note that in older individuals, on average only 1–2 kb of telomere repeats were present in lymphocytes. The figure and its legend are reproduced from the article by Aubert et al. (2012a) distributed under the terms of the Creative Commons Attribution License. Copyright: © 2012 Aubert et al.

in bone marrow. Therefore, the proportion of newly released leukocytes may be enhanced in such conditions in total leukocyte population. Newly differentiated (naïve) leukocytes have TLs similar to those of hematopoietic stem cell progenitors, whereas much shorter telomeres are in mature leukocytes (Kimura et al., 2010). These processes can likely influence LTL. Since LTLs are

clearly affected by the leukocyte turnover, it makes it difficult to determine whether variations in LTL can be attributable to a blood-sample leukocyte composition or to a particular condition of interest (Hastings et al., 2017; Krasnienkov et al., 2018). It is also necessary to take into account that age-related variation in LTL could reflect different histories of the individual's immune responses under differing environmental conditions. As the proliferative potential is compromised and TLs are shorter in memory compared to naïve cells (Weng et al., 1995), LTL might be relatively shorter when a large proportion of memory CD8+ T cells is present in the leukocyte sample. Evidence is obtained that, for a given age, those individuals whose blood contains comparatively less naïve CD8+ T cells and more memory CD8+ T cells may display relatively shorter LTLs and more old DNAm age (Chen et al., 2017). Thus, since age-related TL attrition in adult T cells reflects the history of their antigen-mediated induction, this process may mirror, at least partly, the aging of the immune system of an individual.

The clonal hematopoiesis of indeterminate potential (CHIP) also may be a factor potentially influencing age-related dynamics of TL. CHIP is a common aging-related phenomenon in which hematopoietic stem cells (HSCs) and other early progenitors of blood cells contribute to the formation of genetically distinct subpopulations of blood cells in aging humans (Jaiswal and Ebert, 2019). Recently, it has been found in epidemiological studies that human aging is commonly associated with an increased frequency of somatic mutations in the hematopoietic system. In this process, particular mutant cells receive a competitive advantage allowing their clonal expansion, a process referred to as clonal hematopoiesis (Evans et al., 2020). Thus, each of such arising subpopulations is clonally derived from a single founder progenitor cell, thereby representing a genetic clone of this founder. These cell subpopulations are characterized by shared unique DNA mutations which occur most commonly in the transcriptional regulators DNMT3A, TET2, and ASXL1 (Jaiswal and Libby, 2020). CHIP was repeatedly found to be associated with a variety of adverse health outcomes, including hematological cancers, and also with a significantly elevated risk of atherosclerotic cardiovascular disorders which are a leading cause of death in the elderly (Evans et al., 2020; Jaiswal and Libby, 2020). The incidence of clonal hematopoiesis was observed to rise dramatically with individual's age. Clonal hematopoiesis was found in only 1% of persons younger than 50 years of age but it was evident in about 10% of persons older than 65 years of age), and about 20% of octogenarians were shown to be CHIP carriers (Genovese et al., 2014; Aviv and Levy, 2019).

The dynamics of TL in the cellular components of "hemothelium" (a vascular endothelium as a single entity) was proposed to be a central factor in the genesis of both CHIP and atherosclerosis (Aviv and Levy, 2019). This hypothesis is primarily based on findings from the whole genome sequencing and genome-wide-association analyses indicating that those subjects who display the features of CHIP have relatively short LTLs and often harbor a certain variant of the gene encoding telomerase (Zink et al., 2017). Moreover, CHIP was found to be common in patients with dyskeratosis congenita, a disease resulting from mutation in TL maintenance genes, including those that encode telomerase subunits and thereby causing critically short telomeres. In addition, the prevalence of CHIP was shown to be higher in the general population in subjects with relatively short LTLs, including males, who are known to have shorter LTLs than females, and elderly, who have shorter LTLs than younger individuals. Finally, age-associated rise in the prevalence of CHIP also provides supporting evidence. According to this hypothesis, age-related telomere shortening influences all proliferative tissues, but particularly the hematopoietic system characterized by a high proliferation rate. With age, TLs in hematopoietic stem/progenitor cells become progressively shorter, gradually approaching a "telomere brink," i.e., critically short TLs thereby compromising the function of these cells and increasing the risk of death in the near future (Steenstrup et al., 2017; Aviv and Levy, 2019). If these considerations are correct, then it can be assumed that emergence of hematopoietic clones with increased replicative potential due to *de novo* mutations in hematopoietic stem/progenitor cells may enable several individuals to postpone reaching the critically shortened telomere threshold ("telomere brink").

Methodological Aspects

There are also some methodological issues that hamper a wider application of TL analysis in clinical and epidemiological research. An important limitation of most existing TL measurement approaches is their reliance on producing a measure of average TL, which is not representative of the mechanisms linking TL to aging; indeed, the senescence process can be triggered by a single shortest telomere (Hemann et al., 2001). Some techniques have been developed to measure TL; each of these approaches, however, has distinct advantages and disadvantages. A comprehensive overview of the most useful TL measurement methods can be found in the review by Lai et al. (2018).

The Terminal Restriction Fragment (TRF) method is regarded as the gold standard for TL measurement. This method uses the Southern blotting or in-gel hybridization with a labeled probe specific for telomere DNA, providing an average TL value for the total cell population (Jenkins et al., 2017). The applicability of this technique is, however, substantially limited by the requirement of large (about 3 μg) amounts of DNA for analysis. Another important limitation of the TRF technique is that restriction enzymes used in this method lead to the inclusion of sub-telomeric DNA that is contiguous to the telomere, thereby resulting in an overestimation of the true TL (Montpetit et al., 2014). Moreover, this assay presents a relatively laborious procedure. In addition, very short telomeres (up to 2 kb) are difficult to detect with this method. Although reproducibility of TRF method within the same laboratory is rather good, the data obtained cannot be easily compared between different laboratories. The possibility of inter-laboratory comparability, however, emerged after the appearance of commercial kits for the TRF assay on current market.

Average cell TL and other TL-related parameters may be determined by the fluorescent *in situ* hybridization (FISH) technique by using flow cytometry (flow FISH) or digital microscopy (quantitative FISH, Q-FISH) (de Pedro et al.,

2020). This technique is highly accurate and allows detect even subtle changes in TLs. It, however, requires fresh cell samples and is technically demanding. Recently, high-throughput Q-FISH method was developed for examining many variables of individual telomeres. This method represents the combination of high-throughput imaging and software workflows. It allows simultaneous collection of a large number of telomere-associated variables in a large number of peripheral blood mononuclear cells. It should be noted, however, that although FISH-based methods produce highly reliable results, they are quite labor intensive and require expensive equipment. Importantly, the frequency of short telomeres (< 3 kbp) can be accurately evaluated with this method. This is an important point because relative frequency of shortest telomeres which may trigger a cell cycle arrest is a parameter critical for cell viability and chromosome stability, and it is highly associated with mortality (Hemann et al., 2001). Due to these properties, this technique may be useful in epidemiological and clinical studies. Furthermore, reliable measurement of shortest telomeres with this method could provide new opportunities in assessing biological age.

Recently, a new method called Telomere Shortest Length Assay (TeSLA) was developed for measuring the distribution of the shortest telomeres in heterogeneous telomere backgrounds (Lai et al., 2017). This method improves the efficacy of TL measurements after Southern blot analysis by using specific image-processing software aimed to automatically detect and annotate band sizes, and also calculate average TLs and percentages of shortest telomeres. With TeSLA, it is possible to detect telomere dynamics in a range from <1 to ∼18 kb in both normal aging processes and in telomere-related disorders in humans. In particular, TeSLA is capable of measuring different cellular subpopulations in peripheral blood mononuclear cells, e.g., CD28(–) T cells, which have a limited capability for cell division, shorter telomeres, and more high rate of telomere shortening relative to other subtypes of mononuclear cells (Weng et al., 2009). Therefore, this method can provide an opportunity to identify critically short telomeres in specific subsets of immune cells which are known to be largely contributed to age-related decline of immune function.

The quantitative polymerase chain reaction (PCR) assay is most widely used now. This assay is easy to perform, allows high throughput and requires small amount of DNA (Lin et al., 2019). Due to its high throughput, this technique has been often applied in large population studies. This method allows determine the number of copies of telomeric repeats (T) compared to a single copy gene (S) and its results are expressed as a T/S ratio. However, information about the distributing long and short telomeres and also regarding the differences between individual cells and chromosomes cannot be obtained with this approach. Moreover, the variability of data obtained with this technique may be substantial and exceed 10% within and between samples (Aubert et al., 2012b).

Concluding, although many innovative technological approaches were developed to measure TL, there still are substantial inter-laboratory variations and the results obtained by one method can substantially differ from those obtained by other which makes it difficult to use them in epidemiological and clinical studies (Dagnall et al., 2017). It should also be noted that accuracy and reproducibility of TL measurements depend, along with assay procedures, on many other factors including the sample collection, processing and storage, DNA extraction, etc. (Lin et al., 2019). Therefore, further efforts should be made to optimizing these techniques in order to improve their sensitivity, repeatability and throughput.

TL: A Single Biomarker or a Part of Composite Biomarker Panel?

Since the discovery of the phenomenon of age-related telomere attrition, TL has attracted a great deal of attention in gerontological research as one of the most promising biomarkers of aging. However, despite well-founded theoretical background for that, the available empirical evidence is rather contradictory. Therefore, the measurement of LTL is not yet widely-used in routine clinical diagnostics. In most epidemiological and clinical studies, only weak relationships were observed between TL and age-sensitive indices of physical functioning such as blood pressure, grip strength and lung function; more strong association was found for indices of cognitive performance, although the results were not unequivocal as well [for review, see Mather et al. (2011)]. Results from these studies indicate that TL does not reflect underlying aging processes and therefore cannot be considered as universal marker of biological aging. Many authors, however, acknowledge that biomarkers of aging can change over the life course. Moreover, a single biomarker could not sufficiently reflect the aging process across various biological systems. Contradictory findings from these studies could be likely explained, at least partly, by small sample sizes (and, consequently, by limited statistical power to detect associations) and also by narrow age ranges investigated (Der et al., 2012). Moreover, a cross-sectional design is an important limitation of most these studies. Such design may provide only limited inferences regarding causality.

In many studies, TL was examined as a single measure of aging rate. It is an important point because a question still remains whether TL can be used as a single biomarker of aging or as a part of composite biomarker panel only. To answer this question, Der et al. (2012) conducted a large community-based prospective cohort study in a Scottish population. In this research, two composites from the measures of functioning have been formed by principal components analysis, one of which included the TL and the other did not include it. Both these composite biomarkers of aging have been found to be better predictors of overall health outcomes than chronological age. There were, however, several differences between these two composites. TL was shown to be significantly associated with age and with eight measures of physical and cognitive functioning known to be related to normal aging. These measures included indices of physical functioning (pulse pressure, lung function and grip strength), cognitive functioning (a general mental ability test and a four-choice reaction time scores) as well as overall measures of health status (registered disability, self-rated health and the total number of chronic conditions). In most cases, TL added predictive power to that of age though, according to the authors, it was found to be not nearly as good a predictor overall. More specifically, when adjusted for age, the association with TL

remained statistically significant in five out of eight cases, thereby indicating that it may add predictive power over and above that of age. It, however, accounted for a very small proportion of the effect of age only and therefore does not satisfy itself the strict criterion of being a better predictor than age *per se*. These findings, collectively, suggest that TL, as a single biomarker, does not quite satisfy the criteria commonly applied for biomarkers of aging, but it does add predictive power to that of chronological age. The authors emphasize, however, that the fact that TL only modestly contributed to the composite biomarker of aging can lead to misleading interpretation of the study results, depending on whether they are used for the prediction or explanation of biological aging. Indeed, TL operates at a lower (cellular) level of the biological hierarchy compared to other components operating at various systemic levels. If telomere attrition was a part of the causal process ultimately resulting in an age-associated functional decline at higher levels, then adjusting for these higher level measures of functioning may likely lead to underestimating the effect of TL. In a more recent study by Hastings et al. (2019), composite measures of biological aging along with TL were quantified. All these measures correlated with participants' chronological ages. Three biological aging composite biomarkers were correlated with one another, but none of them was correlated with TL. The authors concluded that TL measures different aspects of the aging process as compared to the patient-level physiological biomarker composites. Moreover, effect sizes for these measures tended to be larger as compared to TL. However, importantly, marginal increases in the effect sizes were observed when TL was integrated into these biomarker composites compared to indices constructed without TL.

Summarizing the results of these studies, it can be stated that the validity of LTL as a single measure of aging rate and as a prognostic tool in clinical settings remain questionable. Indeed, since aging is an extremely complex multivariate process involving multiple molecular pathways operating at many levels of the functional organization, it unlikely may be evaluated with a single biomarker such as TL. Thus, the composite measures definitely have larger predictive value in assessing the aging rate than single measures, including the TL-based ones.

DISCUSSION

During past decades, TL is recognized as one of the most suitable biomarkers of aging. This is because telomeres are well-known to be critically implicated in cellular aging; moreover, many observations indicate that telomeres tend to shorten with age and that accelerated telomere shortening is a sign for many aging-associated pathological conditions. Therefore, LTL is commonly used as conventional biomarker of aging now. However, findings from available epidemiological studies on the links between TL and age-related diseases and mortality are rather inconsistent and contradictory. Moreover, since these associations were observed mostly in cross-sectional studies, no causal inferences can be made. Furthermore, individual LTL is apparently a very dynamic parameter reflecting changes which are often transient (e.g., following induction of immune responses) and have nothing to do with aging process *per se*. It is still far from clear, whether change in TL is a cause or effect of aging (Turner et al., 2019). Longitudinal designs should be certainly used in future research to prove causality (Chen et al., 2011; Hastings et al., 2017).

Based on these considerations, increased doubts are currently expressed by several authors concerning whether TL really plays a causal role not only in cellular senescence but also in aging of multicellular organism and, accordingly, whether LTL could serve as reliable biomarker of aging (Mather et al., 2011; Der et al., 2012). Many research findings do not confirm that TL meets the main criterion established by the American Federation for Aging Research for a biomarker of aging (Johnson, 2006). Indeed, in many studies, TL was not a better predictor of age-dependent functional declines, morbidity and mortality than chronological age. Moreover, results obtained in investigating these relationships may be substantially biased due to mortality selection in older populations. Furthermore, LTL is largely dependent on the blood-sample leukocyte composition. In addition, it is still not clear whether LTL is a reliable surrogate marker for TL changes in other body tissues, particularly in those with low proliferative activity (e.g., central nervous system), which are currently recognized as main drivers of the aging process. Nevertheless, despite these doubts, and even although epigenetic age-based approaches becomes increasingly favored in the aging research, TL remains the most widely used molecular biomarker of aging now. Innovative approaches, such as single-cell TL measuremens (Wang et al., 2013), techniques aimed at the identification of critically short telomeres (Serakinci et al., 2019) and DNA methylation-based methods of TL estimation (Lu et al., 2019) are being developed to improve sensitivity, repeatability and throughput of methods used for determining TL.

An important point in the context discussed is that, since aging is an extremely complex phenomenon involving multiple pathways and operating at various levels of the biological organization of a living system, it can hardly be accurately measured with a single biomarker. Different measurements of biological age apparently measure different aspects of the aging process. Therefore, estimates of biological age obtained with different measuring approaches may not coincide with each other. Considering this, it is reasonable to assume that TL (if included) may improve the predictive power of composite measures of biological age, while its use as a single biomarker of aging may be questionable in many cases. Indeed, each individual measure included in the composite score would likely point to different aspects of aging process, such as the developmental program (DNA methylation), replicative history of a cellular lineage (TL), environmental stress (mitochondria), etc. (Notterman and Schneper, 2020). Various measures can complement each other thereby improving the predictive power of the composite measure. These limitations need to be considered and challenges need to be addressed before wider implementation of TL as an established biomarker of aging in epidemiological research and clinical trials.

AUTHOR CONTRIBUTIONS

AV and DK contributed equally to the drafting, editing, and final composition of the manuscript. All authors contributed to the article and approved the submitted version.

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METHODS
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Method and Computer System for Dialog Optimization of Aging Biomarker Panels for Biological Age Assessment

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A concept, method, algorithm, and computer system (CS) of step-by-step dialog optimization of biomarker (BM) panels for assessing human biological age (BA) according to a number of universal criteria based on incomplete and noisy data have been developed. This system provides the ability to automatically build BM panels for BA assessment and to increase the accuracy of BA determination while reducing the number of measured BMs. The optimization criteria are as follows: high correlation of BMs with chronological age (CA); minimum size of BM panels, obtained by rejecting highly cross-correlated BMs; high accuracy of BA assessment; high accuracy of BA/CA dependency interpolation; absence of outliers in BM values, which reduce the BA assessment accuracy; rejection of panels resulting in a high standard deviation for the BA-CA difference; and possible additional criteria entered by the researcher according to the task specifics. The CS input consists of data on physiological, biochemical, and other BMs that change with age. The CS output is a panel of BMs optimized according to the specified optimization criteria. The CS is user-friendly. It allows the user to add optimization criteria that the researcher considers to be important or to remove criteria that the user considers incorrect. The CS may be used in solving practical problems of anti-aging medicine, such as the treatment and prevention of age-related chronic non-infectious diseases representing the main causes of death. The authors' point of view on the role and place of BA diagnostics in this area is discussed.

Keywords: aging, diagnostics of aging, biological age, biomarkers of aging, dialog optimization, personalized medicine

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INTRODUCTION

The global problem of population aging has significantly increased research interest in both the mechanisms of aging and the search for methods for decelerating and reversing aging (Moskalev et al., 2016, 2017; Vaiserman and Lushchak, 2017; Krut'ko et al., 2018). The fundamental basis of these studies is the methods for quantitative assessment of aging levels both of the body as a whole and of its individual vital organs and systems; in other words, methods for assessing the biological age (BA) of the body as a whole and the partial biological age (BAp) of its vital systems

(Dean, 1988; Mooradian, 1990; Balin, 1996; McClean, 1997; Anstey and Smith, 1999; Krøll and Saxtrup, 2000; DeCarlo et al., 2014; Negasheva et al., 2014; Dontsov and Krut'ko, 2015; Moskalev et al., 2016, 2017; Finkel et al., 2017; Mitnitski and Rockwood, 2019). Moreover, while there are many different methods for BA assessment in literature, there is still no answer to the question: "Which of these methods is the best?" This paper contains an attempt to answer this question.

The purpose of our article is to try to solve several theoretical and practical problems of interest to gerontology, namely, (1) to give one of the possible answers to the key question of Ward Dean (Dean, 1988), which is presently relevant, "It remains unclear which of the panels presented is the best and can be recommended for wide practical use"; (2) to offer, in our opinion, a reasonable criterion for selection of the best panels of aging biomarkers (BMs) for specific research tasks; and (3) to create a computer system (CS) that facilitates the application of this criterion for solving various practical problems of creating optimal (i.e., accurate and easy-to-use) panels of aging BMs for BA assessing.

Different views on the concepts of "biological age" and "aging" are presented in literature and may exist. We adhere to the following views:

- a person ages with years, and it is expressed as a decrease in bodily functions;
- there are average age norms for these functions;
- comparison of function values of a particular person with age norms determines the BAp of these functions;
- and the weighted sum of the BAp of the vital functions of the organism determines the BA of the organism as a whole.

METHODS AND MATERIALS

The standard input data loaded into the CS is an N × M rectangular Excel spreadsheet containing data on N values of BMs for M clients [the set of BMs included the calendar (chronological) age (CA) of the clients]. However, it is not necessary to have all BM data for each client. An example of the practical CS application is based on spreadsheets of size 15 × 160 (for women) and 15 × 33 (for men), containing data for a group of clients examined at the Russian National Gerontological Center (www.ngcrussia.org/) to obtain recommendations for individual anti-aging programs.

These data were processed using the following algorithms:

- calculation of correlations of BMs with CA,
- calculation of BMs cross-correlations,
- calculation of mean values and standard deviations for BMs and BAp in the examined panel for separate BMs and deviations of BA from CA,
- consideration of measurement accuracy and BM age range,
- exclusion of maximum and minimum values of BMs within age ranges.

Abbreviations: CS, computer system; CA, chronological age; BM, biomarker; BA, biological age; Bap, partial biological age (biological age of separate body system).

As a result, an optimized BAp was calculated for each BM.

RESULTS

The method was implemented using the CS, which was developed on Object Pascal software with Delphi 7 application development system. The CS provides step-by-step dialog optimization of BM panels and thereby assists in obtaining formulas for BA and BAp calculation for the optimized panels and the results of calculations by these formulas.

The optimization criteria for this CS are as follows:

- high correlation of BMs with CA,
- minimum size of BM panels, obtained by rejecting highly cross-correlated BMs,
- high accuracy of BA assessment,
- high accuracy of interpolation of BA/CA dependency,
- absence of outliers in BM values, which reduce the BA assessment accuracy,
- rejection of panels resulting in a high standard deviation for the BA-CA difference,
- possible additional criteria entered by the researcher according to the task specifics.

The CS is user-friendly. It allows the user to add optimization criteria that the researcher considers important or remove criteria that he or she considers incorrect (for example, discussion criteria related to CA).

The CS operational algorithm is as follows:

- *At the first step* the correlations of BMs with CA are determined, and BMs with low correlation are rejected. The rejection threshold may be pre-specified in the CS or else determined by the researcher in the dialog mode.
- *At the second step* the BM sets are checked for redundancy among the BMs selected for the same panel using the calculated BM cross-correlations.
- *At the third step* the accuracy of each BM assessment is determined based on the instrument accuracy of the BM measurement procedure, the range of interindividual BM assessment fluctuations in the group studied, the magnitude of BM variation in the reference age interval, and the size of the age interval for which the BM is assessed.
- *At the fourth step* the BM/CA graphs are plotted, and the linear, exponential, and polynomial regression formulas for each BM, as well as the inverse formula of BA determination for a given BM value, are calculated. To correct the graphs, the extreme BM values are rejected.
- *At the fifth step* a BA/CA graph is plotted (a typical BA graph will, in theory, show little deviation of values from the diagonal of the BA/CA square), and a standard deviation σ for the difference (BA-CA) is calculated. In the group of clients examined, there are typically individuals with both higher and lower BA compared to their CA. In our experience, $\sigma < 10$ years is an acceptable value.
- *At the sixth step* additional BM criteria, determined by the specifics of the goals and objectives of the BA study or the practical CS application, may be introduced: for example,

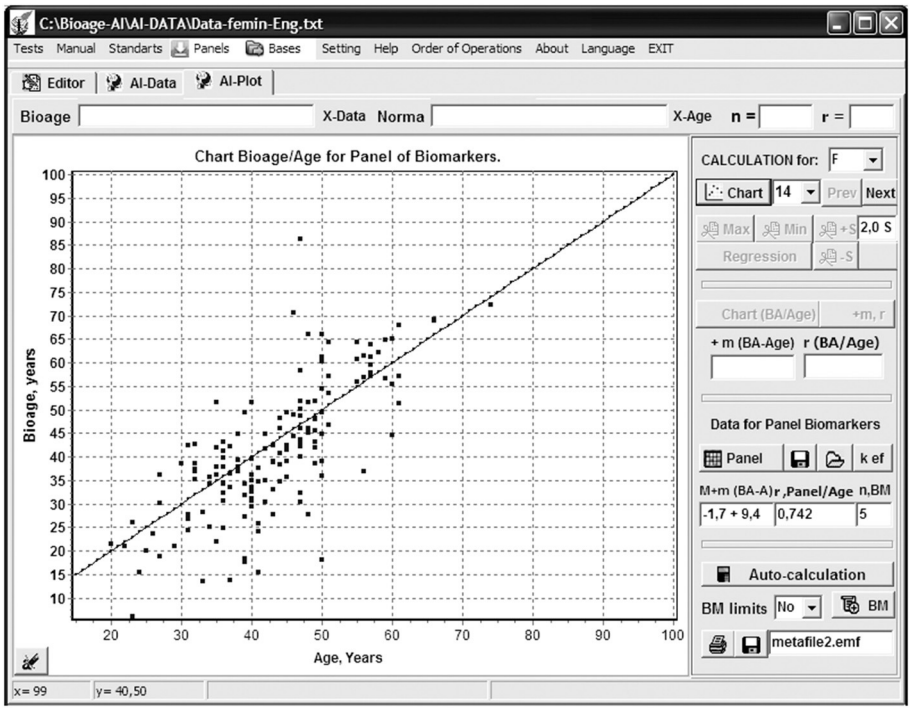

FIGURE 1 | Program "Constructor of aging biomarkers panels": a dialog panel of setting parameters for biomarkers selection and bioage calculation.

the possibility of sharp BM deviations from expected age standards due to sports training or illness may be taken into account.

- *At the seventh step* the selected BMs are used to create panels to determine BA and BAp according to the specifics of the goals and objectives of the BA study or the practical CS application.

The above processes are carried out in dialog mode using the CS dialog box (**Figure 1**). In this mode, the BM selection criteria, determined by the researcher, are introduced: the rejection threshold for BMs demonstrating a low level of correlation with CA; the threshold for BM cross-correlation (when exceeded, a BM may be rejected as superfluous); the BM measurement accuracy, which determines the accuracy of the estimate of the corresponding BAp (in years); thresholds for deviations from mean values (the number of sigmas for each 10-year age period, entered separately), so that exceeding maximum and minimum BM values will be rejected as unrepresentative; the number of maximum and minimum BA values that can be rejected; the precision (i.e., number of decimal places) of the coefficients found in the formulas for BA calculation; and the limits for correlation values of BA with CA for the created panels. Moreover, the significance factors (weight factors w) of each BM (or corresponding BAp) contribution to the resulting BA may be calculated and taken into account for the w values in the range (0, 1). The final BA for the created BM panel is calculated as a weighted mean BAp value for the selected BM set.

The above criteria and limits may be specified before commencement of the CS operation, and then all optimization steps will be carried out by the CS automatically, without human intervention. These criteria and limits may also be introduced at each step, if the researcher wants to consider the results of calculations obtained at previous steps of CS operation, e.g., the significance and accuracy of individual BM assessments.

Example of Computer System Operation

An example of the CS operation is given below. The objective of the study was to determine the BM panel resulting in a maximum correlation of BA with CA while using a minimum number of selected BMs and producing a minimum difference value (BA-CA).

Data for two groups of clients (33 men and 160 women) were examined separately using a set of BM values included in the panel officially approved in the USSR for BA assessment (Vojtenko et al., 1984). This panel includes the following BM:

APs, APd and APp – systolic, diastolic and pulse arterial pressure (mmHg),
PWVe: pulse wave velocity through the artery of the elastic type (m/sec),
PWVm: pulse wave velocity through the artery of the muscular type (m/sec),
LC: lung capacity (ml),
BHT: breath hold time as you exhale (sec),
A: eye accommodation (distance to the closest point of clear vision, expressed in diopters),
HA: hearing acuity or hearing threshold at 4,000 Hz (dB),
SB: static balancing (sec) on the left foot,

BW: body weight (kg),
SAH: self-assessment of health test (scores),
WT: Wechsler's test (scores).

At the stage of assessing the correlations of BMs with CA, extremely low correlations with age were noted for BHT ($r = -0.047$) and SAH ($r = 0.232$); moreover, there were high cross-correlations for the following parameters: APs/APd ($r = 0.795$), APs/APp ($r = 0.803$), PWVe/PWVm ($r = 0.986$), and LC/BW ($r = 0.657$). This provides the need of excluding two of three factors (APd and APp) of blood pressure assessment from the BA assessing panels, as well as one of two parameters (PWVe or PWVm) of artery elasticity assessment. What specific factors should be excluded may be determined by considering their assessment accuracy.

The accuracy of BAp assessment (in years) depends on the accuracy of the BM measurement method, the magnitude of BM change in the measured age interval, the size of this age interval, and the size of interindividual differences of BM in the studied group. There are two classes of the most common and important BA and BAp assessment tasks.

The first class includes tasks of longitudinal assessment of BA and BAp changes in individuals under the influence of personal aging prevention programs. In this case, there are no interindividual differences, and the accuracy of assessment depends on the ratio of BM measurement method accuracy to the BM changes within the reference research range, usually having a value from several months to several years. For example, for APs changing from 120 to 160 mmHg (change = 40 mmHg) in the age range 20–70 years (range = 50 years) with a hardware measurement accuracy 5 mmHg, the calculated accuracy value = 40 mmHg/5 mmHg = 8 units for 50 years, or 50 years/8 registered units = 6.2 years represents the accuracy achievable in determining BAp in years. This is marginally acceptable for BAp, but considering the importance of the parameter and the comparison of its value with an earlier one for the same patient (with no interindividual variation), its application is acceptable. The accuracy of BA assessment within ±5 years is considered to be sufficient for this kind of research. Similarly calculated accuracy for APp equals ±12.5 years, which excludes this parameter from the list of parameters acceptable for BA assessment.

The second class includes tasks of population cross-sectional BA studies. For these tasks, it is necessary to consider the values of interindividual dispersion, which almost always significantly exceeds the error of the measurement method. For example, LC variation within the 50-year age interval from 20 to 70 years reaches a 2-fold value with method accuracy <2–3% from the measured value, but interindividual variation also becomes 2-fold. However, the LC parameter is almost always included in the BA assessment panel, since the high measurement accuracy and the large age-dependent variation allow us to obtain sufficiently accurate and reliable data in population studies.

The BMs with the highest instrumental measurement accuracy, smallest interindividual dispersion, and most evident age-related changes are most acceptable for both classes of tasks. For example, PWV is such a BM. An approximate value of PWVe alteration in the 50-year age interval from 20 to 70 years equals 700 m/s (1,200 m/s−500 m/s) with an instrumental measurement accuracy of about 10 m/s and a small interindividual dispersion; BAp for this BM may be determined with an accuracy of 0.8 years and a correlation value with CA of $r = 0.823$.

When the graphs of BW to BM were plotted, it was clearly seen that the possibility of sharp and rapid fluctuations of this parameter throughout life and the significant interindividual dispersion (from 45 to 135 kg in the considered group) prohibits using this BM in the panel for individual BA assessment.

After a similar step-by-step analysis was carried out for all 15 BMs, it was found that the optimal BM panel contains five BMs for women (APs, PWVe, LC, A, WT) and six BMs for men (APs, PWVe, LC, A, BHT, SB). The formulas for BA-CA difference calculated for these panels are as follows:

For women:

$$\begin{aligned}&\text{BA-CA} = \\&(0.83 \times [-44.384 + 0.1235 \times \text{PWVe} - \text{CA}] + \\&0.65 \times [-7.0318 + 0.333 \times \text{A} - \text{CA}] + \\&0.58 \times [-118.760 + 1.328 \times \text{APs} - \text{CA}] + \\&0.54 \times [204.880 - 2.8573 \times \text{WT} - \text{CA}] + \\&0.54 \times [226.860 - 0.0662 \times \text{LC} - \text{CA}])/5\end{aligned}$$

For men:

$$\begin{aligned}&\text{BA-CA} = \\&(0.77 \times [-50.098 + 0.1325 \times \text{PWVe} - \text{CA}] + \\&0.53 \times [67.658 - 0.632 \times \text{SB} - \text{CA}] + \\&0.43 \times [-38.018 + 0.426 \times \text{A} - \text{CA}] + \\&0.42 \times [319.980 - 0.073 \times \text{LC} - \text{CA}] + \\&0.38 \times [-382.410 + 3.3884 \times \text{APd} - \text{CA}] + \\&0.38 \times [-110.290 + 3.837 \times \text{BHT} - \text{CA}])/6\end{aligned}$$

The accuracy in determining the BA-CA difference was $M \pm \sigma = -1.7 \pm 9.4$ years, with a correlation coefficient $r = 0.741$ for women (**Figure 2**) and $M \pm \sigma = -3.8 \pm 7.7$ years, $r = 0.792$ for men.

For comparison, the official BM panel of the USSR, including 15 BMs, had an accuracy for women $M \pm \sigma = -4.1 \pm 28.2$ years, $r = 0.324$.

The accuracy may still be improved by excluding the maximum and minimum outlier BM values and entering weight factors of the significance of BMs (w) into the formulas, which are expertly evaluated based on the functional significance of the processes and mechanisms referred to by those BMs for the aging processes of the whole body, as well as both the researcher's capabilities and the client's wishes regarding the set of BMs they prefer to use for one or another reason.

All stages of calculations may be carried out automatically or step-by-step in dialog mode. As a result, optimal (according to the above criteria) BMs are selected, which may be saved in the CS database for further use: creating effective new panels or adding to existing panels for BA assessment of individual clients.

The developed CS of step-by-step dialog optimization of BMs for the assessment of BA may work with an unlimited set of data based on incomplete and noisy data and according to the requirements and preferences of the researcher.

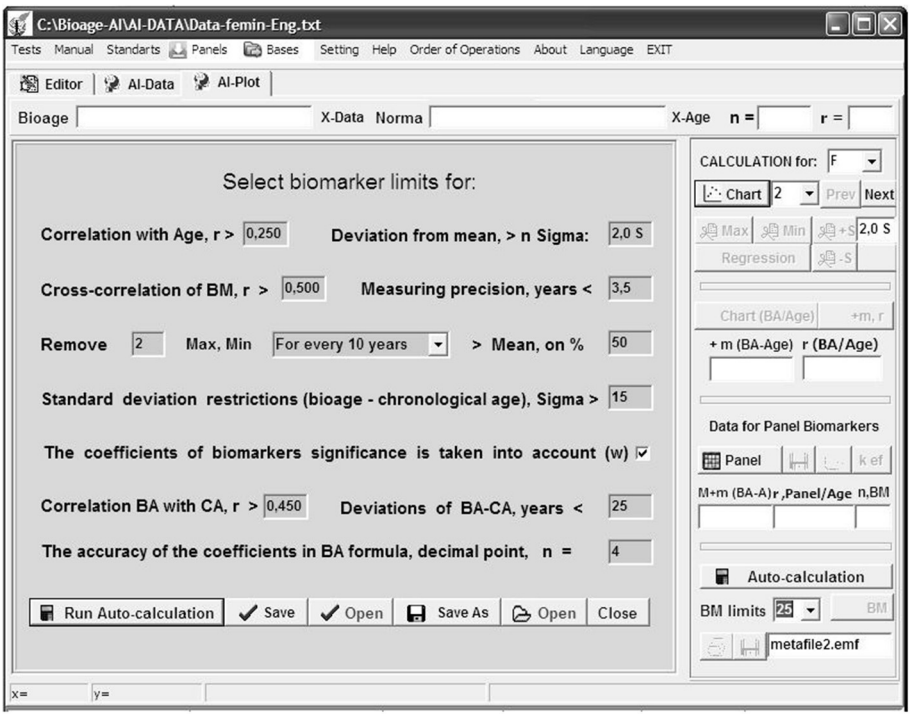

FIGURE 2 | Program "Constructor of aging biomarkers panels": curve Bioage—Chronological age (Age) for women based on five selected biomarkers.

DISCUSSION

Currently, there is a problem of different perspectives in the literature regarding the concept of "biological age" and the quality criteria of various BMs of aging. Our work addresses the practical issues. The general consideration of this problem is beyond the scope of this article.

The first serious generalization of the results of solving the problem of creating panels of BMs of aging for human BA assessing was given in Ward Dean's book *Biological Aging Measurement* (Dean, 1988). The book described about two dozen BM panels and the corresponding formulas for determining BA, which were created by teams of scientists from different countries and which contained from a few to several dozen BMs. After describing all these panels in detail, Ward Dean ended his book with an at-the-time unsolved question: "It remains unclear which of the presented panels is the best and can be recommended for wide practical use."

From the control science it is well-known that the task of finding the optimal solution is correctly set if an optimality criterion or a group of criteria is specified. After the publication of Ward Dean's book, many authors tried to make the best BM panels for BA determination, offering their own optimality criteria (Dean, 1988; Mooradian, 1990; Balin, 1996; McClean, 1997; Anstey and Smith, 1999; Krøll and Saxtrup, 2000; DeCarlo et al., 2014; Negasheva et al., 2014; Dontsov and Krut'ko, 2015; Moskalev et al., 2016, 2017; Finkel et al., 2017).

The conceptual view of the authors of this paper on the problem of BM panels creating for BA assessing, with the resulting method that implements this view, is that posing the problem in terms of finding a single optimal (best) panel is, in general, incorrect, since there are many scientific and practical problems in gerontology and anti-aging medicine, each of which involves its own system of optimality criteria reflecting the specifics of those tasks and the BM sets corresponding to these tasks—in particular, diverse physiological, functional, genetic, epigenetic, and other BMs.

However, among the many criteria, there are several fairly obvious adaptive optimality criteria suitable for any panel and serving to improve the accuracy and ease of use of these panels. In this regard, the authors conceived and implemented the idea (presented in this paper) of creating a tool that automatically takes these criteria into account when developing optimal BM panels for BA assessment, which greatly facilitates the work of a researcher or practitioner working in the field of anti-aging medicine or treatment or prevention of age-related chronic non-communicable diseases (CNCD) that are the main causes of death.

The CS offers a gentle and user-friendly interface. Any optimization criteria may be excluded during the dialogue; additional criteria reflecting the specifics of the user's tasks (e.g., the availability of this or that equipment for BM diagnostics, the cost of BM assessment, time constraints for the whole BA assessment procedure, and others) may be introduced, and the

fully automatic operation mode, convenient for servicing a large flow of clients, may be used.

The use of the CS for the optimization of the BM panel officially approved in the USSR (Vojtenko et al., 1984) allowed 3-fold reduction of the number of BMs, increased the accuracy of BA determination, and permitted the use of a noisy and incomplete BM sampling to calculate BA.

Fundamental degenerative processes of aging are the basis of CNCD—the main cause of death (Blumenthal, 2003; Kipling et al., 2004; Marengoni et al., 2011). These processes may be combined into several aging syndromes (Krut'ko et al., 2018), each of which usually corresponds to one or more CNCD syndromes. In particular, the following main aging syndromes may be selected: tissue sclerosis syndrome, tissue hypoxia syndrome, intoxication syndrome, oxidative stress syndrome, immune deficiency syndrome, maladaptation syndrome, physical senility syndrome, metabolic disorder syndrome, hormonal disorder syndrome, social isolation of elderly persons and psychological age-related changes.

All of these syndromes may be associated with groups of specific BMs characterizing the level of age-related degradation of the body system under consideration and determining the BAp of this system. For example, the following set of BMs may be applied to tissue sclerosis syndrome: pulse wave velocity, systolic arterial pressure, blood oxygen saturation, lung capacity.

In each specific case this set of BMs may be determined by a dedicated physician; naturally, he or she is faced with the task of determining the desired BAp using an optimal method, i.e., in the most accurate and simplest way, without requiring special knowledge of mathematics and computer technology. In this case our CS may be useful. In our opinion, in this case the diagnosis of current BA or BAp is not of the greatest interest, but the determination of the individual changes (ΔBA and ΔBAp) in specific clients subject to personalized anti-aging programs or undergoing the prevention and treatment of CNCD is very interesting.

The initial assessment of BA and BAp is of course not very accurate, as it is determined on the basis of the reference group data where BMs have sufficiently large inter-individual dispersion. The assessment of changes—ΔBA and ΔBAp—is protected from significant errors due to interindividual dispersion depending on individual BM changes during the implementation of anti-aging programs. These changes are much more informative because they show the effect of prevention or treatment programs and indeed the fine structure of this effect, represented by the pattern of changes in specific BMs included in the panel. Another useful task that can be solved here is the use of the "general checkup" method—a comprehensive BAp assessment of the main vital systems of the body, which makes it possible to reveal the most rapidly aging system—a weak link that accelerates the body's approach to death.

Finally, it is interesting to note that the logic of the CS operation is similar to that of deep machine learning of a neural network, where the steps of the CS correspond to the layers of that network and more and more aggregation of the processed information about the object takes place at each step. This opens a way for further CS improvement by adding artificial intelligence and voice assistance to its functions, provided there is a sufficiently large BM database (today these data are rapidly accumulating all over the world) to help the researcher become more effective in solving the problems of aging and risk management for CNCD. Such approaches are currently effectively used to solve BA assessment problems (Putin et al., 2016; Vidaki et al., 2017).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

VK: concept and selection of optimization criteria for biomarker panels. VD: concept and algorithms of computer system realization. NE: preparation of experimental data. VM: the choice of biomarkers and interpretation of the program. OM: examples and scenarios of using a computer system. ES: help with writing an article and assistance in the translation of the article. DS: computer system programming. All authors contributed to the article and approved the submitted version.

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Multimorbidity, Trauma Exposure, and Frailty of Older Adults in the Community

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The aim of this study is to investigate the relation between multimorbidity, traumatic events and frailty among older adults in the community. The studied population consisted of 257 older people who were recipients of the services and active members of Open Care Centers for the Elderly (OCCE) of the Municipality of Grevena and meet a set of selection criteria. The collection of the data was carried out using a fully structured questionnaire, which consisted of two sections: a form of individual features and the Tilburg Frailty Indicator (TFI). The sample consisted of 114 men (44.4%) and 143 women (55.6%) aged between 61 and 96 years with an average of 75.12 years. The results showed that the mean scores were 2.70 for the Physical Frailty (standard deviation = 2.16), 1.43 for the Psychological Frailty (standard deviation = 1.21), 1.32 for the Social Frailty (standard deviation = 0.64) and 5.44 for the total Frailty (standard deviation = 3.02). We took into account the cut-off point five of 54.1% (n = 139) in terms of the participants' frailty. Physical, Psychological, and Total Frailty are related to (a) the presence of two or more chronic diseases or disorders, (b) the experience of a serious illness in the previous year, and (c) the experience of a serious illness of a loved one during the previous year. The outcomes helped to identify frailty syndrome in older people and the factors associated with it.

Keywords: multimorbidity, chronic diseases, trauma exposure, frailty, older adults, community health, health care

INTRODUCTION

European countries are among the countries in the world with the highest population of aging people according to epidemiological data (Kinsella and Philips, 2005). The demography of Greece shows a simultaneous and constant increase in people who are aging and those with chronic diseases (Ntanasi et al., 2018). Greece is second in Europe in aging population after Italy (Sintihaki and Kasabali, 2010). Data from recent literature show that a common phenomenon observed in older adults and especially in those suffering from chronic diseases is the frailty syndrome, which affects not only their physical and mental health but also their social life (Vardaki and Manolitsaki, 2011; Koutsonida et al., 2016).

The frailty syndrome has been defined several times by researchers; however, no common definition has prevailed worldwide (Chen et al., 2014). From Fried et al. (2001), frailty has been defined as a biological syndrome that has detrimental effects on the body's systems due to limited resistance to stressor factors. Other scientists declare that it is a clinical syndrome affecting older people, as it can cause a decrease in functionality, a rise in the probability of falls, and possibly an increase in hospitalization, disabilities, and mortality (Xue, 2011). In the study by Conroy and Elliott (2017), frailty is a multidimensional geriatric syndrome with significant health effects.

The frailty syndrome is featured by five phenotypic criteria, according to Fried et al. (2001), while the occurrence of one or two of them can be characterized as a predisposition syndrome. These criteria include energy loss, decreased physical activity, decreased gait, weakness, and weight loss. The frailty existence is due to biological, genetic, physical, environmental, social, and psychological factors. This reasoning results from the five criteria of Fried et al. (2001). The featured conclusion of the research was that females are prone to frailty syndrome, while other factors that have been associated with the occurrence of the syndrome are low socioeconomic status, education level, lifestyle (alcohol use and smoking), the psychological situation, such as the presence of depressive symptoms, and disability (Rockwood et al., 2004; Theou et al., 2014; Lewis et al., 2019).

Several multidimensional instruments are currently available for measuring frailty in older persons, such as the Tilburg Frailty Indicator (TFI), the Edmonton Frail Scale, the Frailty Index and the Groningen Frailty Indicator. The TFI differs from these instruments in that the score on the TFI results entirely from self-reports and contains no questions on disability. Research has shown that frailty should be distinguished from disability as it is regarded as a pre-disability state (Gobbens et al., 2012).

The syndrome and therefore the modern diseases are caused by way of life and stress despite the continued improvement of hygiene and nutrition. Frailty is associated with chronic diseases, including cognitive impairment, as several studies have remarked that frail older adults were highly affected by some type of dementia (Kulmala et al., 2014; Rogers et al., 2017; Lewis et al., 2019).

Fried et al. (2001) published one of the most valuable papers on the frailty syndrome, researching the prevalence, frequency, accuracy, and prediction of the clinical syndrome. This was a large-scale study that was completed in 7 years. The researchers observing demographic characteristics (lifestyle), health habits, medication intake, weight loss, the presence of cardiovascular events, asthma, diabetes, visual, and hearing impairment in the older people, conducted an assessment of physical activity, mental state, cognitive function as well as morbidity and mortality. After 5 years, in the observed population, the percentage of frailty doubled in women. Moreover, there was an observed 7% increase in frailty after 3 years in people over 90 years old. In frail older people, 27% did not have any kind of disability or comorbidity. The cohort study shows that individuals with the following features are more prone to frailty: low income, comorbidity, chronic illness, and a low educational level (Fried et al., 2001). Frailty can also cause disability and can be associated with obesity (Fried et al., 2001).

Frailty and multimorbidity are considered promising clinical biomarkers for studying mechanisms underlying aging. Both have been shown to be related to the risk of disability in older people, hospitalization, mortality, and escalating health-related costs. A certain degree of overlap between the two conditions is biologically plausible, and bidirectional causality between them is likely. Frailty may predispose individuals to the development of multiple chronic diseases, but it may also stem from the coexistence of multiple conditions (Vetrano et al., 2019). Evidence suggests that frail older people are a clinically highly heterogeneous group and that further risk stratification may allow the identification of subgroups at greater risk. Also, the accumulation of chronic health problems, along with age-related physiologic changes, results in multisystem dysregulation, leading to frailty. Different chronic disease profiles, or patterns of multimorbidity, which is defined as the co-occurrence of two or more chronic conditions, may impersonate clinically different etiologic pathways to frailty and provide additional prognostic information within older adults with the same level of frailty (Nguyen et al., 2019).

Traumatic life events have been associated with having long-term effects on human health and causing early late-life mortality, and it can thus be argued that frailty must be associated with traumatic life events. In the conceptual model of frailty proposed by Freitag and Schmidt (2016). traumatic life events are described as part of sociodemographic factors affecting the development of diseases and frailty. Despite the empirical findings on health and theoretical links with frailty, the association between traumatic life events and frailty has not been investigated in much detail so far (Freitag and Schmidt, 2016).

This study aims to address this research gap and investigate the relationship between multimorbidity, traumatic events and frailty among older adults in the community.

MATERIALS AND METHODS

Research Methodology and Sample

A descriptive epidemiological, cross-sectional study was performed to be considered the correlation between multimorbidity, traumatic events and frailty among older adults in the community. The studied population consisted of the older adults who were recipients of the services and active members of the Open Care Centers for the Elderly (OCCE) of the Municipality of Grevena. We recruited participants for this study by visiting the three OCCE of the Municipality of Grevena, one urban structure and two country centers of Grevena. A sample of 257 older people was collected from the interested population. Individuals participated in this research voluntarily, and all of them provided written informed consent.

The selection criteria of the individuals consisting the study population were as follows:

a. People greater than 60 years old.

b. Active members of (OCCE) of the Municipality of Grevena.
c. Self-service and independent living.
d. Undiagnosed mental or cognitive disorder.
e. Knowledge of the Greek language and fluency of communication.
f. Acceptance of the terms and participation in the research.

Data Collection

The applied sampling method was the non-probability sampling and, in particular, the convenience sampling technique. The collection of the research data by the research team took place in the three OCCE of the Municipality of Grevena following the acquisition of a relevant permit and the approval of the research by the competent service of the Municipality. The questionnaires were completed by the older adults themselves after being informed about the purpose of the research, and the required clarifications were given. The participation of the older people was voluntary and in accordance with the principles and ethical rules of the research.

Questionnaire

The collection of the empirical research material was carried out using a special and fully structured questionnaire, which consisted of the following two sections.

Sociodemographic Variables, Living Status and Health Assessment Questionnaire

The questionnaire is specifically designed for the present study and includes some self-reported questions taken from part A of TFI without any changes and some questions from part A of TFI adjusted to the sociocultural context of Greece; we also added some other questions. In particular, the questionnaire includes questions about the socio-demographic information of older people (gender, age, marital status, number of children, educational level, permanent residence area and individual monthly income) as well as the information mentioned in the self-assessment of health and living status (living alone or with other persons, healthy lifestyle, the existence of chronic diseases or disorders, the experience of psychologically stressful situations and satisfaction from the family environment).

Tilburg Frailty Indicator

The TFI was used to assess the frailty of older people (Gobbens et al., 2010). The TFI was chosen as it is a self-administered instrument for screening for frailty in older adults and also it is an instrument that is economical, efficient and quick to provide answers, which has proven to be reliable and valid (Freitag et al., 2016). The TFI Frailty Scale has satisfactory psychometric characteristics of reliability and validity when tested on samples of older people living in the community. Zhang et al. (2020) examined the internal consistency, convergent and divergent validity, and concurrent validity of the TFI within a diverse community-based sample of older adults in five European countries, including Greece. The Cronbach's alpha of the physical domain in three countries, including Greece, varied between 0.60 and 0.67 while the internal consistency of the psychological and social domains was not satisfactory in none of the studied countries, with the Cronbach's alpha varying between 0.22 and 0.55. Also, regarding the full TFI, it had satisfactory reliability with an internal consistency of Cronbach's $\alpha \geq 0.70$ in the total population and in each country. The authors concluded that the TFI was a reliable and valid psychometric instrument for use in screening for frailty in community-dwelling older people in Spain, Greece, Croatia, Netherlands, and United Kingdom (Zhang et al., 2020). The TFI Frailty Scale is a self-report questionnaire for the detection of frailty in older adults and consists of 15 questions that assess frailty in three sub-scales: "Physical Frailty," which includes eight questions; "Psychological Frailty," which includes four questions and "Social Frailty," which includes three questions. A total of 11 questions accept binary answers (Yes or No), and the remaining four questions provide three options (Yes, Sometimes, and No); they are graded with values of zero or one. The total score of the questionnaire and the sub-scales is the sum of the answers to the individual questions that make them up. Higher score values declare higher levels of frailty in the respondent.

Statistical Analysis

The processing and the statistical analysis of the empirical research material were done using the software package "SPSS 25.0 for Windows," with the methods of Descriptive and Inferential Statistics. In particular, the Descriptive analysis included the frequency distribution for the qualitative variables (absolute and relative% frequency) as well as estimates of position and spreading parameters for the quantitative variables (mean, median, standard deviation, minimum and maximum value). The Kolmogorov–Smirnov test was used for assessing the normality of the distribution, which was $p > 0.05$, and a *t*-test was thus used to exam possible differences of frailty among groups of multimorbidity and traumatic events exposure. The significance levels (p value) were two-sided, and the level of acceptable statistical significance was set at $p < 5\%$.

RESULTS

Sample Characteristics

The sample of 257 older people consisted of 114 men (44.4%) and 143 women (55.6%). Their age ranged from 61 to 96 years with an average value of 75.12 years. Of these, 65.4% were married and 30.4% were widowed. Three out of five older people had one to two children (60.7%). Most of the older people were primary school graduates (68.5%) and lived in a non-urban area (54.9%). Regarding their monthly personal income, 55.6% of the studied population received between 500 and 1,000 euros. Approximately 3/4 of the older people lived with family members or others (75.9%). According the older people's answers, 61.9% described their lifestyle as healthy, and 54.9% did not have two or more chronic diseases or disorders. In terms of experiencing stressful psychological experiences, 27.2% had experienced the death of a loved person, 19.1% a serious illness, 23.0% a serious illness of a loved one, 7.0% a divorce or the end of an important relationship, 0.8% a car accident and 2.3% a crime. Finally, the

vast majority of older people (93.0%) reported that they were satisfied with the family environment. The **Table 1** summarizes the aforementioned findings.

Frailty

The internal consistency reliability of the Frailty Scale (TFI), determined by the Cronbach's Alpha coefficient, was for the total Frailty $\alpha = 0.75$, while in the three sub-scales it was $\alpha = 0.74$ for the Physical Frailty, $a = 0.68$ for Psychological Frailty and $\alpha = 0.21$ for Social Frailty. Consequently, the Social Frailty reliability was low, while the other dimensions of the TFI Scale showed very good reliability of internal consistency (**Table 2**).

The score for the total Frailty ranged from zero to 13 with an average value of 5.44 (standard deviation = 3.02) and a median value of 5.00. The findings of the study showed that the majority of older people had relatively low values of overall Frailty, as the total scores of the 50% of the patients were below 5.00 (median), which is less than 7.5—the middle point of the theoretical range of responses (**Table 2**). Taking into account cut-off point 5, around 54.1% ($n = 139$) of the participants displayed frailty.

A study of the individual dimensional outcomes of the TFI Scale shows that the Physical Frailty score ranged between zero and eight with an average value of 2.70 (standard deviation = 2.16), the Psychological Frailty score ranged from zero to four with an average value of 1.43 (standard deviation = 1.21) and the Social Frailty score was between zero to three with an average value of 1.32 (standard deviation = 0.64) (**Table 2**).

Chronic Diseases, Trauma Exposure and Frailty

The presence of two or more chronic diseases or disorders is related to the Physical ($p < 0.001$) and Psychological Frailty ($p < 0.001$) as well as to the Total Frailty ($p < 0.001$) of older people. In particular, the older people with two or more chronic illnesses or disorders have a higher mean value of Frailty levels than the older persons with one or no chronic illness or disorder (**Table 3**).

The experience of a serious illness in the previous year is related to the Physical ($p = 0.002$) and Psychological Frailty ($p = 0.002$) but also to the Total Frailty ($p < 0.001$) of older people. Particularly, older people who experienced a serious illness in the last year have a higher mean value of Frailty than older adults who had no such experience (**Table 3**).

The experience of a serious illness of a loved one during the previous year is related to Physical ($p = 0.002$) and Psychological Frailty ($p = 0.015$) but also to the Total Frailty ($p = 0.002$) of older people. Specifically, older persons who experienced a serious illness of a loved one in the last year have a higher average value of Frailty levels than older people who did not live such an experience (**Table 3**).

TABLE 1 | Individual features of the older adults ($n = 257$).

Characteristics	*n*	%
Gender		
Male	114	44.4
Female	143	55.6
Age (years)		
Mean ± SD		75.12 ± 8.39
Min–Max		61–96
Marital status		
Single	4	1.6
Married	168	65.4
Divorced	7	2.7
Widowed	78	30.4
Number of children		
0	8	3.1
1–2	156	60.7
≥3	93	36.2
Education level		
Primary education	176	68.5
Secondary education	61	23.7
Tertiary education	20	7.8
Permanent residence area		
Urban	116	45.1
Non-urban	141	54.9
Individual monthly income (Euro)		
<500	63	24.5
500–1,000	143	55.6
>1,000	51	19.8
Cohabitation		
Alone	62	24.1
Family members or others	195	75.9
How would you describe your lifestyle?		
Healthy	159	61.9
Neither healthy nor unhealthy	90	35.0
Unhealthy	8	3.1
Do you have two or more chronic diseases or disorders?		
Yes	116	45.1
No	141	54.9
Did you experience any of the following during the previous year?		
Death of a loved one	70	27.2
Serious disease	49	19.1
Serious illness of a loved one	59	23.0
Divorce or the end of an important relationship	18	7.0
Car accident	2	0.8
Criminal action against you	6	2.3
Are you satisfied with your family environment?		
Yes	239	93.0
No	18	7.0

DISCUSSION

The Greek population is aging rapidly and especially in the provinces. The aging of the population is a global fact that leads scientists to further engage and conduct research to address issues related to an older demographic. The purpose of this paper was to study the relationship between chronic diseases, traumatic experiences and frailty in older people living in the community.

TABLE 2 | Frailty levels of the older adults.

Tilburg frailty indicator (TFI)	Cronbach's alpha	Mean ± SD	Median	Min–Max values
Physical frailty	0.74	2.70 ± 2.16	2.00	0–8
Psychological frailty	0.68	1.43 ± 1.21	1.00	0–4
Social frailty	0.21	1.32 ± 0.64	1.00	0–3
Total frailty	0.75	5.44 ± 3.02	5.00	0–13

The results of the present study presented that the existence of two or more chronic diseases or disorders is related to physical, psychological and overall frailty in the older people living in the community. Particularly, comorbidity is associated with higher frailty rates. A similar result was obtained by the study of Vergara et al. (2019) in which 865 people over the age of 70 participated, and they presented that comorbidity is associated with high frailty. The most common chronic diseases of older people, in the Vergara et al. (2019) study, were diabetes mellitus, heart failure and chronic obstructive pulmonary disease. The meta-analysis of 30 studies conducted by Skela-Savic and Gabrovec (2018) on the prevention of frailty syndrome and its management at the individual level, showed that comorbidity, being female and age were the most important factors related to the occurrence and prognosis of the syndrome.

The experience of traumatic events in the last year shows a positive statistically significant relationship with frailty. Specifically, older persons who in the previous year either experienced a serious illness themselves or experienced a serious illness of a loved one collected a higher average value of frailty levels than older people who did not experience such a traumatic event. Aguayo et al. (2018) in a study of 5,294 older people, researched the correlation between experiencing a previous traumatic event or serious illness and frailty. The study showed that loss, diseases such as cancer and cardiovascular disease and lifestyle are positively correlated with the frailty score (Aguayo et al., 2018). The relationship between frailty, disability, and the experience of a significant illness was also studied by Greco et al. (2014) who found that chronic kidney disease in aging is significantly associated with physical, psychological, and social frailty, which is mainly due to depressive symptoms and cognitive deficits. Freitag and Schmidt (2016) found that resilience is significantly associated with frailty, as participants in their study with high levels of quality of life and resilience were less likely to be frail. Another study found that frail adults have greater difficulty adjusting to and recovering from stressful situations and that, compared with non-frail adults, frail adults are maybe more likely to use less suitable strategies or to have less resources to deal with the perceived stress (Desrichard et al., 2018).

TABLE 3 | Differences of frailty (TFI) among groups of chronic diseases and traumatic events exposure.

Features	TFI Scale			
	Physical Frailty	Psychological Frailty	Social Frailty	Total Frailty
More than 2 chronic disease or disorders				
Yes	3.56 ± 2.22	1.72 ± 1.21	1.29 ± 0.61	6.58 ± 2.92
No	1.99 ± 1.83	1.18 ± 1.16	1.33 ± 0.66	4.51 ± 2.78
T	6.098	3.642	0.504	5.802
p value	**<0.001**	**<0.001**	0.615	**<0.001**
The experience of death of a loved person				
Yes	2.97 ± 2.15	1.50 ± 1.15	1.36 ± 0.68	5.83 ± 2.95
No	2.60 ± 2.16	1.40 ± 1.23	1.30 ± 0.62	5.30 ± 3.04
T	1.234	0.583	0.647	1.253
p value	0.218	0.561	0.518	0.211
The experience of a serious illness				
Yes	3.55 ± 2.26	1.90 ± 1.26	1.35 ± 0.63	6.80 ± 3.19
No	2.50 ± 2.09	1.32 ± 1.17	1.31 ± 0.64	5.13 ± 2.89
T	3.122	3.071	0.388	3.566
p value	**0.002**	**0.002**	0.698	**<0.001**
The experience of a serious illness of a loved person				
Yes	3.47 ± 2.23	1.76 ± 1.17	1.29 ± 0.53	6.53 ± 2.89
No	2.47 ± 2.08	1.33 ± 1.21	1.32 ± 0.67	5.12 ± 2.99
T	3.199	2.444	0.372	3.193
p value	**0.002**	**0.015**	0.711	**0.002**
The experience of a divorce or the end of an important relationship				
Yes	2.61 ± 1.91	1.33 ± 0.97	1.50 ± 0.71	5.44 ± 2.64
No	2.71 ± 2.18	1.44 ± 1.23	1.30 ± 0.63	5.44 ± 3.05
T	0.182	0.344	1.281	0.001
p value	0.856	0.731	0.201	0.999

Bold is used to emphasize the statistically significant results.

This study presents limitations, which makes it difficult to draw general conclusions; these include limitations such as the small sample and the sampling method. The percentage population of older people in the Municipality of Grevena is not great, and so the sample of the study includes only 257 people, and convenience sampling was performed. Many older persons refused to participate in the study, and many of them had difficulty completing the questionnaire due to health problems or a low level of education. The limitations of the present research imply the design and implementation of new studies in a larger sample, by selecting the members of the sample by random sampling, for greater validity of the results and their optimal verification. This will contribute to drawing valid conclusions so that, in the future, the implemented plans could improve the quality of care provided to older people.

However, despite the above limitations and considering that the results of the present study are in accordance with the outcomes of similar international studies. The findings are considered, by researchers, to be reliable and safe, both overall and in the individual parameters, and they are suitable for use in Greece as well as internationally, contributing to the appropriate and effective assessment of frailty and its relationship with comorbidity and the experience of traumatic events.

Moreover, further investigation is needed regarding the correlation between multimorbidity, traumatic events and frailty among older adults in the community, and, also, it would be useful to investigate the types of strategies frail and non-frail adults use to cope with stressors (Desrichard et al., 2018). Research evidence on frailty should be translated into clinical practice and health care policymaking to improve quality care and promote healthy aging. This would also reduce the impact of aging on health care systems and strengthen their sustainability. Among others, the current challenges related to frailty research include the further understanding of interventions to reverse frailty and the best timing for intervention as well (Kojima et al., 2019).

CONCLUSION

In conclusion, the study showed that the older people who participated in the research were not very frail. Nevertheless, the comorbidity and the experience of a serious illness, in the past, of both the older people and a loved one are important factors that are statistically and significantly associated with higher levels of frailty. The results of the study helped to identify the frailty syndrome in older people as well as the factors associated with it. However, conducting new studies on a larger sample in the Greek region will derive more reliable and accurate results. In addition, the necessity to provide new data to healthcare professionals will lead to their faster information, awareness, and education and training regarding the geriatric patients, and this will contribute to the optimization of healthcare.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Center of Social Solidarity and Sports of the Municipality of Grevena. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IP, EF, and KG: conceptualization. DP: methodology and data curation. DM: software and formal analysis. IP, AR, and DM: investigation. AR: resources. IP and AR: writing—original draft preparation. IP, EF, DM, and KG: writing—review and editing. IP and EF: visualization. IP and KG: supervision. All authors read and approved the final manuscript and agree to be personally accountable for the authors' own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work—even ones in which the authors were not personally involved—were appropriately investigated.

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REVIEW
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Ranking Biomarkers of Aging by Citation Profiling and Effort Scoring

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Aging affects most living organisms and includes the processes that reduce health and survival. The chronological and the biological age of individuals can differ remarkably, and there is a lack of reliable biomarkers to monitor the consequences of aging. In this review we give an overview of commonly mentioned and frequently used potential aging-related biomarkers. We were interested in biomarkers of aging in general and in biomarkers related to cellular senescence in particular. To answer the question whether a biological feature is relevant as a potential biomarker of aging or senescence in the scientific community we used the PICO strategy known from evidence-based medicine. We introduced two scoring systems, aimed at reflecting biomarker relevance and measurement effort, which can be used to support study designs in both clinical and research settings.

Keywords: aging, biomarker, health, senescence, survival

INTRODUCTION

Aging can be described as a time-dependent multifactorial functional decline which affects the majority of living organisms (López-Otín et al., 2013). Very generally, it includes all processes that reduce health and survival of an individual (Fuellen et al., 2019). Notably, the chronological age and the "biological age" of an individual can differ remarkably (Kudryashova et al., 2020).

In this review, we give an overview of commonly mentioned and frequently used potential biomarkers of aging in clinical and research settings. A biomarker is a measurable feature (also called a marker) that predicts a biological state or condition (Siderowf et al., 2001). Biomarkers of aging are suggested to predict future health and survival better than chronological age. Further, they should be reproducible and they should also cause minimal trauma for the proband (Baker and Sprott, 1988; Fuellen et al., 2019). Until now no universally acceptable single-measurement biomarker of aging is known, and due to the complexity of the aging process, it is unlikely that a single universal biomarker of aging can be found. Many researchers believe that sets of

biomarkers must be considered to predict aging-related outcomes with confidence (Earls et al., 2019; Kudryashova et al., 2020). Multiple markers may complement each other thereby improving the predictive power. In fact, recent comparisons have shown that composite biomarkers (also dubbed biomarker signatures) are potentially useful as biomarkers of aging. Belsky et al. (2018), Hastings et al. (2019) have both shown composite measures to be superior in predicting age-related outcomes. Belsky et al. (2018) tested the association of 7 different methods (3 epigenetics clocks, 3 composite biomarkers, and telomere length) with outcome measures such as physical functioning, cognitive decline, and subjective signs of aging, including aged facial appearance. The measures had a low agreement with each other. Nonetheless, one of the epigenetic clocks and all composite biomarkers were consistently, albeit modestly, related to the aging-related outcomes. In turn, Hastings et al. (2019) compared four different biomarkers of aging, three composite markers and telomere length, for their association with age-related outcomes such as physical, cognitive and perceptual functioning. Effect sizes tended to be larger for the composite biomarkers, compared to simple markers such as telomere length (Belsky et al., 2018; Hastings et al., 2019). Most bio markers including telomere length lack specificity regarding the mechanisms of aging processes (Hastings et al., 2017) and furthermore, the identification and validation of new biomarkers is important, which track aging-related changes in humans already by young adulthood and may also vary in their rate of change over time (Hastings et al., 2017).

Biomarkers of aging can be based on laboratory measurements (e.g., telomere length, epigenetic clocks) or phenotypic data (e.g., hand grip strength). Routine laboratory biomarkers are commonly measured in accredited clinical laboratories based on standardized methods, e.g., complete blood count, inflammation markers, or surrogate markers for the functional and structural status of organs such as creatinine (kidney function), bilirubin and alkaline phosphatase (liver and bile metabolism), liver transaminase (liver function and integrity), NT-proBNP (heart function), and troponin (heart structural integrity). Other molecular biomarkers are based on high-throughput analyses, which are often of unknown predictive value and are primarily used in a research context only. As molecular biomarkers we consider, in particular, all genome-level ("omics") biomarkers. Non-molecular phenotypic biomarkers describe physiological functions of the body, specifically physical capability and organ function. Diagnostic biomarkers help to diagnose, confirm, or exclude a disease. In addition, biomarkers can be "prognostic," for death or for the progression of disease or dysfunction, as well as "predictive," for monitoring success or failure of some treatment. We do not explicitly distinguish biomarkers by this scheme, though we are mostly interested in prognosis.

We investigated the citation profiles of potential biomarkers of aging to gauge their "relevance." This "relevance" is intended to be a proxy for their accuracy in predicting future health and survival. Longitudinal human studies investigating the usefulness of biomarkers of aging are lacking. Without such studies, there are no head-to-head comparative data that allow any direct ranking of markers by accuracy. We doubt that such longitudinal studies will become available soon, given the increasing number of new biomarker candidates, for which, in the short-term, validation is only possible in short or non-prospective studies, and given technical problems (lack of standardization and sampling under distinct conditions). Furthermore, each potential biomarker was assigned an effort score (e-score). The effort score should help to estimate the effort required by the measurement of a biomarker.

Many researchers have gone to great lengths to identify potential biomarkers of aging (Engelfriet et al., 2013; López-Otín et al., 2013; Xu and Sun, 2015; Wagner et al., 2016; Khan et al., 2017; Xia et al., 2017; Bai, 2018; Justice et al., 2018; Sahu et al., 2018; Dodig et al., 2019; Kudryashova et al., 2020). Most of the reviews in the field are focusing on specific subgroups of markers (e.g., senescence markers, molecular markers, omics-based markers, epigenetic markers, etc.). Our goal was to summarize the most often mentioned potential biomarkers of aging, and to suggest effort scores. The effort scores are to a certain degree subjective and dependent on circumstances such as experimental setting and location. The citation profiling scores are to some extent subjective, too, since publishing is influenced by scientific as well as other (sociopsychological, political etc.) considerations. The purpose of this review is not to propose a new comprehensive composite marker that measures reliably all aspects of aging. Nevertheless, this review may facilitate the selection of aging-related biomarkers for specific study objectives in terms of relevance and effort.

METHODS

We considered reviews, and other articles, listed at NCBI-PubMed, which contain listings of potential biomarkers. Based on these listings, we established a set of "potential biomarkers" to begin with. We were interested in biomarkers of aging in general, and in biomarkers related to (cellular) senescence. To answer the question as to whether a "potential biomarker" is indeed an aging or (cellular) senescence biomarker we used the PICO strategy known from evidence based medicine (da Costa Santos et al., 2007). The following research question was formulated:

> "Is the ***"potential biomarker"*** a **biomarker** mentioned in the field of **aging** research?"

In this context, the term "potential biomarker" is a placeholder for the reviewed biomarker. Given this question, a PICO-Scheme was built (**Figure 1**) and a Pubmed query was done. By the way of query processing by the Pubmed search engine, the pertinent MeSH-terms (Medical Subject Headings, NLM controlled vocabulary thesaurus used for indexing articles for PubMed) are automatically included. Due to the high number of alias names of the term "potential biomarker," the specific MeSH-terms used by the PubMed search engine are not listed here explicitly.

The number of publications returned by the search, the count-score, is referred to as the "c-score." As a last step the results of the PubMed search were filtered for "review;" this results in the "review count score" (rc-score). The rc-score displays how often a potential biomarker was referred to in reviews, based on the

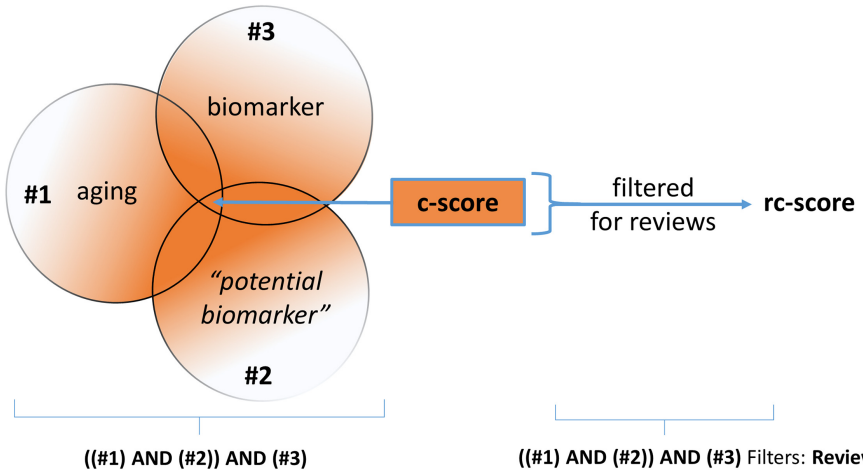

FIGURE 1 | Simplified graphic of the PICO-based queries employed at NCBI-PubMed. The search strategy was conducted as "((aging) AND (*potential biomarker*)) AND (biomarker)" for establishing the "c-score" and "((aging) AND (*potential biomarker*)) AND (biomarker)," filtered for reviews, for establishing the "rc-score."

above-described NCBI-PubMed queries. For senescence markers, the first query term (#1, "aging") was replaced by "senescence." Potential biomarkers often mentioned in reviews are assigned a high rc-score. Potential biomarkers of aging with a high PubMed citation count are not necessarily frequently mentioned in reviews, which results in a low rc-score. The rationale behind this strategy is that reviews may fulfill at least a partial filter function for relevance. The usefulness of the rc-score is expected to be the higher, the more source articles are included. By the use of this strategy we may miss, or disvalue potential biomarkers of aging published in recent studies. Additionally, widely used and long known disease markers may be overrepresented. Further, reviews have biases such as overvaluation of some high-profile studies and undervaluation of studies published in journals with low impact. Moreover, we did not normalize for date of publication. Then again, the results of recent studies are mostly not yet further confirmed. In a final step, the potential biomarkers were sorted according to their properties in the following categories: "routine laboratory," "research laboratory" (not epigenetic), "research laboratory" (epigenetic), "physical capability and organ function" and "senescence-related" biomarkers.

We then added an effort score for each potential biomarker. We consider the following three values:

- A low e-score (-) describes a potential biomarker which is easy to sample, to handle *and* to process (usually automated fast and reliable measurements are available from known sources). For example, blood counts from venous blood qualify, since such blood can be sampled easily, the plasma/serum can be stored at room temperature (up to 3 h for many analytes) or in a standard freezer (≥3 month for most analytes) and be processed by equipment that is regularly available in a standard diagnostic/clinical unit or research laboratory. The costs are low to moderate (≤10 €).
- A moderate e-score (--) is assigned if one step (sampling, handling, or processing) is associated with substantial extra effort for routine laboratories. This includes sampling under special conditions, a requirement for prompt sample handling, or the need for elaborate validation.
- A high e-score (---) implies elaborate sampling (e.g., biopsy, lumbar puncture, etc.), handling (e.g., storage in liquid nitrogen) and/or processing (e.g., non-routine nucleotide or protein sequencing). The financial costs are usually high.

In detail, the e-score is based on six main attributes that is, 1: easy sampling; 2: easy sample handling (storability at room temperature (up to 3 h) and/or frozen (≥3 month)); 3: automation availability; 5: routine laboratory availability; 5: costs ≤ 10 € per test; 6: degree to which the method is oblivious to confounding and interfering factors. For each attribute that is true, we incremented the score by 1, for each potential biomarker examined (see **Supplementary Data 1**). The following scoring system was then used:

- 6 – 5 (T): low e-score (-)
- 4 – 3 (T): moderate e-score (s.o.)
- 2 – 0 (T): high e-score (s.o.)

Just like the rc-score, the e-score is in part subjective.

A RANKING OF BIOMARKERS OF AGING

Routine Laboratory Biomarkers

We define a "routine laboratory" biomarker as a biomarker which is commonly analyzed in accredited laboratories based on standardized methods. It may also help to diagnose, confirm, or exclude a disease. In addition, biomarkers can be prognostic, to determine disease progression, as well as predictive, for monitoring success or failure of some treatment. In this category, cytokines such as interleukins (IL) and tumor necrosis factor alpha (TNFα) and other proteins such as C-reactive protein (CRP) belong to the

TABLE 1 | Frequently mentioned potential "routine laboratory" biomarkers.

Potential biomarkers	Material	Age linked processes[#]	e-score	rc-score*	c-score
Lymphocytes/WBC [CDC] [PA]	blood/EDTA	Inflammation autoimmune disorders	-	202	2240
Insulin	blood/serum	Diabetic state	--	148	1143
Glucose/glucose fastened [PA]	blood/glucose monovette	Diabetic state	-	111	1175
C-reactive protein (CRP/hsCRP) [IA] [PA]	blood/plasma	Inflammation, cancer, cardiovascular disease	-	71	1146
Cholesterol	blood/plasma	Cardiovascular disease	-	67	896
Albumin [PA]	blood/plasma	Kidney and liver dysfunction	-	65	1062
IL6 [IA]	blood/plasma	Inflammation	-	58	979
Tumor necrosis factor alpha (TNFα) [IA]	blood/serum	Inflammation, cancer	--	51	751
Hemoglobin [CDC]	blood/EDTA	Anemia, other hematopoietic disorders	-	39	471
Insulin-like growth factor 1 (IGF-1)	blood/serum	Metabolic disease	--	29	263
LDL-cholesterol	blood/plasma	Cardiovascular disease	-	24	280
Triglycerides	blood/plasma	Cardiovascular disease	-	23	498
HDL-cholesterol	blood/plasma	Cardiovascular disease	-	23	349
Creatinine [PA]	blood/plasma	Kidney dysfunction	-	19	479
Monocytes	blood/EDTA	Inflammation	-	16	378
Glycated hemoglobin (Hba1c)	blood/EDTA	Diabetic state	-	13	220
Cystatin C	blood/plasma	Kidney dysfunction	-	12	142
N-terminal prohormone of brain natriuretic peptide (NT-proBNP)	blood/EDTA	Heart failure	-	10	119
Alkaline phosphatase [PA]	blood/plasma	Liver damage, bone disorder	-	9	252
Hematocrit/RBC [CDC]	blood/EDTA	Anemia	-	8	159
D-dimer	blood/citrate monovette	Hypercoagulable state	-	8	91
IL8 [IA]	blood/plasma	Inflammation	--	7	164
Plasminogen activator inhibitor-1 (PAI1)	blood/EDTA	Prothrombotic state in cancer and other acute phases	--	6	72
Bilirubin	blood/plasma	Liver dysfunction	-	5	46
Urea	blood/plasma	Renal dysfunction	-	3	137
IL15	blood/plasma	Inflammation	--	3	55
Mean corpuscular volume/MCV [CDC] [PA]	blood/EDTA	Anemia, other hematopoietic disorders	-	2	42
Mean corpuscular hemoglobin concentration/MCHC [CDC]	blood/EDTA	Anemia, other hematopoietic disorders	-	2	32
CD4/CD8 ratio	blood/EDTA	Immune deficiency, autoimmunity	--	1	103
C-peptide (preferable to insulin)	blood/serum	Diabetic state	-	1	32
IL1-β [IA]	blood/plasma	inflammation	--	1	5

** rows are sorted by rc-score.*
[#] frequently mentioned general or disease-linked processes.

[IA] = inflammaging
[PA] = Phenotypic Age
[CDC] = complete blood count

most often mentioned biomarkers of aging (see **Table 1**). Interleukins and chemokines are secreted by leukocytes and other cell types and play a major role for the function of the immune system (Brocker et al., 2010; Ben Menachem-Zidon et al., 2011). **IL-6**, **IL-8**, **IL-15,** and **IL-1**β are often associated with aging-related inflammation, chemotaxis, and production of natural killer cells. Other inflammation-linked biomarkers of aging are high sensitive **CRP** (hs-CRP) and **TNF**α (Engelfriet et al., 2013; Wagner et al., 2016; Khan et al., 2017; Niedernhofer et al., 2017; Sebastiani et al., 2017; Bai, 2018; Justice et al., 2018; Levine et al., 2018; Tanaka et al., 2018; Dodig et al., 2019; Kudryashova et al., 2020). Elevated levels of these inflammation-related biomarkers in the blood of older individuals are risk factors for age related conditions and are often subsumed with the term "***inflammaging***" (marked in **Table 1** with [IA]) (Ferrucci and Fabbri, 2018). hs-CRP is an additional less specific marker for age-dependent atherothrombosis, featuring increasing levels in advanced stages of disease. These biomarkers are influenced by inflammation-related diseases such as coronary artery disease and (type 1) diabetes mellitus and immunological diseases. The specificity of established laboratory inflammation markers is low, considering latent as well as temporarily infections as a reason for their elevation. Another frequently mentioned group of biomarkers are related to lipids, which are cardiovascular risk factors in particular, such as **total cholesterol**, **HDL-cholesterol**, **LDL-cholesterol** and **triglycerides** (Engelfriet et al., 2013; Putin et al., 2016; Wagner et al., 2016; Niedernhofer et al., 2017; Sebastiani et al., 2017; Levine et al., 2018; Tanaka et al., 2018; Dodig et al., 2019; Mamoshina et al., 2019; Kudryashova et al., 2020). Blood lipid measurement levels vary with age. Lipid levels may influence aging and are themselves influenced by aging (Walter, 2009; Johnson and Stolzing, 2019). Studies have shown that effective treatment of dysregulated

lipid levels reduces mortality and morbidity. Other routine laboratory biomarkers correlate to the function and integrity of organs, which undergo age-dependent alterations. **Creatinine**, **cystatin C**, **urea,** and **albumin** are markers of renal and liver function which declines with old age (Lindeman et al., 1985; Weinstein and Anderson, 2010). Still other biomarkers discussed here are related to glucose, and are risk predictors for metabolic age-dependent conditions**: glycated hemoglobin (Hba1c)** and **glucose** (fastened or tolerance) are indicators for diabetic risk (Dubowitz et al., 2014). **Insulin**, despite high fluctuation, is an often-mentioned potential biomarker of aging. In the clinical setting, the **C-peptide** (the cleaved part of proinsulin) is most frequently measured instead of insulin due to simpler handling, less fluctuation and its equimolar amount compared to insulin. Additionally, C-peptide levels are not influenced by any insulin injections (Chandni et al., 2013). Some more routine laboratory biomarkers of aging are listed in **Table 1**. Taken alone, most biomarkers do not predict aging-related outcomes with high accuracy. Several studies have shown that some specific combinations of blood-based biomarkers result in more reliable predictions for "biological age" or mortality (Levine, 2013; Liu et al., 2018a). An example is the "***Phenotypic Age***," which is based on a linear combination of chronological age and nine multi-system clinical chemistry biomarkers (marked in **Table 1** with [PA]) (Liu et al., 2018a,b). With the aid of these nine blood-based biomarkers, an estimation of an individual's "biological age" is aimed for. Further blood-based biomarkers that are part of the complete blood count are used as input for software predicting "biological age," e.g., "**aging.AI**," in different versions (Zhavoronkov et al., 2019).

Non-epigenetic Research Laboratory Biomarkers

A research laboratory biomarker is a laboratory biomarker lacking the routine validation and/or standardization of a clinical laboratory biomarker. These markers often use nucleic acids (DNA and RNA). A frequently discussed biomarker of aging is the **telomere length** (López-Otín et al., 2013; Wagner et al., 2016; Khan et al., 2017; Niedernhofer et al., 2017; Xia et al., 2017; Bai, 2018; Dodig et al., 2019; Vaiserman and Krasnienkov, 2020). Telomeres are repeats of a hexametric DNA sequence capping the end of chromosomes preventing DNA damage (Aubert and Lansdorp, 2008). Telomeres shorten with each cell division or due to cell stress. This attrition ultimately leads to cellular senescence and can thus function as a biomarker for replicative aging in mitotic cells. Specifically, leukocyte telomere length is used as a potential biomarker for healthy aging (Bekaert et al., 2005; Lulkiewicz et al., 2020), whereby shortened telomeres may represent cellular exhaustion and/or increased cell stress for leukocytes or other cells in the body as a surrogate; however the usefulness of telomere length as a biomarker of aging is discussed critically. Kahl & Allen et al. summed up and described different methods measuring telomere length, covering imaging based methods (TCA, TRF, Q-FISH, Flow-FISH) and PCR-based methods (qPCR) (Kahl et al., 2020). Another very good overview is provided in the review of Lai et al. (2018), comparing known methods and additionally describing methods to measure the shortest telomeres (STELA, TeSLA). Telomere length measurements are characterized by poor standardization and limited comparability. Moreover, telomere length in leucocytes is only partially a surrogate marker for telomere length in other organs. Telomere length is classified as highly relevant according to the rc-score, which exemplifies the limitations of such a score. The telomere length is certainly very interesting from a physiological point of view, but whether it can serve as a relevant (or even accurate) biomarker is questionable. Telomere shortening can only be counteracted in a few cells (germ cells, stem cells, some immune cells) by **telomerase**, an enzyme that is able to lengthen the telomeres (Shay, 2016). Telomerase is induced in most tumor cells, which renders its induction risky. Nevertheless, telomerase activity plays an important role in longevity. A study showed that centenarians have a particularly active telomerase in T-cells compared to healthy 67 – 80 year old donors (Tedone et al., 2019), which raises the question if telomerase activity could be a biomarker for aging. Another DNA-linked potential biomarker of aging is the degree of **DNA damage** (Wagner et al., 2016; Khan et al., 2017; Niedernhofer et al., 2017; Dodig et al., 2019). DNA damage accumulates with age, fostering the development of age-related pathologies such as malignancies, cellular senescence and inflammation (Da Silva and Schumacher, 2019). In particular, mitochondrial DNA damage is a factor leading to **mitochondrial dysfunction**, which is also often related to aging processes (López-Otín et al., 2013; Khan et al., 2017; Niedernhofer et al., 2017; Sahu et al., 2018; Dodig et al., 2019). Mitochondrial DNA has less repair capacity and a higher mutation rate compared to nuclear DNA (Haas, 2019). Mitochondrial, metabolic and respiratory dysfunction can, in addition to exogenous stress, lead to the production of excess **reactive oxygen species (ROS)** (López-Otín et al., 2013; Wagner et al., 2016; Santos et al., 2018; Dodig et al., 2019; Kudryashova et al., 2020). Potential negative effects of excess ROS are dysregulated protein homeostasis, accumulation of oxidative modified proteins and advanced glycation/lipid peroxidation end products and loss of function of cellular protein maintenance systems. It has been shown that **autophagy** is another modulator of aging processes. A tissue-specific overexpression of autophagy genes can be sufficient to extend lifespan by preventing the accumulation of dysfunctional cellular components (Simonsen et al., 2008; Hansen et al., 2018; Kumsta et al., 2019). Such hallmarks of aging as discussed here were observed in various organs and tissues (Stadtman and Berlett, 1997; Baraibar and Friguet, 2013; Davies et al., 2017). In response to mitochondrial dysfunction, **growth differentiation factor 15 (GDF15)** may be generated, protecting tissues against inflammation by suppressing T-cell activation and mediating release of cytokines (Moon et al., 2020). On this basis, GDF15 was suggested as a potential aging biomarker (Justice et al., 2018; Tanaka et al., 2018; Basisty et al., 2020; Sebastiani et al., 2021). **TGF-β** and **GDF11** (from the same protein superfamily) are also regarded as proteins playing a role in aging-associated cellular senescence, frailty,

TABLE 2 | Frequently mentioned potential "research lab" biomarkers based on non-epigenetic measurements.

Potential biomarkers	Material	Methods	Age linked processes[#]	e-score	rc-score*	c-score
Telomere length (TL):			Morbidity, mortality, cell stress		191	932
Average TL	DNA	Q-PCR, TRF, TCA		--		**
TL structure	DNA	Q-FISH, Flow-FISH		---		**
Shortest TL	DNA	STELA, TeSLA		---		**
DNA damage	DNA	Various methods	Morbidity, mortality	--	174	713
Reactive oxygen species (ROS)	Tissue mitochondria	Various methods	Morbidity, cell stress, DNA/protein damage	---	168	712
Mitochondrial dysfunction	living cells, mitochondrial DNA	Various methods	Morbidity, mortality, neurodegenerative diseases	---	86	289
EVs (extracellular vesicles)	blood/plasma, liquor, cell culture supernatant	Immuno-histochemistry Western Blot, FACS	Cellular senescence, cancer	---	65	194
Autophagy	cells, cell extract	Electron microscopy immunoblotting flow cytometry	Morbidity, cancer, Parkinson's and Alzheimer's disease	---	46	207
Transforming growth factor beta (TGF-β)	blood/serum	ELISA	Inflammation, fibrosis, cellular senescence, cancer	--	45	315
Telomerase activity	cell extract, DNA	PCR-ELIDA, TRAP	Morbidity, mortality, tumor progression	---	41	169
Gut microbiome	fecal specimen	Next generation sequencing	Morbidity, mortality	--	29	101
α-Klotho	blood/plasma tissue	Immuno-histochemistry ELISA	Morbidity, mortality, renal function	--	20	107
Adiponectin	blood/plasma blood/EDTA	ELISA	Morbidity, mortality, frailty, metabolic syndrome, liver cirrhosis, diabetes type 2	-	14	217
Sirtuin 1 (SIRT1)	blood/serum	ELISA immuno-histochemistry PCR	Morbidity, mortality, inflammation, cancer	--	12	112
Growth differentiation factor 15 (GDF15)	blood/plasma	Proteomics immunoassays	Morbidity, organ damage (liver, heart, kidney)	--	12	63
Sirtuin 6 (SIRT6)	blood/serum	ELISA immuno-histochemistry PCR	Morbidity, mortality, diabetic risk, arthritis	--	4	50
Growth differentiation factor 11 (GDF11)	blood/plasma	Proteomics immunoassays	Morbidity	--	3	22
CXCL1	blood/plasma	Immunoassays, ELISA	Immune response, inflammation, cancer, Alzheimer's disease	--	0	15
Skin microbiome	skin swab	Next generation sequencing	Morbidity, mortality	--	0	4

** rows are sorted by rc-score.*
*** included in the c-score of TL.*
[#] frequently mentioned general or disease-linked processes.

stem cell aging and fibrosis as well as surgical risk in older adults (Schafer et al., 2016; Khan et al., 2017; Niedernhofer et al., 2017; Bai, 2018; Dodig et al., 2019; Tominaga and Suzuki, 2019). Circulating biomarkers based on **extracellular vesicles (EVs)**, including exosomes, microvesicles and apoptotic bodies, are moving into focus for the prediction of age-related diseases (Yáñez-Mó et al., 2015; Kalluri and LeBleu, 2020; Noren Hooten, 2020). Moreover, changes in the community composition of the **skin microbiome** have been related to age (Kim et al., 2019), and more precise information is obtained when considering the **gut microbiome** (Maynard and Weinkove, 2018; Guest, 2019; Askarova et al., 2020). In general, there is now ample evidence that microbiome dysbiosis is associated to aging and longevity (Kim and Benayoun, 2020). Other frequently mentioned potential research laboratory biomarkers of aging are listed in **Table 2**.

Epigenetic Research Laboratory Biomarkers

The epigenome is a dynamic system playing a major role in aging. Methylation of the DNA (DNAm) and histone modifications ensure appropriate high fidelity gene expression; both change with chronological age and with chronic diseases over time. Even if it is currently not known to what extend these changes cause aging, they can be useful, e.g., for chronological age prediction (Ashapkin et al., 2019). Generally, aging is associated with global hypomethylation and local hypermethylation. For the

analysis of DNA methylation, various so-called epigenetic clocks were developed. Famous examples for first generation epigenetic clocks are the **Horvath clock**, **Weidner Clock,** and **Hannum clock** (see Levine, 2019). Basically, these clocks are considering specific sets of CpG sites with respect to their DNA methylation status, as a molecular correlate to predict chronological age (Horvath and Raj, 2018; Bell et al., 2019). Hannum's clock is based on blood samples and uses 71 CpG sites measured from the Illumina 450k array. Age-related shifts in blood cells are *per se* informative for changes in chronological age (Hannum et al., 2013; Bell et al., 2019), but they are also considered by some epigenetic clocks. The Horvath clock is specifically designed to be used across multiple tissues, whereby it captures 353 CpG sites on a Illumina 27k array (Horvath, 2013; Bell et al., 2019). Second-generation epigenetic clocks learn to associate clinical data with methylation status, e.g., the **DNAm PhenoAge** (Levine et al., 2018) or **DNAm GrimAge** (Lu et al., 2019). More specifically, second-generation epigenetic clocks such as GrimAge and PhenoAge were developed to learn biological endpoints (which in turn are suggested to relate to "biological age") directly. Recently, it was shown that cytosines, whose methylation levels change with age across mammalian species, are involved in mammalian developmental processes, suggesting that aging is indeed evolutionarily conserved and coupled to developmental processes (Lu et al., 2021). Histone modifications such as **H4K16 acetylation**, **H4K20 methylation**, **H3K4 methylation**, **H3K9 methylation** and **H3K27 methylation** were also proposed as epigenetic chronological age predictors (López-Otín et al., 2013; Moskalev, 2019), and these modifications can be influenced by ROS (Wu and Ni, 2015). However, data regarding specific outcomes are scarce. Histone modifications and DNA methylation are closely related to **chromatin remodeling** and changes in chromatin architecture (Oberdoerffer and Sinclair, 2007; López-Otín et al., 2013). The above mentioned EVs also carry **extracellular RNA (exRNA)** which changes with age (Dluzen et al., 2017; Noren Hooten, 2020). Other types of RNA such as **microRNAs (miRNA)**, that function in RNA silencing and posttranscriptional regulation of gene expression, and which are often isolated from peripheral blood mononuclear cells (PBMCs), are also reflecting aging and are used to predict age-related diseases (Evans et al., 2010; Noren Hooten et al., 2010; Reid et al., 2011; Kumar et al., 2017). Examples for age-related miRNAs are: miR-34a, miR-9, miR-132, miR-212, miR-21, miR-96, miR-145 (Halper et al., 2015; Budzinska et al., 2016; Owczarz et al., 2017; Hadar et al., 2018). Frequently mentioned epigenetic based biomarkers are covered in **Table 3**.

Other Aging Biomarkers: Physical Capability, and Organ Function

Physical and cognitive function are important markers for aging processes (Fuellen et al., 2019), as are anthropometric measurements. For example, brain function and integrity are influenced by aging and aging-associated diseases. Aging encompasses changes at the structural, functional, and molecular levels of most cells, tissues and organ systems. Gradual loss of the maintenance functions of tissues is a characteristic of aging (López-Otín et al., 2013). Non-blood aging markers are not the primary focus of this review. However, due to the ease of implementation, certain analyses can supplement studies in which general metabolic and physiological age aspects need to be measured. These tests may include **grip strength** or easy to perform locomotor function tests as **walking speed**, **timed up and go test** or the **standing balance test**. As aging is associated with body composition, biomarkers such as **BMI** or fat and muscle indices should be recorded. **Bone mass** declines with age in both men and women and may be analyzed (Khosla et al., 1996; Khosla et al., 2006; Riggs et al., 2008; Keaveny et al., 2010). Frequently used other anthropometric markers are **muscle mass**, **waist circumference** (Wagner et al., 2016), and **(systolic) blood pressure** (Pinto, 2007; Crimmins et al., 2008). These measurements are mostly carried out without much effort. Most of these markers are also closely related, and they predict frailty in particular. Frailty is age-dependent and often associated with chronic disorders, resulting in an increasing need for diagnostic, nursing, and therapeutic interventions. Grip strength is a predictor of frailty, all-cause mortality and morbidity (Syddall et al., 2003). Strength itself may provide protection against mortality (Rantanen et al., 2000). With age, a decline in physical and cognitive function is frequently observed, as can be seen in the lifespan data of athletes in comparison to controls (Donato et al., 2003; Baker and Tang, 2010; Harridge and Lazarus, 2017). This is observed for muscle mass but also for physiological changes in organ systems leading to age-related diseases (Boss and Seegmiller, 1981). Additionally, various non-invasive methods were proposed to monitor the cardiovascular system and the vascular wall structure and elasticity, including electrocardiogram (ECG), intima media thickness ultrasonography and ultrasound techniques to evaluate endothelium-dependent vasodilation (EDV). Many if not all aspects of cognitive function change with age, which can be measured in complex tests or in rather simple questionnaires. Frequently mentioned non-blood and biomarkers are covered in **Table 4**.

Senescence-Related-Biomarkers

Cellular senescence is a cell state characterized by the cessation of cell division, reached through a combination of telomere shortening, oxidative stress and oncogenic stress. It can also be induced by each of these factors alone, and by DNA damage signaling pathways, with ATM and ATR as primary sensors of DNA double and single-strand damage. As a species-specific aging mechanism, telomere attrition limits the number of divisions. The successive shortening of the chromosomal telomeres with each cell cycle (caused by the so-called end replication problem and often referred to as replicative senescence) is observed in large long-lived species and cooperates with other aging mechanisms to activate the senescence program. These signaling pathways are funneled down to activate the p53 protein, the Rb protein, or both. Once the senescence program is activated, a series of changes in morphology, function, and gene expression takes place, associated with autocrine and paracrine effects of secreted cytokines, macromolecular

TABLE 3 | Frequently mentioned potential "research lab" biomarkers based on epigenetic measurements.

Potential biomarkers	Material	Methods	Prediction	e-score	rc-score	c-score*
DNA methylation and aging clocks:					n.a.	2158
Horvath's clock	DNA (broad spectrum of tissues)	DNA methylation analysis	Chronological age	--	n.a.	214
Hannum's clock	DNA (blood)		Chronological age	--	n.a.	190
DNAm GrimAge	DNA (blood)		Biological age	--	n.a.	31
DNAm PhenoAge	DNA (blood)		Biological age	--	n.a.	26
Weidner clock	DNA (blood)		Chronological age	--	n.a.	8
EpiTOC	DNA (blood)		Biological age	---	n.a.	2
miRNA (microRNA)	RNA (blood/plasma PBMCs)	Next generation sequencing microarrays	Morbidity, mortality	---	198	635
Non-coding RNA expression profiles	RNA	RNA sequencing	Chronological age	---	167	602
exRNA (extracellular RNA)	blood/plasma	Next generation sequencing	Morbidity, mortality	---	25	119
Histone modifications:					36	73
H4K20 methylation		DNA methylation analysis mass spectrometry, HPLC, ChIP Immunohisto-chemistry	Cell stress	---	n.a.	n.a.
H4K16 acetylation				---	n.a.	n.a.
H3K4 methylation	protein extract			---	n.a.	n.a.
H3K9 methylation	from tissue DNA			---	n.a.	n.a.
H3K27 methylation				---	n.a.	n.a.
Chromatin remodeling	DNA	Chromatin remodeling assays	Chronological age	---	13	26

** rows are sorted by c-score.*
n.a.: not assigned due to high variation of terminology in literature.

TABLE 4 | Frequently mentioned potential non-blood physical capability and organ function biomarker.

Potential biomarkers	Method	Age linked processes[#]	Domain	e-score	rc-score*	c-score
Physical capability						
Grip strength	Physical exam	Mortality, morbidity	Strength	--	11	229
Walking speed	Physical exam	Mortality, morbidity	Locomotor function	--	3	106
Standing balance	Physical exam	Mortality, morbidity	Balance	--	1	26
Timed up and go test	Physical exam	Mortality, morbidity	Locomotor function	--	0	11
Organ function						
Atherosclerotic lesions	IMT, ultrasound	Mortality, CAD	Cardiovascular system	--	158	680
Muscle mass	MRI	Mortality, cardiovascular risk	Body composition	--	81	495
Systolic blood pressure	Auscultatory method	Mortality, cardiovascular risk	Cardiovascular system	--	65	844
Cognitive function	Various	Mortality, morbidity	Brain function	---	56	581
Body mass index	Calculated	Mortality CAD	Body composition	--	24	1280
Bone density	Bone density test	Mortality, morbidity	Body composition	--	17	84
Lung function	Spirometry	Mortality, morbidity	Respiratory system	--	16	84
Waist circumference	Tape measure	Mortality, cardiovascular risk	Body composition	--	3	202
General well being						
Health assessments	Questionnaire	Mortality, morbidity	General	--	n.a.	n.a.

** rows are sorted by c-score.*
[#] frequently mentioned general or disease-linked.
n.a.: not assigned due to high variation of terminology in literature.

damage, and altered metabolism (Gorgoulis et al., 2019). The limitation of investigating "senescence-related" biomarkers lies in the cumbersome extraction of appropriate patient samples. Furthermore until now, there is no clinically validated senescence-related biomarker available. Indirect biomarkers of cellular senescence can be measured in blood samples, such as markers of proliferation status or components of the SASP (Castleab et al., 1999). However, these measurements are non-specific and only a proxy of the senescence status of the cells providing the sample. Moreover, they cannot reflect the senescence status of the entire organism (Schafer et al., 2020; Yousefzadeh et al., 2020). Additionally, often it is not clear from which tissue(s) the markers originate, since markers found in blood could originate from almost anywhere. The proliferation status of senescent cells can also be gauged by the expression of various cell-cycle-related markers (p16, p21, p53) (Jung et al.,

2010; Baker et al., 2011; Burd et al., 2013; Qian and Chen, 2013). On one hand, cellular senescence is a cause or bystander of many age-related diseases contributing to inflammation and/or tumorigenesis (Milanovic et al., 2018; Song et al., 2020). On the other hand, cellular senescence and its generally irreversible loss of proliferative potential is considered necessary for tissue remodeling during development, tissue homeostasis, wound healing as well as for tumor prevention (Ovadya and Krizhanovsky, 2018). Identification and characterization of cellular senescence markers receives more and more attention. However, the difficulty in characterizing cellular senescence by biomarkers and the lack of blood markers or other easily accessible specimen still limit the usefulness of the concept (Khan et al., 2017; Hernandez-Segura et al., 2018).

The senescence-associated secretory phenotype (SASP) is characterized by an enrichment of various inflammatory markers (Ghosh and Capell, 2016; Hernandez-Segura et al., 2018; Basisty et al., 2020) and can be detected in serum or EDTA plasma of probands using ELISA (Tanaka et al., 2018), which is readily available. Some important SASP markers are: interleukins (IL-6, IL-7, IL-8, and IL-15); chemokines (CCL3, CCL4) as well as growth factors (GDF-15 and activin A) (Schafer et al., 2020). Nevertheless, the secretion of these markers is highly heterogeneous and regulated at many levels, making it difficult to consider them as well-standardized biomarkers of cellular senescence (Hernandez-Segura et al., 2017). Assignment of inflammatory markers to the SASP or to another aging-related physical or physiological status (e.g., inflammaging) as well as to other diseases with permanent inflammation status (e.g., infections, tumors) is often difficult (Serrano et al., 1997; Barbé-Tuana et al., 2020). For the investigation of some cellular senescence markers, it is advantageous to have cells in culture, e.g., fibroblasts or PBMCs (Migliaccio and Palis, 2011; Riedhammer et al., 2016; Wang and Dreesen, 2018). For fibroblast isolation, a punch skin biopsy (Zuber, 2002; Vangipuram et al., 2013) may be taken, which may represent a notable trauma for the probands (Vangipuram et al., 2013). Another disadvantage of fibroblast cell culture is the long period of culturing of around 50 days, before investigations can be completed (Vangipuram et al., 2013), the difficult standardization, and other confounders such a population doublings in cell culture conditions. Consequently, this procedure is only used for research purposes. Much more easily done is the isolation and cultivation of PBMCs from blood samples including lymphocytes (T-cells, B-cells, NK-cells) (Migliaccio and Palis, 2011; Riedhammer et al., 2016). Senescent cells, especially fibroblasts in culture, become larger in size, flattened in shape (**Figure 2**) and can display a disorganized nuclear envelope mediated by reduction of lamin B1 expression (Nishio et al., 2001; Freund et al., 2012). These features of cellular morphology are features of senescent cells in general. Moreover, progerin (a mutated form of lamin A) associated with the premature aging syndrome Hutchinson-Gilford (DeBusk, 1972) can be detected in fibroblasts, also at low levels due to non-premature aging (Scaffidi and Misteli, 2006). A recent study has shown that elevated blood levels of progerin can be detected in people with obesity, suggesting a cause for premature aging of the cardiovascular system. Therefore, progerin might be measured in blood samples and be adapted for diagnostic measurements (Messner et al., 2019). Another marker of cellular senescence, which can be investigated in cultured cells (e.g., based on punch skin biopsy), are senescent-associated histone foci (SAHF). These darker regions within the nucleus of senescent cells can be detected as compacted DNA foci. While DNA staining of healthy and young human cells is relatively uniform, senescent cells show up to 50 punctuated DAPI-stained DNA foci (Narita et al., 2003; Aird and Zhang, 2013). Additionally, SAHF are enriched in markers of heterochromatin (H3K9Me3 and HP1γ) (**Figure 2**; Sharpless and Sherr, 2015). Furthermore, senescent

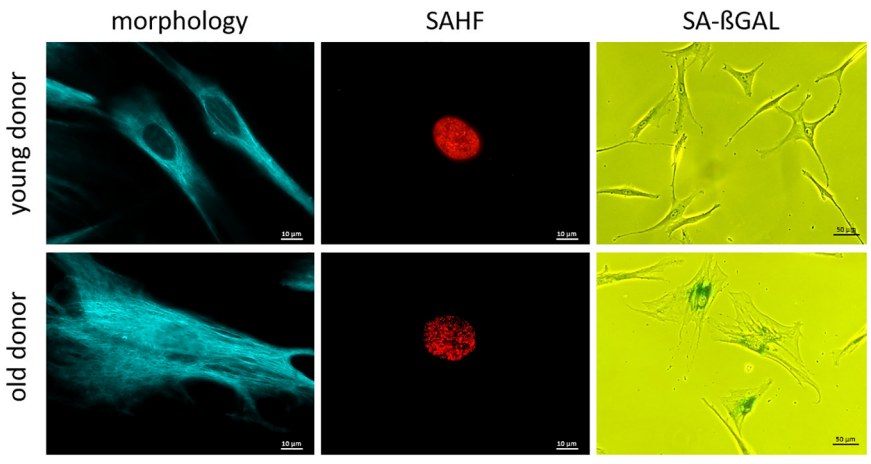

FIGURE 2 | Representative microscopy pictures of cellular senescence biomarkers. Note the clear difference in overt morphology due to age of the respective individual at biopsy (cells are larger in size and more flattened; cells were immunostained for vimentin.) **SAHF** are formed in old fibroblasts and are enriched in heterochromatin markers. (Cells were immunostained for H3K9Me3.) **SA-ßGal** activity increases with age at pH 6.0. (Cells were treated with X-Gal to make SA-βGal visible in senescent cells.); scale bar: 10 μM for morphology and SAHF column, 50 μM for SA-βGAL column.

TABLE 5 | Frequently mentioned biomarkers (routine or research laboratory) associated with cellular senescence.

Potential biomarker		Material and Methods	e-score	rc-score*	c-score
SASP				442	2646
	Cytokines (e.g., IL-6, IL-7, IL-15)	ELISA from Serum or EDTA plasma samples proteomics	--° --° --° --	n.a.	n.a.
	Chemokines (e.g., IL-8, CCL3, CCL4)			n.a.	n.a.
	Growth factors (e.g., GDF-15, activin A)			n.a.	n.a.
Cell cycle arrest	p53	qPCR from blood samples/staining of cultured cells/flow cytometry NGS/microarray	-- -- --	66	561
	p16			27	422
	p21			21	435
SA-βGal		Microscopy/flow cytometry	---	9	359
SAHF	Histone fragments (H3K9Me2, HP1γ)	DAPI/heterochromatin staining	---°	3	19
Lamin B1		Immunohistochemistr Western Blot	---	0	12
Cell morphology (e.g., progerin)	Cell shape	Microscopy of cultured cells	---	n.a.	n.a.

** rows are sorted by rc-score.*
*° on average (detailed in **Supplementary Table 1, 5**).*
n.a.: not assigned due to high variation of terminology in literature.

TABLE 6 | criteria to select biomarkers of aging in clinical trials.

Cohort Size:	Large cohorts require biomarkers that are easy to extract and process at low cost (e.g., serum markers). Studies with smaller cohorts and more specific aging-associated questions may require (and can afford) difficult-to-use and/or more expensive markers (e.g., fibroblast cultures, measurement of telomere length or the methylation level of CpG islands).
Cohort type:	Depending of the aims of the trial, usually reflected by inclusion criteria, it is often not appropriate to consider biomarkers which are usually used as markers for specific tissue damage or organ failure (e.g., creatinine, cystatin C, Pro-BNP) or markers that reflect a general activation of immunological processes such as CRP and IL6 or markers that reflect a higher risk for typical age-related diseases such as lipids, HbA1c or other cardiovascular risk factors. Additionally, organ specific markers should be controlled because these can be strong confounders in a study. A possible strategy to increase the informative value for all aging aspects could be the combination of organ-specific and more general markers.
Compartment of disease:	If the disease (or dysfunction) that is specifically considered in a trial features strong effects not in general but in distinct compartments (organs, tissues, combinations of these, or parts thereof), e.g., the brain, the overall question is which compartment to sample for biomarker analysis, e.g., peripheral blood vs. cerebrospinal fluid. Markers in the blood can often but not necessarily be attributed to more general aging processes.
Assessment of potential pitfalls:	Even if easy-to-handle biomarkers have a high sensitivity for aging-related processes, they often lack clinical specificity. This is true for many inflammatory markers (e.g., CRP, interleukins), which are more valuable markers of aging in populations without an overrepresentation of infections. For most questions acute infection must be ruled out by standard criteria (fever, feeling unwell, B-symptoms, etc.). Specific tissue/organ checks (e.g., physical examination, echocardiography etc.) can be added to rule out acute diseases. Strictly speaking, the biomarkers excluded on this basis may also reflect some acceleration of aging-related processes. However, they are less relevant than biomarkers reflecting more general aspects of aging, and, more importantly, they would lead to misinterpretations in individual patients. Furthermore, in addition to standard preanalytics precautions such as control of patient's position, application of the tourniquet, fasting vs. non-fasting and diurnal fluctuations, special aspects must be taken into account. For example, measurements that may be influenced by habits such as exercise should not be done on Mondays; exercise on weekends may influence cytokine levels, etc. In general, the same days should be used for all participants and all longitudinal time points.
Future directions:	There is a strong need to investigate biomarkers of aging more systematically. This should include promising markers such as the methylation of CpG islands and the standardization for specific sampling procedures (e.g., of peripheral blood cells for specific measurements) and the clarification as to whether and in what context acute disease markers, which at the same time can also reflect chronic processes of aging, are useful biomarkers of aging. Furthermore, biomarkers might be put together into composite markers, also known as "aging panels." Finally, the assessment of very sophisticated but highly informative measures with high potential validity to monitor aging such as MRI ("Brain age") or PET-Scans (e.g., TAU-PET, detecting the continuous increase of TAU deposition in temporo-parietal-occipital lobes) should be considered (Sowell et al., 2003; Cole et al., 2015; Schöll et al., 2016).

cells usually have an increased lysosomal content, which can be detected cytochemically by measuring senescence-associated β-galactosidase (SA-βGal) activity at a pH of 6.0 (Debacq-Chainiaux et al., 2009; Hernandez-Segura et al., 2018; **Figure 2**). Frequently mentioned senescence related biomarkers are covered in **Table 5**.

In summary, there is currently no biomarker for cellular senescence that can easily be used for diagnostics or prediction. Therefore, in the future, more attention should be paid to the research and establishment of diagnostically applicable biomarkers of cellular senescence. Potentially, measuring senescence markers after cell isolation by flow

cytometry can be further developed for use in diagnostics (Adewoye et al., 2020).

Transcriptomic Biomarkers

Transcriptomic (gene expression) changes also accompany the aging process, and transcriptomic clocks were trained to predict chronological age. Some clocks were trained on samples belonging to a specific tissue, e.g., the clocks by Peters et al. and Huan et al. are based on whole blood or PBMC, the clocks by Fleischer et al. and LaRocca et al. are based on skin fibroblasts, while the one by Mamoshina et al. (2019) is based on muscle tissue, and the clocks by Ren and Kuan (2020); Shokhirev and Johnson (2020) are based on multiple tissues (Peters et al., 2015; Fleischer et al., 2018; Huan et al., 2018; Mamoshina et al., 2018; LaRocca et al., 2020; Ren and Kuan, 2020; Shokhirev and Johnson, 2020). Most of these clocks are taking mRNA as input, while some employ microRNAs (Huan's clock) or repetitive elements (LaRocca's clock). Shokhirev and Johnson's pan-tissue transcriptomic clock is based on a large-scale meta-analysis of transcriptomic data from the Sequence Read Archive. 3060 multi-tissue samples were used as input, and a random forest model was able to learn chronological age with high accuracy (Shokhirev and Johnson, 2020). On a practical level, the e-score of transcriptomic clocks strongly depends on the tissue used for the analysis. Perhaps the best specimen in this context is whole blood or its components. Peripheral blood mononuclear cells (PBMC) are easily accessible as well, and PBMC transcriptomes were measured in the context of nutrigenomic interventions and proved to be sensitive to these (Afman et al., 2014; Herrera-Marcos et al., 2017; Bottero and Potashkin, 2020).

Preanalytics and Methodology Reporting

Appropriate preanalytics and exact description of sampling and methodology are critical for reliable results and reproducibility, particularly the use of validated tests and procedures. Details are beyond the scope of this review and are summarized elsewhere (Rai and Vitzthum, 2006). These include blood draw, blood collection, blood processing and storage. We recommend, whenever possible, a morning (before 10 am, and fasting) blood draw to control diurnal fluctuation; Mondays should be avoided due to weekend-specific confounders. For blood collection a 21-gauge needle and slow aspiration is preferred to avoid the activation of coagulation. Needle material and tube material may impact assays, e.g., in the measurement of trace elements. A standardized description of venipuncture and sampling is strongly recommended. There are advantages and disadvantages for serum and plasma respectively (Rai and Vitzthum, 2006). If possible, both serum, heparin plasma, double centrifuged citrate plasma and, if necessary, EDTA blood (e.g., for DNA preparation, telomere length measurements, etc.) should be collected and prepared in fractions, and, if necessary/possible, frozen. Blood counts and other highly standardized routine methods should be performed immediately. In general, tests with high inter-assay variability should be processed batch-wise based on initially frozen samples, provided the samples can be frozen for the respective test. Data on the stability of the frozen samples and on potential confounding by the freeze thawing procedures should be recorded. The position of the probands during blood drawing (standing, lying, sitting) influences almost all analyte values (due to water shifts between vessel and interstitial space). Sampling from probands at a stable position (horizontal or sitting for some minutes) is recommended. Serum/plasma should not be in contact with blood cells for more than 2 h (Kiechle, 2010). Long-term storage should be at −80°C or in liquid nitrogen (Rai et al., 2005).

CONCLUSION

Aging is a complex process and not fully understood. In this review we propose the "rc-score" and the "e-score" as tools for gauging the suitability of biomarkers of aging, with a focus on clinical settings. Together the two scores reflect the presumed relevance of a potential biomarker of aging, and the effort needed for its measurement. The "e-score" must be seen in relation to the equipment and possibilities of the laboratory performing the measurements. Routine blood biomarkers and easy-to-measure phenotypic markers such as blood pressure often correlate well with age-dependent organ/metabolic dysfunction, including cardiovascular, renal, or diabetic risk. The most cited non-epigenetic biomarkers are telomere length, amount of DNA-damage and mitochondrial dysfunction, reflecting aging-related changes on the genome and cellular level. Telomere length is classified as the most relevant according to rc-score, which exemplary shows the limitations of such a score. Telomere attrition is very interesting from a physiological point of view, but whether it can serve as an accurate biomarker is still questionable due to methodological problems. Novel telomere analyses such as TESLA have yet to be validated. Molecular markers such as cytokines/chemokines and sirtuins show a relatively low rc-score but are of clinical and scientific interest. The strong presence of BMI in reviews might also be attributed to the fact that potentially better markers such as the formal criteria of metabolic syndrome were not considered in many studies because the effort is higher for these. Methylation of CpG sites is among the most interesting candidate biomarkers of aging, but it awaits further validation in longitudinal studies. Functional decline affects all types of tissues and has a negative effect on grip strength and mobility, which can both be used as biomarkers of aging. Cellular senescence is a fundamental part of the aging process. However, it is difficult to analyze to date in a clinical setting due to difficulties in sampling and specificity. A combination of routine laboratory, epigenetic, non-epigenetic and physical capability and organ function biomarkers, and possibly senescence markers, may be the key to a valid composite biomarker of aging. Yet, a standardized (composite) biomarker of aging that specifically measures all important aspects of the aging process has not yet been found.

Clinical Get Home Message

The general question of which biomarkers should be used in clinical trials to study aging and (cellular) senescence remains difficult and clearly requires further systematic longitudinal studies. Nevertheless, there are numerous potential markers,

which differ concerning their difficulty to sample, to handle and to process, including significant differences in costs. Several parameters can be used to select biomarkers of aging for clinical trials, and we point out some specific issues in **Table 6**.

AUTHOR CONTRIBUTIONS

AHa, CH, and GF wrote the manuscript with input of RS, AHe, and MW. AHa did the analyses underlying the preparation of the tables. All authors approved the final version of the manuscript and made substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.686320/full#supplementary-material

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A Compendium of Age-Related PheWAS and GWAS Traits for Human Genetic Association Studies, Their Networks and Genetic Correlations

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The rich data from the genome-wide association studies (GWAS) and phenome-wide association studies (PheWAS) offer an unprecedented opportunity to identify the biological underpinnings of age-related disease (ARD) risk and multimorbidity. Surprisingly, however, a comprehensive list of ARDs remains unavailable due to the lack of a clear definition and selection criteria. We developed a method to identify ARDs and to provide a compendium of ARDs for genetic association studies. Querying 1,358 electronic medical record-derived traits, we first defined ARDs and age-related traits (ARTs) based on their prevalence profiles, requiring a unimodal distribution that shows an increasing prevalence after the age of 40 years, and which reaches a maximum peak at 60 years of age or later. As a result, we identified a list of 463 ARDs and ARTs in the GWAS and PheWAS catalogs. We next translated the ARDs and ARTs to their respective 276 Medical Subject Headings diseases and 45 anatomy terms. The most abundant disease categories are neoplasms (48 terms), cardiovascular diseases (44 terms), and nervous system diseases (27 terms). Employing data from a human symptoms-disease network, we found 6 symptom-shared disease groups, representing cancers, heart diseases, brain diseases, joint diseases, eye diseases, and mixed diseases. Lastly, by overlaying our ARD and ART list with genetic correlation data from the UK Biobank, we found 54 phenotypes in 2 clusters with high genetic correlations. Our compendium of ARD and ART is a highly useful resource, with broad applicability for studies of the genetics of aging, ARD, and multimorbidity.

Keywords: aging, age-related disease, age-related trait, biomarker, GWAS

INTRODUCTION

In humans, physiological deterioration starts to occur at a young age (26–38 years) with loss of bone, cartilage, muscle mass and strength, and gain of abdominal fat (Belsky et al., 2015). Consequently, the incidence of age-related diseases (ARDs) increases exponentially with advancing age (Niccoli and Partridge, 2012). For example, the risk of developing Alzheimer's disease doubles

every 5 years after the age of 65 (Kaeberlein, 2013). Additionally, at least half of those individuals that reach 70 years of age suffer from 2 or more chronic diseases, a state known as multimorbidity (Barnett et al., 2012). However, an extended period of disease and dysfunction in late life is not an inevitable outcome, as studies on extremely long-lived individuals (i.e., centenarians), have found that they exhibit a significantly delayed age of onset of ARDs, resulting in a substantial compression of late-life morbidity (Partridge et al., 2018). This finding supports the recently widely embraced "geroscience hypothesis" (Kennedy et al., 2014), which posits that chronic diseases (i.e., ARDs) share a common underlying mechanism, the aging process itself, and that by targeting this process for intervention one can target multiple ARDs simultaneously. Thus, in order to uncover suitable targets for longevity interventions, it is important to comprehensively identify and characterize the relevant age-related traits (ARTs), including ARDs and their respective biomarkers. Once precisely defined, ARTs can then be used as proxy phenotypes of aging, providing a useful basis for both the quantification of the health status of aged cohorts (Fried et al., 2001; Mitnitski et al., 2001; Evert et al., 2003; Terry et al., 2008; Andersen et al., 2012; Belsky et al., 2015; Cieza et al., 2015; Caballero et al., 2017), as well as for studies that aim to identify the shared genetic architectures of ARDs and longevity (Belsky et al., 2015; Fortney et al., 2015; Johnson et al., 2015; Zenin et al., 2019; Melzer et al., 2020).

Although the common definition of an ARD is an increased rate of disease morbidity (i.e., incidence or prevalence) with age (Barnett et al., 2012), the specific criteria used to identify ARDs differs among studies. For example, Chang et al. (2019) identified 92 ARDs using a two-step linear regression framework and data from the Global Burden of Disease Study 2017 (GBD). Restricting their analysis to the adult population (25+ years), they used a linear model to test for whether the incidence rates of diseases increased with age. The authors additionally tested whether the incidence rates followed a convex relationship with age by way of a quadratic model. In another example, by utilizing self-reported disease data from the UK Biobank (UKBB), Donertas et al. (2020) generated age-of-onset profiles of common diseases ($\geq$2,000 cases) and grouped the profiles into 4 clusters by using the partition around medoids algorithm. Out of 116 common diseases, the authors identified 25 ARDs which display a rapid increase in incidence after middle age (40+ years) and 51 ARDs that show a slow increase in incidence after early age (20+ years). However, the authors considered a limited age range, up to only 65 years old.

Human genetics research currently benefits from the wealth of publicly-available genetic association data compiled in the genome-wide association studies (GWAS; Buniello et al., 2019) and phenome-wide association studies (PheWAS) catalogs (Denny et al., 2010). However, for researchers of the genetics of aging and ARDs to fully take advantage of these highly valuable resources a comprehensive and well-defined list of ARTs is required, but to our knowledge, such a list has not yet been reported. Therefore, in this study, we aimed to identify a comprehensive list of ARTs, including ARDs and related biomarkers, using the whole traits and phenotypes present in the GWAS and PheWAS catalogs. As a result, we identified 463 ARTs, which we annotated with 100 international classification of diseases (ICD)-10 codes from the Gene ATLAS database. These traits map to 294 unique terms from Medical Subject Headings (MeSH) metadata, including 276 disease terms and 45 anatomy terms. Moreover, by overlaying our ARDs onto a human symptoms-disease network (HSDN), we identified 6 ARD subnetworks that represent disease groups with shared symptoms. Lastly, by translating our list of ARTs to UKBB phenotypes and clustering them by their genetic correlations, we found multiple ARTs with potentially shared genetic architectures. These results support the robustness of our methodology and the utility of the created resource for the field.

METHODS

Definition of Age-Related Traits

Google health cards[1], which use data that is manually sourced from the Mayo Clinic and the Center for Disease Control and Prevention (CDC), were queried using 1,358 electronic medical record (EMR)-derived phenotypes (phecode) retrieved from the PheWAS catalog (accessed April 2017) to obtain prevalence profiles with age (**Figure 1**). TheWolfram| Alpha search engine[2] was also used to compile "disease and patient-level statistics" data, sourced from CDC-conducted surveys, the National Ambulatory Medical Care Survey and the National Hospital Ambulatory Medical Care Survey, including the visitation data of 131,748 patients of United States healthcare providers from 2006 to 2007. From the disease prevalence profiles, ARTs were selected by the following criteria: (1) a unimodal distribution with prevalence increasing after mid-life (>40 years old); (2) a maximum peak at around 60 years old or later in the patient population size or prevalence rates. Non-disease traits such as biomarkers or endophenotypes associated with lifespan, aging, and ARTs were found and included through literature mining in PubMed, and mortality data for the United States (Vital Statistics NCHS' Multiple Cause of Death Data, 1959–2016; http://www.nber.org/data/multicause.html). The ARTs were queried to find the equivalent traits/diseases from the GWAS and PheWAS catalogs.

Annotation of International Classification of Diseases Codes and Gene Atlas

The GWAS Catalog database[3] includes manually curated GWAS data, with >70,000 variant-trait associations from >3,500 publications (Buniello et al., 2019). As a reverse GWAS concept, the PheWAS catalog[4] is a repository of phenome-wide association scans for associations between 3,144 single-nucleotide polymorphisms and 1,358 EMR-derived phenotypes (i.e., phecodes). This database is a powerful cross-validated source of replicated gene-phenotype associations from GWAS (Denny et al., 2010). The 399 phecodes of PheWAS ARDs were

[1]https://www.google.com, accessed September 2017.
[2]https://www.wolframalpha.com, accessed October 2017.
[3]https://www.ebi.ac.uk/gwas/home, accessed June 2020.
[4]https://phewascatalog.org

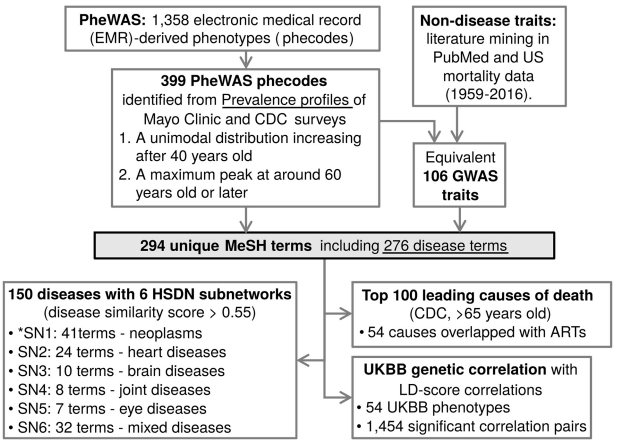

FIGURE 1 | Identified age-related traits and diseases annotations for genetic resources. A schematic of the analysis flow of this study. From the PheWAS and GWAS catalogs, ARTs were identified, converted to Medical Subject Headings (MeSH) terms, and overlaid onto a human symptom-disease network (HSDN), top 100 leading causes of death, and UKBB genetic correlation data. *SN means subnetwork.

mapped to ICD-9[5] (Denny et al., 2013) as well as ICD-10[6] and ICD-10-CM[7] (Wu et al., 2019). We merged the ICD codes to our ARD phecodes for external data annotation from the Gene ATLAS database. The Gene ATLAS database is a repository of genetic associations for 778 traits (self-reported or clinical diagnoses, tabulated as ICD-10 codes) that are found in at least 500 of the >452,000 UKBB participants (Canela-Xandri et al., 2018). Using the PheWAS ICD-10 codes, the equivalent ICD-10 codes or trait names from Gene ATLAS were annotated.

Translation of Trait Terms to MeSH

The MeSH metadata[8] provides a standardized vocabulary of medical terms with hierarchical categories, including biomedical information, including diseases, anatomy, chemicals and drugs, phenomena and processes, etc. (Lowe and Barnett, 1994). The 463 ARTs were queried to both the "Search"[9] and "MeSH on Demand"[10] functions on the MeSH webpage. The ART-MeSH term pairs are equivalent 1-to-1 matches with the following 4 non-equivalent pair instances; (1) some specific traits with no equivalent MeSH terms are mapped to parental MeSH terms (e.g., "Fracture of hand or wrist" mapped to "fracture" in MeSH terms); (2) some non-disease ARTs with no equivalent MeSH terms are alternatively mapped to MeSH anatomy, chemical, diagnosis, or phenomena terms (e.g., "Abnormal chest sounds" and "Stiffness of joint" mapped to "thorax" and "ankle joint" in MeSH terms, respectively); (3) co-morbidity traits with a common cause are mapped to the causal MeSH disease (e.g., "Hypertensive heart and/or renal disease" mapped to "hypertension" in MeSH terms); and (4) some ambiguous co-morbidity traits are mapped to one of those equivalent MeSH disease terms (e.g., "Cardiac arrest & ventricular fibrillation" mapped to "heart arrest" in MeSH terms). The ARTs were also independently mapped to relative MeSH anatomy terms by a 1-to-1 match. Some co-morbidity traits are mapped to one selected disease tissue (e.g., "cancer of kidney and urinary organs" mapped to "kidney" in MeSH terms).

Previously reported ARDs from Chang et al. (2019) and Donertas et al. (2020), as well as the top 100 leading causes of death according to data from the CDC[11] were downloaded. These disease names were translated to equivalent MeSH terms in order to identify overlap with our list of 276 ARDs.

Age-Related Disease Network

The HSDN data was extracted from the original study, which was constructed using data on 4,219 MeSH diseases, and their shared symptoms (Zhou et al., 2014). To identify the ARDs network of shared symptoms, the 276 MeSH diseases were overlapped with the HSDN. The disease similarity score criteria for the ARD network was empirically optimized as >0.55. The filtered ARD network is displayed in a yFiles Circular Layout with different colors for disease categories, unique node shapes for tissues/organs, and edge thickness for similarity scores by using Cytoscape software (Shannon et al., 2003).

Annotation of UK Biobank Phenotypes and Identification of Genetic Correlations

Linkage disequilibrium (LD)-score correlations of 677 UKBB phenotypes were calculated and published electronically by the Neale lab[12]. In this study, the Neale lab's UKBB genetic correlation data was downloaded (data accessed Dec. 29, 2020) and the UKBB phenotypes were mapped to the MeSH terms by equivalent 1-to-1 matches. The mapped MeSH terms were overlapped with the ART-derived MeSH terms to extract the age-related UKBB phenotypes. The raw *p*-values of genetic correlations were corrected to false discovery rate (FDR) by using the *p*.adjust function with method = "BH" option in R (Benjamini and Hochberg, 1995). To identify significant genetic correlations between the ARTs, a threshold of FDR < 0.05 was utilized. The genetic correlation r_g values were displayed as a heatmap by using the ComplexHeatmap library in R. To identify clusters of phenotypes, hierarchical clustering and the dynamic tree cut algorithm were applied by using hclust and cutreeDynamic functions (method = "tree" and cutHeight = 0.99 options) of dynamicTreeCut library in R.

RESULTS

276 Age-Related Diseases Across 45 Tissues Were Identified

In this study, we are using the terms ARDs to refer to diseases associated with age, and ARTs to refer to both ARDs and

[5]https://phewascatalog.org/phecodes
[6]https://phewascatalog.org/phecodes_icd10
[7]https://phewascatalog.org/phecodes_icd10cm
[8]https://meshb.nlm.nih.gov/
[9]https://meshb.nlm.nih.gov/search
[10]https://meshb.nlm.nih.gov/MeSHonDemand
[11]http://wonder.cdc.gov/ucd-icd10.html
[12]https://ukbb-rg.hail.is

their biomarkers. To identify ARDs, first we obtained the prevalence profiles with age of 1,358 EMR-derived phecodes from the PheWAS catalog, and defined ARDs by these criteria; (1) a prevalence showing a unimodal distribution, increasing after mid-life (>40 years old); and (2) a maximum peak of prevalence occurring at around 60 years old or later. In addition to the PheWAS phecode-based diseases, non-disease traits were also manually identified through mining of PubMed literature and United States mortality data. ARTs corresponding to these phecodes and non-disease traits were then identified in the GWAS catalog. As a result, we found that 106 GWAS traits and 399 PheWAS traits (463 total traits) have age-associated prevalence profiles and are identified as ARTs (**Supplementary Table 1**).

In many instances the GWAS and PheWAS catalogs use different terminologies for an equivalent disease trait (e.g., "Dementia" in the GWAS catalog and "Dementias" in the PheWAS catalog). To remove the redundancies and standardize the terms, we mapped the 463 ARTs to MeSH metadata and found 294 unique MeSH terms (**Figure 1**; see details in section "Methods"). The MeSH terms include 276 disease terms, 7 anatomy terms, 5 chemical terms, 3 diagnosis terms, 2 psychiatry terms, and a phenomena term. Of the 276 diseases, the top 5 most abundant disease categories were neoplasms (48 terms), cardiovascular diseases (44 terms), nervous system diseases (27 terms), male urogenital diseases (24 terms), and musculoskeletal diseases (22 terms; **Figure 2A**).

To identify the tissue distribution of the MeSH diseases (i.e., ARDs), we also mapped the 463 ARTs to MeSH anatomy terms. As a result, out of the total 45 tissues, the top 5 disease-specific tissues were heart (25 diseases), brain (20 diseases), eye (17 diseases), blood vessels (17 diseases), and blood cells (15 diseases; **Figure 2A**). And the top 5 MeSH anatomy categories were cardiovascular system (43 diseases), urogenital system (32 diseases), nervous system (29 diseases), digestive system (27 diseases), and sense organs (23 diseases; **Figure 2B**).

Cancers and Diseases of the Heart, Brain, Joint, and Eye Are Grouped as Major Symptom-Shared ARDs

The HSDN was constructed from disease symptoms as well as shared genetic associations between diseases by calculation of disease similarity scores (Zhou et al., 2014). To identify the symptom-shared ARD groups, we overlapped the ARDs to the HSDN (**Figure 1**). Of the 276 diseases, we found that 144 ARDs are found in 6 primary subnetworks when using a disease similarity score criteria of >0.55 (**Figure 3**). We labeled the 6 subnetworks according to disease category and tissue type, with subnetworks 1–6 representing cancers, heart diseases, brain diseases, joint diseases, eye diseases, and various mixed diseases, respectively.

In subnetwork 1 (41 diseases), the biggest category of ARDs, there are the 30 neoplasm terms as well as 3 digestive system diseases (gallstones, inflammatory bowel diseases, and chronic pancreatitis), and 8 other diseases such as aneurysm and venous thrombosis (cardiovascular diseases), bronchiectasis and pneumothorax (respiratory tract diseases), neutropenia (hemic and lymphatic diseases), urinary incontinence (male urogenital diseases), psoriasis (skin and connective tissue diseases), and hemorrage (unspecified). The 30 neoplasm terms are distributed in various tissues such as gastrointestinal (5 terms; colonic, colorectal, stomach, digestive system, and gastrointestinal), blood (3 leukemia terms; precursor cell lymphoblastic leukemia-lymphoma, lymphoid leukemia, and acute myeloid leukemia; 2 lymphoma terms; lymphoma and non-Hodgkin lymphoma; a myeloproliferative neoplasm term, polycythemia vera), skin (3 terms; skin neoplasms, melanoma, and squamous cell carcinoma), esophagus (2 terms; esophageal neoplasms and Barrett esophagus), cervix (2 terms; cervical intraepithelial neoplasia and uterine cervical neoplasms), kidney (2 terms; renal cell carcinoma and kidney neoplasms), liver (2 terms; hepatocellular carcinoma and liver neoplasms), lung (2 terms; non-small-cell lung carcinoma and lung neoplasms), and other 6 sites (head and neck neoplasms, breast neoplasms, lipoma, pancreatic neoplasms, prostatic neoplasms, and thyroid neoplasms).

In subnetwork 2 (24 diseases), 20 heart disease terms can be further sub-divided into heart rhythm-related (7 terms; sick sinus syndrome, tachycardia, cardiac arrhythmias, atrial fibrillation, ventricular fibrillation, atrioventricular block, and bundle-branch block), myocardial ischemia-related (3 terms; angina pectoris, coronary artery disease, and myocardial ischemia), heart arrest (2 terms; cardiac sudden death and heart arrest), cardiomyopathy (2 terms; cardiomyopathies and dilated cardiomyopathy), and other 6 heart disease terms (heart diseases, myocardial infarction, unstable angina, cardiomegaly, heart failure, and heart valve diseases). Additionally, there are 2 blood disease terms (hypotension and orthostatic hypotension), a thorax disease term (Tietze's Syndrome), and a blood vessel disease term (aortic aneurysm).

Brain diseases are found in subnetwork 3 (10 terms) including a brain cancer term (brain neoplasms), cerebral ischemia (2 terms; brain ischemia and transient ischemic attack), cerebral arteries occlusion (2 terms; cerebral infarction and cerebrovascular disorders), and other 5 brain disease terms (brain disease, intracranial hemorrhages, intracranial aneurysm, cerebral hemorrhage, and stroke). Joint diseases are clustered in subnetwork 4 (8 terms) such as arthritis, rheumatoid arthritis, joint diseases, and osteoarthritis as well as other 4 disease terms (testicular diseases, psoriatic arthritis, breast diseases, and bone fracture). Eye diseases are grouped in subnetwork 5, including glaucoma (2 terms; glaucoma and open-angle glaucoma), retina-related diseases (2 terms; retinal degeneration and retinal drusen), and 3 other eye diseases (choroid diseases, macular degeneration, and blindness).

In subnetwork 6 (32 terms), various diseases are clustered, including kidney diseases terms and related diseases (6 terms; kidney diseases, kidney calculi, chronic kidney failure, lupus nephritis, nephrotic syndrome, and renal insufficiency), male urogenital diseases (5 terms; renal hypertension, male infertility, erectile dysfunction, hematuria, and proteinuria), diabetes (2 terms; diabetes mellitus and type 2 diabetes mellitus) and diabetes complications (3 terms; diabetes complications, diabetic

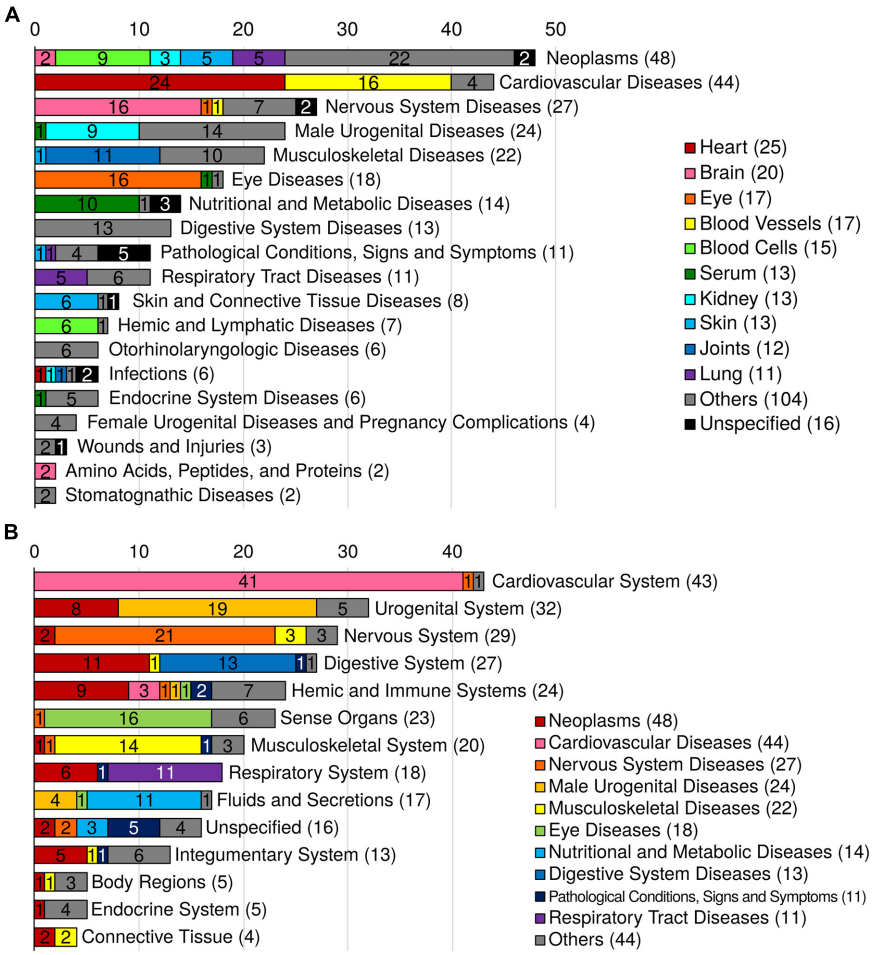

FIGURE 2 | Summary of 276 MeSH disease and anatomy terms. **(A)** Distribution of the ARD MeSH disease categories and their corresponding tissues. The colors indicate the corresponding tissue. **(B)** Distribution of the ARD MeSH anatomy categories and their corresponding MeSH disease categories of ARDs. The colors indicate the corresponding MeSH disease category.

retinopathy, and diabetic nephropathies), nutritional and metabolic diseases (4 terms; hypercholesterolemia, lipid metabolism disorders, metabolic diseases, and obesity), cardiovascular diseases (4 terms; atherosclerosis, venous thromboembolism, hypertension, and acute coronary syndrome), musculoskeletal diseases (3 terms; metabolic bone diseases, osteoporosis, and gout), female urogenital diseases (2 terms; female infertility and endometrial hyperplasia), and 3 other diseases (e.g., multiple myeloma, back pain, and systemic lupus erythematosus).

Half of the Top Causes of Mortality in the Aged Population Are ARDs

To evaluate the mortality of our ARDs, data on the top 100 clinical diagnoses (i.e., ICD-10 codes) of the leading cause of death for those over the age of 65 in the United States in 2018 was downloaded from the CDC, translated to MeSH terms, and overlapped with our 315 MeSH terms (**Figure 1**). As a result, 54 causes of death overlapped with our ARDs, including 18 cancers, 14 cardiovascular diseases, 10 nervous system diseases, 5 male urogenital diseases, 3 respiratory tract diseases, and 4 other diseases (gastrointestinal hemorrhage, myeloproliferative disorders, type 2 diabetes mellitus, and emphysema; "Rank_100" column in **Supplementary Table 2**). Intriguingly, the 54 high-mortality ARDs represent 85% of the total deaths from the top 100 causes of mortality.

High Genetic Correlations Are Observed Between ARDs in Different Disease Symptom Subnetworks

To identify shared genetic architectures that might exist between our ARTs, we downloaded genetic correlation data calculated between 677 UKBB phenotypes[13] and translated these phenotypes to MeSH terms by equivalent 1-to-1 matches (**Figure 1** and **Supplementary Table 1**). As a result, 54 UKBB phenotypes overlapped with 74 ARTs with 32 MeSH terms, and 1,454

[13]https://ukbb-rg.hail.is/rg_browser/

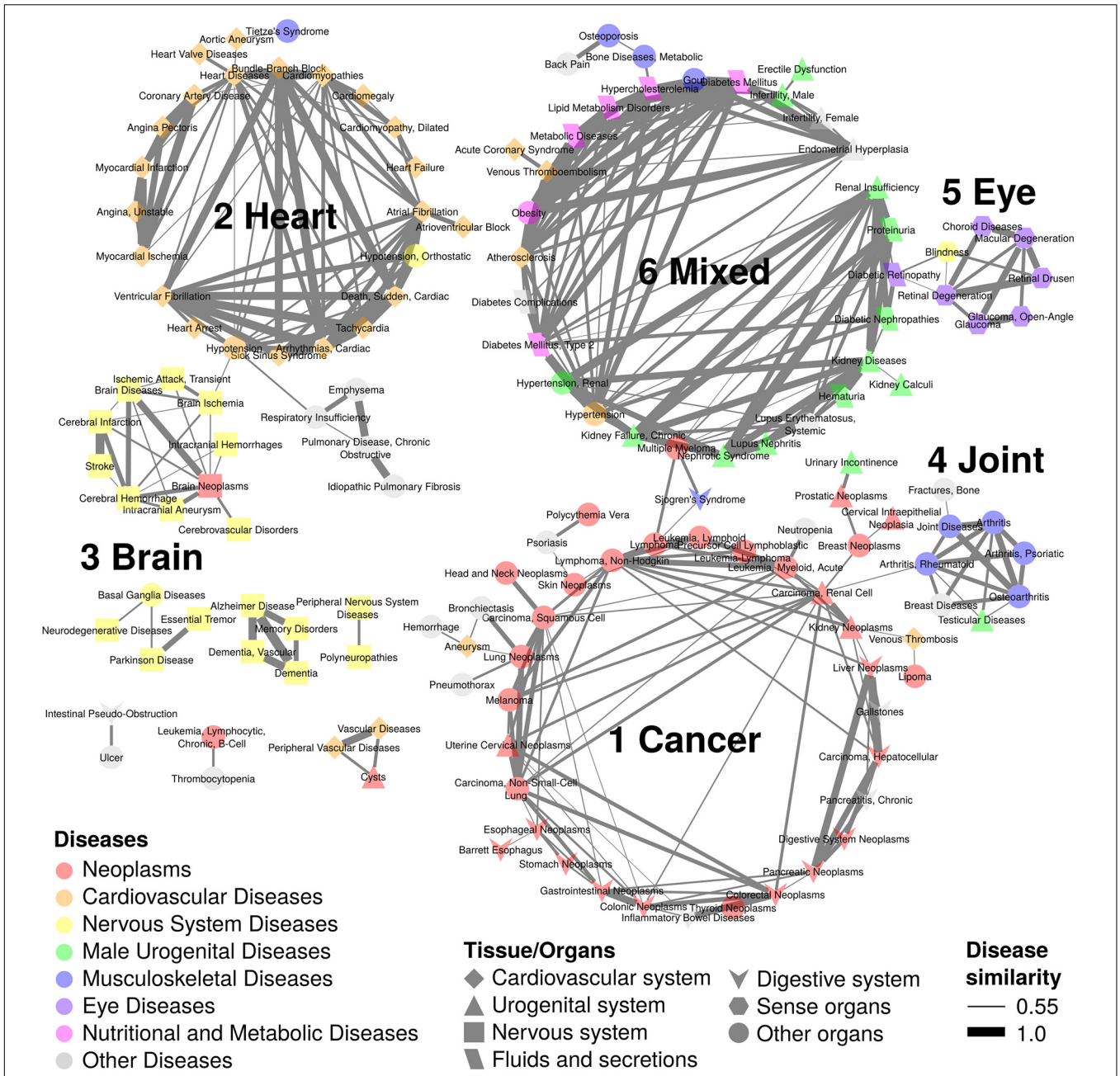

FIGURE 3 | Disease network of 144 ARDs with 6 subnetworks. The ARD subnetworks of disease pairs with similarity scores > 0.55 are shown. There are 6 subnetworks; subnetwork 1 with 41 ARDs is composed of cancers; subnetwork 2 with 24 ARDs includes heart diseases; subnetwork 3 with 10 ARDs is composed of brain diseases; subnetwork 4 with 8 ARDs includes joint diseases; subnetwork 5 with 7 ARDs is composed of eye diseases; and subnetwork 6 with 32 ARDs contains mixed diseases. Node colors indicate different disease types. Node shapes represent different tissue/organs. Edge thickness indicates disease similarity score, with the thickest edge representing a disease similarity score = 1.

pairs of phenotypes showed significant genetic correlation values (FDR < 0.05). By hierarchical clustering and using the dynamic tree cut algorithm, 29 and 25 phenotypes were grouped into 2 respective clusters (**Figure 4**).

The 29 phenotypes of cluster 1 have 746 significantly positive correlations (r_g > 0.11), with 2 main subclusters visible by examination of their tree structure. The first subcluster contains 14 phenotypes (r_g > 0.18) including 2 phenotypes of deep venous thrombosis (IDs: 6152_5 and 2002_1094), diverticular disease of intestine (K57), unspecified haematuria (R31), emphysema/chronic bronchitis (6152_6), wheeze or whistling in the chest in last year (2316), 4 phenotypes of backpain/dorsalgia (6159_4, M13_DORSALGIA, M54, and 3571), and 4 phenotypes of joint disorders (M13_ARTHROSIS, 20002_1465, M23, and

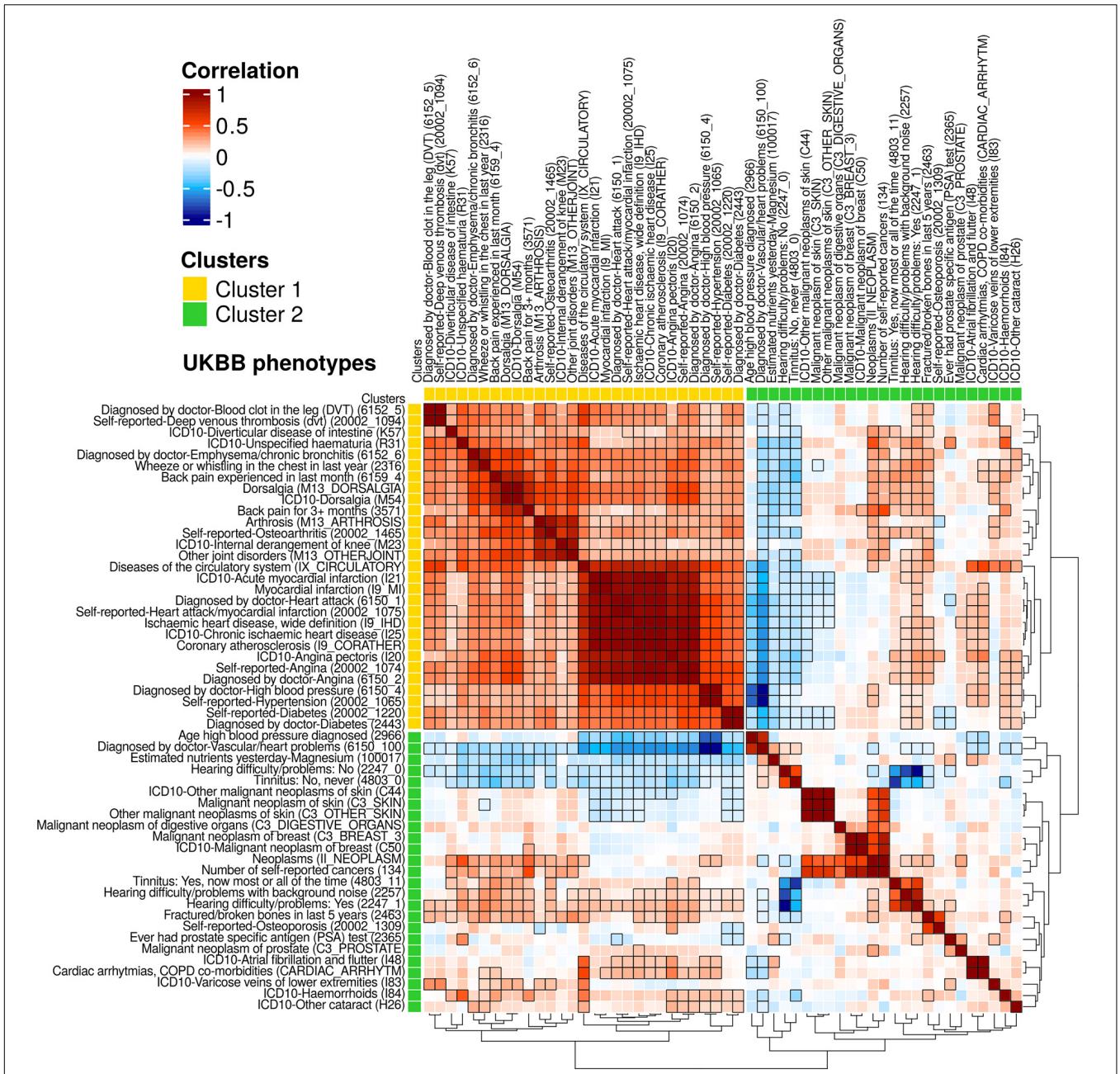

FIGURE 4 | A heatmap for genetic correlations (r_g) of 54 UKBB phenotypes. From the publicly available UKBB genetic correlation data (https://ukbb-rg.hail.is/rg_browser/), the genetic correlations (r_g) of 54 UKBB phenotypes are displayed. By hierarchical clustering with the dynamic tree cut algorithm, 2 clusters of phenotypes were identified; Cluster 1 is composed of 17 phenotypes with positive correlations; cluster 2 includes 26 phenotypes with both positive and negative correlations. Unique ID of the UKBB phenotypes is noted in parentheses. Colors represent the values of genetic correlations (r_g); dark red is $r_g = 1$, white is $r_g = 0$, and dark blue is $r_g = -1$. Significant genetic correlation pairs (FDR < 0.05) are indicated with black edged squares.

M13_OTHERJOINT). The other subcluster shows positive correlations ($r_g > 0.36$) of 14 phenotypes than the first subcluster, of which, the 11 phenotypes are highly correlated ($r_g > 0.71$) including diseases of the circulatory system (IX_CIRCULATORY), heart attack/myocardial infarctions (4 phenotypes; I21, I9_MI, 6150_1, and 20002_1075), ischaemic heart diseases (2 phenotypes; I9_IHD and I25), coronary atherosclerosis (I9_CORATHER), and angina (3 phenotypes; I20, 20002_1074, and 6150_2). Hypertension (2 phenotypes; 6150_4 and 20002_1065) and diabetes (2 phenotypes; 20002_1220 and 2443) are also present in the second subcluster.

In cluster 2, 25 phenotypes have 114 significant correlation pairs. Of these 25 phenotypes, 8 cancer phenotypes ($r_g > 0.38$), including 3 skin cancers (C44,

C3_SKIN, and C3_OTHER_SKIN), a digestive organ cancer (C3_DIGESTIVE_ORGANS), 2 breast cancers, C3_BREAST_3 and C50), and 2 other cancers (II_NEOPLASM and 134) appear subclustered from examination of the tree structure, as do 3 hearing loss-related phenotypes ($r_g > 0.44$; 4803_11, 2257, and 2247_1). The 4 phenotypes including vascular/heart problems diagnosed by doctor (6150_100), magnesium (100017), hearing difficulty/problems: No (2247_0), and tinnitus: No, never (4803_0) are negatively correlated ($r_g < -0.08$) with phenotypes in cluster 1. The latter 2 normal hearing phenotypes (2247_0 and 4803_0) also show negative correlations ($r_g < -0.47$) with the 3 hearing loss phenotypes. The phenotype age high blood pressure diagnosed (2966) and the 3 skin cancers (C44, C3_SKIN, and C3_OTHER_SKIN) displayed negative correlation ($r_g < -0.10$) with phenotypes in the second subcluster of cluster 1.

This result indicates that the ARDs grouped in different HSDN subnetworks (**Figure 3**) might share genetic associations. For example, venous thrombosis (subnetwork 1), myocardial infarction and angina pectoris (subnetwork 2), osteoarthritis and joint diseases (subnetwork 4), hematuria, back pain, hypertension, and diabetes mellitus (subnetwork 6) are included in cluster 1. For future analysis of genetic correlations, we additionally annotated 100 ICD-10 codes from the UKBB that have summary statistics stored in the Gene Atlas database (Canela-Xandri et al., 2018; **Supplementary Table 1**). In total, we annotated 144 UKBB phenotypes (including 93 ICD-10 codes) to 165 ARTs that have GWAS summary statistics either made available by the Neale lab[14] or present in the Gene ATLAS database, or both.

DISCUSSION

In this study, we identified 463 ARTs in the GWAS and PheWAS catalogs. The disease prevalence profiles of these ARTs exhibit a unimodal distribution, increasing in prevalence after 40 years of age, and reaching a maximum peak at 60+ years of age. The 463 ARTs were annotated with clinical diagnosis code sets such as ICD-9, ICD-10, and ICD-10-CM from PheWAS data (**Figure 1**). To remove redundancies, the 463 ARTs were also translated to 294 unique MeSH terms, including 276 diseases. A combined analysis with both the MeSH disease terms and MeSH anatomy terms showed that the leading ARD categories are neoplasms (blood cells, skin, lung, kidney, and brain, etc.), cardiovascular diseases (heart, blood vessel, etc.), and nervous system diseases (brain, peripheral nerves, and spinal cord, etc.; **Figure 2A**). We also found that symptom-shared ARD subnetworks include cancers and diseases of the heart, brain, joint, eye, as well as others (**Figure 3**). Previously, we reported that shared biological pathways exist among the genes found to be associated with five major categories of ARDs using data from GWAS, including cancer, cardiovascular disease, neurodegenerative disease, metabolic disease, and other ARDs (Johnson et al., 2015). In this study, we report additional genetic correlations among ARDs in different subnetworks (**Figure 4**).

[14]https://github.com/Nealelab/UK_Biobank_GWAS

Since GWAS and PheWAS catalogs use different terminologies for the same trait (GWAS uses the experimental factor ontology and PheWAS uses ICD-9-CM billing codes), integrating data from these resources can prove difficult. Only 9% (42/463) of ART terms are shared between both resources, which would lead to high redundancy in any list of ARTs that relied on simple integration of the two datasets. Our solution was to use the standardized terminology provided by MeSH metadata, which allowed for the creation of a non-redundant set of ARTs, and also allowed us to expand our annotation to other resources, such as the HSDN and UKBB. However, 1-to-1 matching from traits to MeSH terms can present a separate set of difficulties, such as traits with no equivalent MeSH terms and co-morbidity traits that share a common cause. These issues can be partially circumvented by using alternative terms, such as parental terms, alternative category terms, or causal terms. However, ambiguous co-morbidity terms (e.g., "Cancer of kidney and urinary organs") has to be translated to only one MeSH term (e.g., "Kidney Neoplasms"), which then leads to a biased distribution of diseases and tissues. For future studies, the development of a hub for multiple clinical-genomic resources which uses standardized terms (i.e., MeSH or ICD codes) should be prioritized and would be a great benefit for the field.

Recently, Donertas et al. (2020) and Chang et al. (2019) reported 76 ARDs, using UKBB data, and 92 ARDs, using GBD data, respectively. Compared with our list of ARDs, 61% of the diseases (46/76 diseases) and 49% (45/92 diseases) overlap (**Supplementary Table 2**). Surprisingly, only 7 MeSH diseases are shared between all 3 lists of ARDs, including 4 cardiovascular disease terms (hypertension, cardiovascular diseases, cardiac arrhythmias, and heart valve diseases), 2 eye disease terms (cataract and glaucoma), and stroke. These results suggest that there is a need to develop a consensus with regard to defining what constitutes an ARD, one that can be universally applied to different populations. In this study, by starting with EMR-derived phecodes in the PheWAS catalog and extensive text mining, the largest number of traits considered thus far, we were able to provide a comprehensive list of ARDs and ARTs available in the GWAS and PheWAS databases. When combined with population-scale clinical diagnosis data, for example those of the UKBB, our list can help identify shared genetic mechanisms of co- or multi-morbidity in the elderly. Furthermore, our tissue-specific ARD list can be useful in the investigation of the underlying tissue-specific mechanisms of aging, and can also be used as proxy phenotypes of aging.

Major ARDs (e.g., cancer, cardiovascular disease, dementia, hypertension, osteoporosis, and stroke) show rates of mortality and morbidity that are strongly associated with age (Andersen et al., 2012). Indeed, our study showed that out of the top 100 leading causes of death for individuals over the age of 65, 54 diseases are present in our list of ARDs and account for 85% of total deaths. This result indicates that our ARDs might be useful as proxy phenotypes of life expectancy.

In this study, we provide a resource for the fields of aging and ARD genetics, identifying 499 ARTs, and reporting their respective ICD codes, MeSH terms, and symptom similarity networks, as well as their genetic correlations. The data sets

generated by our study represent important, but currently lacking, resources for the aging research community.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.nber.org/data/multicause.html, https://www.ebi.ac.uk/gwas/home, https://phewascatalog.org, https://phewascatalog.org/phecodes, phewascatalog.org/phecodes_icd10, https://phewascatalog.org/phecodes_icd10cm, gene atlas.roslin.ed.ac.uk/, https://meshb.nlm.nih.gov/, meshb.nlm.nih.gov/search, meshb.nlm.nih.gov/MeSHonDemand, wonder.cdc.gov/ucd-icd10.html, and https://ukbb-rg.hail.is/rg_browser/.

AUTHOR CONTRIBUTIONS

YS designed and instructed the study. S-SK analyzed the results and wrote the first draft of the manuscript. AH provided useful suggestions in methodology and wrote the first draft. BG generated the raw data. SM, NB, JV, and ZT provided useful suggestions in methodology. All authors contributed to the article and approved the submitted final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.680560/full#supplementary-material

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REVIEW
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Sarcopenia: What Is the Origin of This Aging-Induced Disorder?

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We here review the loss of muscle function and mass (sarcopenia) in the framework of human healthspan and lifespan, and mechanisms involved in aging. The rapidly changing composition of the human population will impact the incidence and the prevalence of aging-induced disorders such as sarcopenia and, henceforth, efforts to narrow the gap between healthspan and lifespan should have top priority. There are substantial knowledge gaps in our understanding of aging. Heritability is estimated to account for only 25% of lifespan length. However, as we push the expected lifespan at birth toward those that we consider long-lived, the genetics of aging may become increasingly important. Linkage studies of genetic polymorphisms to both the susceptibility and aggressiveness of sarcopenia are still missing. Such information is needed to shed light on the large variability in clinical outcomes between individuals and why some respond to interventions while others do not. We here make a case for the concept that sarcopenia has a neurogenic origin and that in manifest sarcopenia, nerve and myofibers enter into a vicious cycle that will escalate the disease progression. We point to gaps in knowledge, for example the crosstalk between the motor axon, terminal Schwann cell, and myofiber in the denervation processes that leads to a loss of motor units and muscle weakness. Further, we argue that the operational definition of sarcopenia should be complemented with dynamic metrics that, along with validated biomarkers, may facilitate early preclinical diagnosis of individuals vulnerable to develop advanced sarcopenia. We argue that preventive measures are likely to be more effective to counter act aging-induced disorders than efforts to treat manifest clinical conditions. To achieve compliance with a prescription of preventive measures that may be life-long, we need to identify reliable predictors to design rational and convincing interventions.

Keywords: muscle weakness, muscle mass, aging, demography, heritability, phenotype, genotype

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INTRODUCTION

Aging is commonly used to name the progressive decay of organism's function in later life. Aging has an indistinct onset and a highly variable inter-individual progression. Today as well as in all recovered marks of human culture the inevitable end of life has provided a strong incentive to find measures to postpone it. While substantial progress has been made in extending the average expected lifespan, rejuvenation (or preserving youthfulness) still remains a concept more than a reality. The extension of lifespan among those we consider long-lived is not very impressive compared with the gain for those that previously died at a younger age. The latter

accomplishment came by improving living conditions and by preventing as well as combating major health threats until modern times, poor nutrition, and infectious diseases. Throughout the post-industrial nations and now also in developing countries, this has led to a dramatic change in the population composition by age (demography). The inflation of the aged over the younger cohort and the exponential growth of care expenditures, lead to an economic and social stress on society that needs to be addressed. Focus has therefore shifted from lifespan to healthspan, to extend the years that older people can continue to be independent, participate and contribute to society rather than experience life quality deterioration and consume resources because of poor health. To meet these challenges, the WHO recently classified aging-induced disorders as diseases (World Health Organization (WHO) 2019, 2020), an action intended to pave the way for the development of rational interventions to impede or even halt the aging-induced decline of health (The Lancet Diabetes Endocrinology (LDE), 2018).

In this *future perspective* we will touch upon the recent major demographic changes and biological processes considered to be involved in aging, and then devote the main part to sarcopenia, i.e., the dissipation of muscle function and mass, a conspicuous trait of the aged human phenotype. We chose sarcopenia because it is a facultative decay occurring also in healthy aging individuals yet it is accelerated by comorbidity, hospitalization, and a sedentary lifestyle. According to World Health Organization (WHO) (2000), sarcopenia is one of the major causes of loss of independence and a very significant risk factor for developing other morbidities at older age.

LIFESPAN AND HEALTHSPAN

Over the past one and a half centuries, the average human life span has almost doubled in high-income countries (HICs) and middle-and-low-income countries (MILOICs) are following. According to the Gerontology research group[1], a low boundary estimate of the number of people alive today at older age than 109 years is close to 500. They are all born early in the 20th century and since then the world population has increased from 1.6 to 7.8 billion (United Nations (UN), 2019). Due to this enormous increase in human phenotypes (molded out of the genotypes), it is believed that the number of supercentenarians will grow rapidly and that the apparent ceiling of human longevity around 120 years will be pushed forward. However, this raises issues with the interpretation of the lifespan extension during the 19th–20th centuries and the scarcity of reliable more distant historic data as well as to what extent longevity is heritable (Oeppen and Vaupel, 2002; Crimmins, 2015; Dong et al., 2016; Kontis et al., 2017; Ben-Haim et al., 2018; Kaplanis et al., 2018). As Sweden has one of the best backlogging population statistics, we will use this here as a framework to discuss changes to lifespan and mortality across the past centuries and, in addition, how health care intervention is consumed by age and sex in the year 2019 (**Figure 1**).

[1]https://grg.org/

Since 1751, life expectancy at birth has more than doubled in Sweden (**Figure 1A**). The increase was particularly fast in the 20th century (**Figure 1B**) but as we approach and surpass 80 years in lifespan expectancy at birth (**Figure 1A**) and in agreement with several other HICs, lifespan extension is now slowing down (**Figures 1A,B**; Dong et al., 2016). By comparing mortality by age and sex we can see that the extended lifespan is due to the curbing of early life mortality (see inset in **Figure 1C**; <5 years; 3–5% 1751–1760 down to 0.01–0.32% in 2019) meaning that today with a few exceptions all those born alive will reach puberty. At menarche/puberty and throughout a major portion of the reproductive period (here defined as the period between 15 and 49 years), mortality remains low and remarkably stable at 0.6–1.2% in 1751–1760 and 0.2–0.6% in 2019 until age 35–39. This "reproductive mortality plateau" present in the Swedish population statistics both from 1751–1760 and 2019, is in no way exclusive to Sweden (Perls et al., 2002; Crimmins, 2015) and suggests that the human population has been selected from those that had good health through a sufficiently long period to reproduce and nurture a surplus of children also under more challenging and impoverished conditions[2]. The second major change is the shift from ∼50 to ∼70 years of age as the breakpoint when mortality start to increase rapidly (**Figure 1A**); at above 75–80 years of age the mortality is high still today (*idem*). When comparing the changes in mortality between 1751–1760 and 2019 (**Figure 1C**) with health care interventions by age and sex in 2019 (**Figure 1D**), we can conclude that the much-improved survival in early life and at older age both are reflected in a comparatively larger consumption of health care interventions of these age groups. This indicates that we have been more successful in curbing mortality than to decrease morbidity (here defined as an instance of intervention with a treatment diagnosis) over the past centuries and consequently that aging is a key co-factor for the concurrent and future health provisions by society (Ben-Haim et al., 2018; Partridge et al., 2018). Lifespan extension and the parallel change to the fertility rate (**Supplementary Figure 1A**) have altered the demographic profile toward older ages (**Supplementary Figure 1B**), forcing society to recognize aging as a major factor for dependence and morbidity. In order to facilitate the development of effective preventive medicine that can postpone morbidity and increase healthspan, we need reliable predictive biomarkers of morbidity that also consider the impact of aging (Beekman et al., 2010; Deelen et al., 2013).

BIOLOGY OF AGING

A very simplistic description is that aging is the accumulation of non-degraded waste (e.g., myelin bodies, lipofuscin, and amyloid) and unrepaired damage to cellular/extracellular constituents with a run-down of cellular energy production, and the gradual exhaustion of the replicative capacity forcing cells into state of non-responsiveness/senescence (**Figure 2**).

[2]Assuming menarche at 12 years, a 50–75% mortality of children and need of parental protection until puberty, and that starvation and prolonged breast feeding limited the frequency of pregnancies to every other year; a life span of 35–39 years is still enough to accomplish a net growth of the human population.

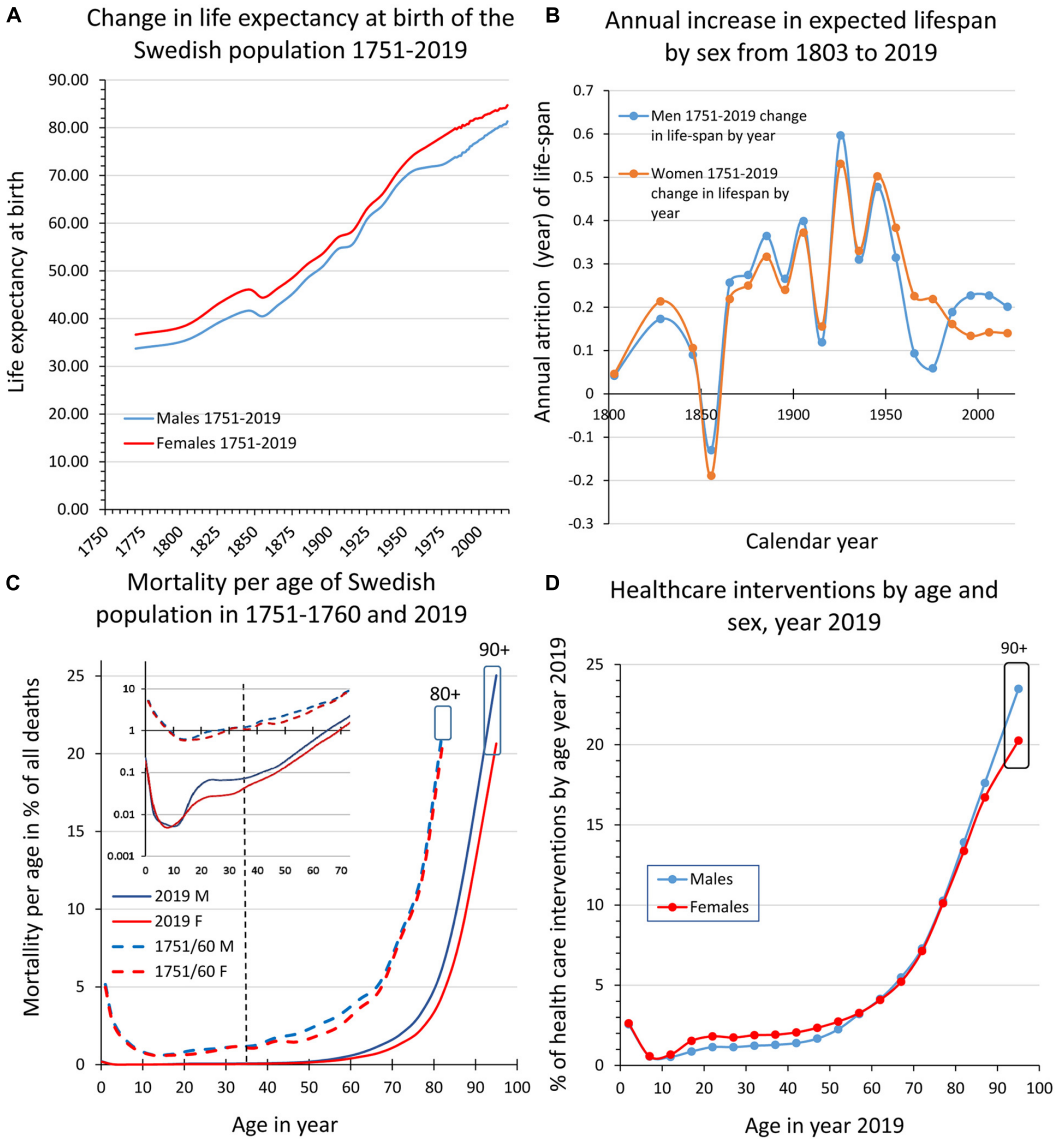

FIGURE 1 | Swedish population statistics 1751–2019. **(A)** Shows the increase in expected lifespan at birth for women and men. **(B)** Diagram illustrating the increase (averaged across 10 years bins) of expected lifespan at birth by year and sex 1803–2019. **(C)** Shows the mortality (%) by age and sex in 1751–1760 and in 2019, respectively. Inset in **(C)** is part of the main diagram but with a logarithmic scale on the ordinate. The curves illustrate that the major impacts on lifespan for women and men are the decreased mortality in early life and at ages 50–80 years, while mortality today is still high at >80 years and corresponds to the leveling-off of lifespan increase evident in more recent data (c.f., **A,B**). **(D)** Shows health care interventions by age and sex in 2019 where the decreased mortality in early and late ife **(C)** corresponds to higher levels of health care consumption. (**A–D** are data replotted from Statistics Sweden (1999) and http://www.statistikdatabasen.scb.se/pxweb/en/ssd/).

This gradual decay has a variable pace both between tissues within individuals as well as between individuals and lead to the disintegration and finally death of the organism. Why aging occurs is a much debated issue (Kirkwood and Melov, 2011; Azzu and Valencak, 2017) but humans in HICs currently live more than twice the time it takes to reproduce and nurture a growing human population and the older ages beyond the reproductive era make up an increasingly large part of the lifespan strongly associated with morbidity and decay of function. While any benefit for the human species of growing old remains to be evidenced, closing the gap between health- and lifespan is not an issue of opposing views. Studies devoted to shed light on the heritability of growing old indicate that only a modest 25% of the variability in lifespan can be accounted for by heritage (Wijsman et al., 2011; Kaplanis et al., 2018; Partridge et al., 2018). However, as we push the expected lifespan toward the ages of those that are most long-lived (100+) and who did not experience the same increase in lifespan over the past centuries, the genetic constraint of human lifespan may become increasingly important (Barzilai et al., 2010;

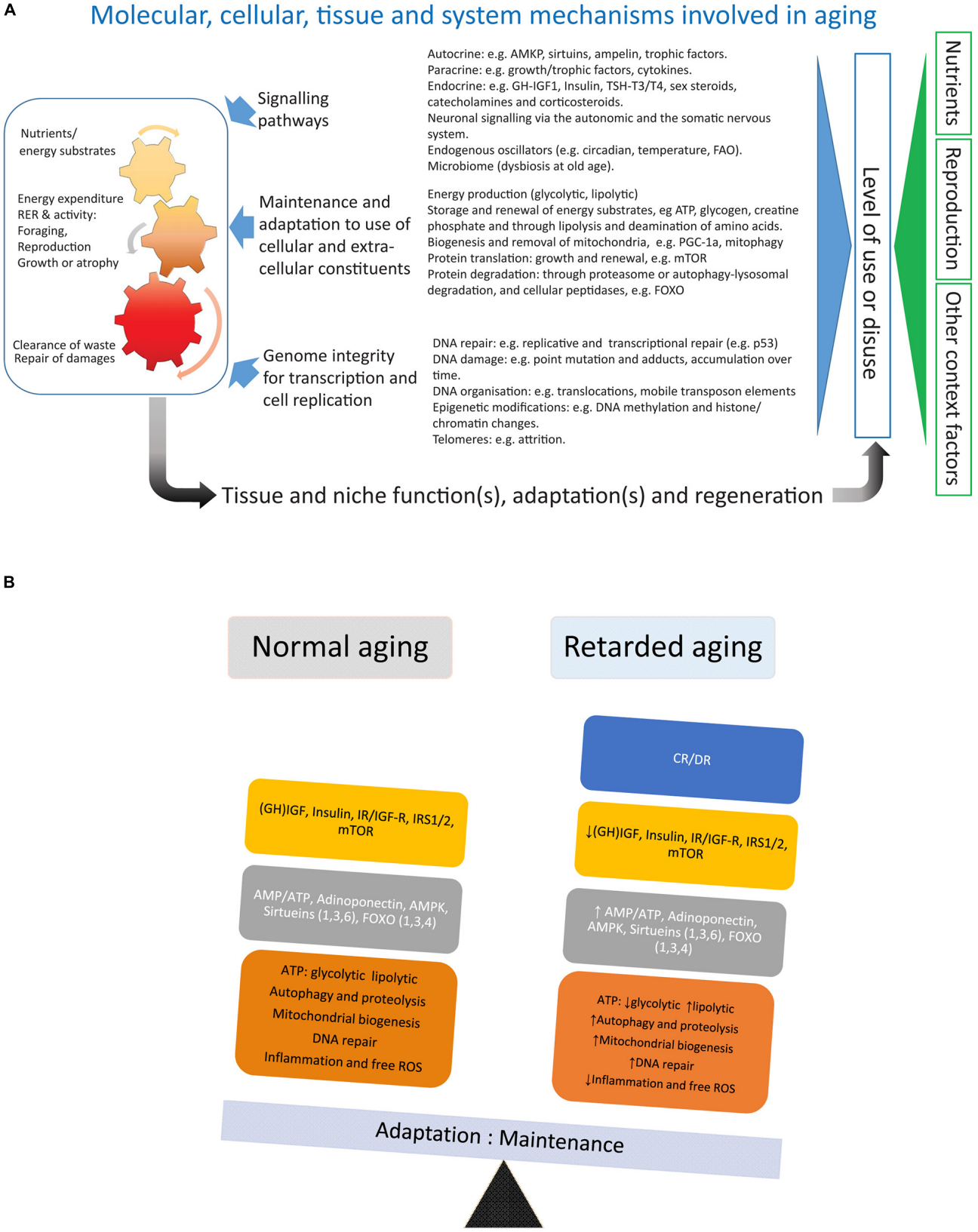

FIGURE 2 | (A) Depicts factors involved in the aging of an organism. **(B)** Summarizes the main effects of calorie/dietary restriction.

Beekman et al., 2010; Kenyon, 2010; Rajpathak et al., 2011). This assumption is supported by the fact that most long-lived humans appear to be protected against some of the hazards conferring morbidity and death among more short-lived humans (Beekman et al., 2010; Rajpathak et al., 2011). Aging is a complex polygenic trait and already the non-exhaustive listing of 24 genes with a strong association with longevity in humans (Partridge et al., 2018) is extensive enough to generate a very large number of different genotypes and, when coupled with environmental impact, an even larger set of phenotypes. To close some of the gap between health- and lifespan at older ages, a deeper understanding of the genetics of aging is needed (Atwood et al., 2003; Kirkwood et al., 2011).

Calorie/dietary restriction (CR/DR) is the most robust and general (across mammalian and non-mammalian species) intervention known to retard aging and is therefore a useful tool to dissect the processes underlying aging and aging-induced traits like sarcopenia (an extended review of CR with references is available in the **Supplementary Material**). Laboratory work in the early 20th century (for references see Speakman and Mitchell, 2011) showed that reducing calorie or dietary (CR/DR restriction) intake by 10%-45% increased lifespan by up to 50% in many invertebrate and vertebrate species, implicating that this intervention intercepted with highly conserved mechanisms of systems biology involved in the aging of organisms. In mammals, CR reduces the incidence of common morbidities such as cardiovascular diseases and cancer (main causes of death at older ages in HICs) and, thus, seems to extend healthspan to a similar extent as lifespan. In mammals like small rodents CR will retard body growth and, if initiated in adulthood, lower body weight, an adaptation which will affect both lean body mass and, in particular, adipose tissue (Duffy et al., 1997; Cameron et al., 2011). Rodents are endothermic and respond to CR with a lowering of body temperature and torpor (*idem*), accompanied by alterations in the endocrine system with decreased levels of TSH, T4, and T3 (Araujo et al., 2009) (HPT axis), growth hormone and IGF-1, and lower levels of blood glucose and insulin as well as increased insulin sensitivity (Cameron et al., 2011; Azzu and Valencak, 2017). Metabolically, there is a shift toward β-oxidation of fatty acids (Bruss et al., 2010). This metabolic shift has also been argued to be the main reason for the lower levels of oxidative (ROS) stress in subjects under CR (Speakman and Mitchell, 2011). The increase in metabolic activity of white adipose tissue (WAT) under CR is accompanied by an upregulation of key lipid enzymes and a browning of the white fat (Bruss et al., 2010; Fabbiano et al., 2016). The decrease in amount of WAT under CR also lowers levels of leptin and increases levels of adiponectin (Hardie, 2011). The decreased production of ATP (higher AMP:ATP ratio) and increased adiponectin signaling will both induce AMP-activated protein kinase (AMPK) (Hardie, 2011), which will depress the anabolic drive by inhibition of mTOR (*idem*). mTOR (mammalian/mechanistic target of rapamycin) is a key protein of the TORC complex (TORC1/2) regulating cell growth by promoting protein translation and inhibiting the FOXO family of transcription factors (*idem*). In addition to inhibiting mTOR through inducing AMPK and decreasing insulin/IGF-1 signaling, CR induces also another family of proteins, the sirtuins (Sirt 1-6) (Guarente, 2011). The sirtuins [nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases] are metabolic sensors that stimulate mitochondrial biogenesis, lower oxidative stress, inhibit mTOR and induce both FOXO and autophagy-lysosomal activity, reduce oxidative stress and DNA damage, and increase p53 activity (*idem*). Thus both actions by the sirtuins and the AMPK-driven inhibition of mTOR will activate the FOXO family of transcription factors, paving the way for increased regulated proteolysis through the ubiquitin-proteasome system (UPS) and increased degradation of proteins, molecular complexes, and organelles through the autophagy-lysosomal pathway conferring a more rapid turnover of cellular constituents (Martins et al., 2016; Maiese, 2021). FOXO proteins also upregulate cellular oxidative defense and have an inhibiting function on proteins involved in cell cycling (*idem*). Combined, the CR-mediated decrease in oxidative stress, increased biogenesis of mitochondria, and accelerated turnover of cellular constituents may slow the wear and tear that generates the cumulative build-up of unrepaired damage during aging. Trials with chemical compounds that show the promising extension of lifespan in model organisms are all partial mimetics of CR and targets mTOR (rapamycin, Miller et al., 2014), AMPK (metformin, Weiss et al., 2018), or indirectly both sirtuins and AMPK by inhibiting mitochondrial function (resveratrol, Price et al., 2012).

Trials with CR on long-lived species such as primates (Colman et al., 2009; McKiernan et al., 2011) and humans (Most et al., 2017) remain limited, for obvious reasons. However, those to date show promising outcomes, suggesting that CR mediates its effects through the same evolutionary conserved mechanisms as described above for small rodents. Furthermore, several of the gene variants associated with human longevity (Partridge et al., 2018) are genes implicated in the beneficial outcome of CR, such as FOXO(3A), the growth hormone-IGF-1 signal transduction pathway, and proteins involved in cell cycling (Guevara-Aguirre et al., 2018).

SARCOPENIA

What Is Sarcopenia?

A conspicuous trait of the aged phenotype is the reduced function and mass of skeletal muscles, which became a clinical entity (Evans, 1995) coined sarcopenia in 1989 because of the dramatic increase in incidence and prevalence brought about by the demographic changes of the 20th century (see above). Sarcopenia was recognized by the WHO (World Health Organization (WHO), 2000) in 2000 as a major threat to independence and as a risk factor for multiple morbidities associated with advanced age (**Figure 3A**) and targeted because it may be modifiable by rational lifestyle interventions (Roubenoff, 2000; **Figure 3B**). As mentioned above, sarcopenia is now a disease entity with an ICD-10-CM code (M62.84). Later, sarcopenia became a corner stone of the clinical concept "frailty" introduced to define more broadly the vulnerability of old individuals (Roubenoff, 2000). As a clinical entity, sarcopenia needs a rigorous operational definition to facilitate the coherent design of epidemiological,

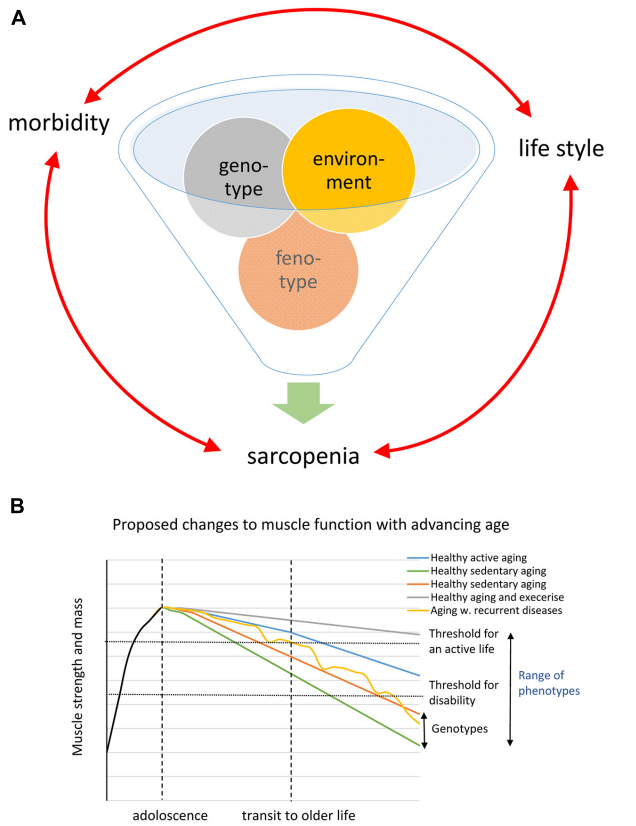

FIGURE 3 | (A) Schematic of the inferred interaction genotype and environment generating the phenotype. **(B)** Shows a schematic drawing based on the WHO diagram illustrating different possible trajectories caused by genotype and phenotype in the loss of muscle strength and mass during aging.

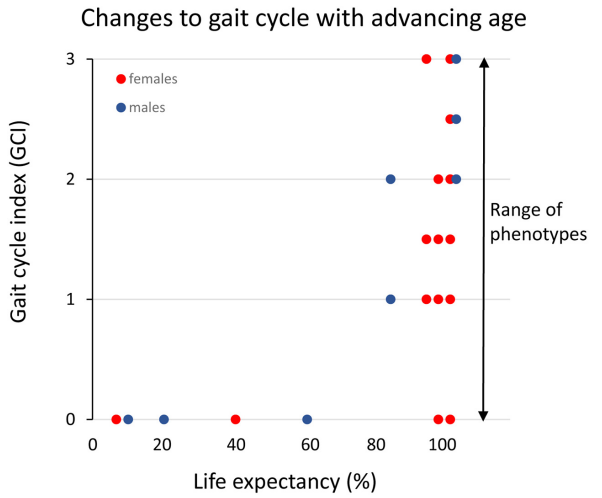

FIGURE 4 | Clinical assessment of gait cycle aberrations in small rodents across the median lifespan in percent (data replotted from Altun et al., 2007 and unpublished data). The different gait cycles phases and the coordination between limbs are preserved (index = 0) until the final 20% of the life-span when these functions progressively deteriorate. However, note that there is a large variance among individual rats in the progression of this aging-induced impairment and that some very old rats still have an intact gait cycle.

clinical, and experimental studies as well as assessment of interventions. However, this issue was not addressed in full until about a decade ago. The two most well-known sets of operational criteria for sarcopenia today are the EWGSOP2 (Cruz-Jentoft et al., 2019) and the FNIH (Studenski et al., 2014). Although these two definitions differ to some extent, they both define primary sarcopenia (in aging of healthy subjects) as the graded loss of skeletal muscle mass, decrease in muscle strength (most commonly static handgrip strength), and performance (usually gait speed with a set distance of 400 m) (*idem*). The latter is highly relevant because of the claimed link between sarcopenia and high risk of (serious) fall accidents among the elderly. While gait is a locomotor behavior relying on timed integration of sensory input and motor command is a clinically relevant assessment of muscle coordination and function; critique of the concurrent definitions of sarcopenia [as will be discussed below] has been raised in particular toward the emphasis on muscle mass but also the use of static muscle strength rather than dynamic muscle force measures (McGregor et al., 2014; Tieland et al., 2018). Furthermore, gait translates well to corresponding read outs in experimental animal models of sarcopenia (Altun et al., 2007; Fahlstrom et al., 2012; Bair et al., 2019; **Figure 4**).

In the literature, the incidence, prevalence, and grading of severity as well as the clinical progression of the condition varies substantially, but studies are reasonably coherent on the evidence that primary sarcopenia becomes clinically assessable during the 5th–7th decades of life with a prevalence of >5%, with an annual progression of 0.5–2% per year (using above mass and strength criteria), and an accelerated progression beyond 70 years with a prevalence of >10%. Confounding factors, apart from differences in operational definitions and the relevance of the metrics used to define and grade sarcopenia, are problems associated with study design, either cross-sectional or longitudinal (Reid et al., 2014; Skoglund et al., 2020), and whether the studied populations are representative of the general population (Mitchell et al., 2012).

Concepts of Sarcopenia

There is still an ongoing debate about whether sarcopenia has a neurogenic or myogenic origin (Gutman and Hanzlikova, 1972). At more advanced stages of the disease, a multitude of alterations are present in the peripheral innervation as well as in the target tissue, which makes it quite challenging to determine cause and effect.

Neurogenic Mechanisms of Sarcopenia

Loss and re-organization of motor units during aging and impact on muscle mass, force, and power

A century ago, Liddell and Sherrington (1925) established that the grading of muscle force is by the recruitment of sets of myofibers, each innervated by one α-motoneuron (MN)—collectively referred to as a motor unit (MU). Subsequently, MUs were categorized according to differences in mechanical properties. At one end of the spectrum, are the slow twitch

fatigue-resistant MUs (type S) with type I myofibers and at the other end, fast twitching rapidly fatigable MUs with type II (IIb; type FF) myofibers (Burke, 1999). Intermediate types include the transitional forms that are fast twitching but resistant to fatigue with type II (IIa and IIx; types FR and FI) myofibers (*idem*). Small muscles comprise usually proportionally fewer (<100) MUs than a large muscle (up 700–800 MU) (Feinstein et al., 1955; Gath and Stålberg, 1981). The MU populations hold MUs with a highly variable number of myofibers (from <10 up to >1000, depending on muscle type). S-type MU may have 30–120 fibers while fast fatigable (FF) MUs have several hundred up to more than one thousand fibers (Feinstein et al., 1955). Regardless of MU type, the fibers of an individual MU are all of the same type and for mechanical reasons dispersed across a larger territory of the muscle cross-section (Edström and Kugelberg, 1968; Burke, 1999; Larsson et al., 2019). In the seminal paper by Buller et al. (1960), evidence was provided that the innervating MNs exert a powerful influence on the phenotype of myofibers (being slow or fast twitch). This organizational design where a skeletal muscle contains a pool of MUs with different mechanical properties including size, provides for a fine tuning of muscle force output adequate and economical for each locomotor task. As recorded from live animals (Hennig and Lomo, 1985), everyday motor tasks including walking and stairclimbing with long duty cycles involve recruitment of mainly S type MU, while moving with higher speed and running expand the recruitment more toward fast fatigue-resistant (FR) MUs and only in brief ballistic movements also the fast fatigable MUs are recruited in mixed muscles (Hennig and Lomo, 1985; Burke, 1999). Thus, in contrast to the S type MU, duty cycles are brief and quite infrequent among fast and fatigable MUs (*idem*).

Recordings from aged animals and humans alike, consistently show that the number of MUs decreases with advancing age but importantly, also that MUs are re-modeled. The latter is due to a re-innervation of denervated fibers similar to the process that is invoked by accidental or experimental severance of the muscle innervation and referred to as collateral re-innervation (McNeil et al., 2005; Power et al., 2016). Based on changes in peripheral nerve conduction velocity, latency, amplitude, and time course of the myofibers response recorded by EMG and muscle histology, available data show that aging-induced atrophy preferentially but not exclusively affects type II myofibers (Larsson, 1978; Lexell et al., 1983). Type II fibers display additional signs of denervation by re-expressing the $Na_v1.5$ sodium channel, the CHRN-γ subunit, and embryonic and neonatal isoforms of myosin. If such a fiber is re-innervated by an S type MN the fiber will co-expresses slow MyHC (hybrid fibers) and is spatially clustered to nearby slow fibers (fiber type grouping) enlarging S type MUs at the expense of functional F type MUs (Rowan et al., 2012; Piasecki et al., 2018). Slow muscles (e.g., m. soleus) and type I myofibers are, however, not spared from sarcopenia (Purves-Smith et al., 2012).

As the sarcopenia process progresses, the collateral re-innervation mechanism will be exhausted and the loss of force and mass will become clinically overt (McNeil et al., 2005; Kawabuchi et al., 2011; Piasecki et al., 2018). Thus, clusters of severely atrophic—probably completely dysfunctional—fibers are a frequent observation in advanced stages of sarcopenia (Altun et al., 2012; **Figure 5**). Combined, the alterations of the MU pool composition and size will affect several qualities of motor behavior that should be possible to assess in the aging subject: a decline in power and force (preferential loss of fast MUs), decrease of maximum speed of contraction (increasing predominance of S MUs), and because of the increasing size of S MUs (through collateral re-innervation), fewer fine-tuned adjustments of locomotor tasks (McNeil et al., 2005; Piasecki et al., 2018). A distinctive difference between the denervation taking place in sarcopenia during normal aging and that occurring in motor neuron diseases like ALS, is the absence of fibrillations in sarcopenic muscles; suggesting that the denervation and re-innervation processes may be different (Wohlfart, 1957).

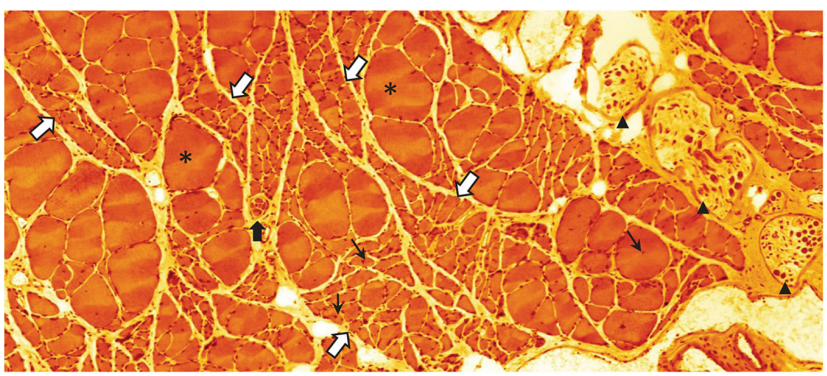

FIGURE 5 | Eosin-hematoxylin stained sections through m. soleus of a 30-month-old rat (average life expectancy for this strain) suffering from severe sarcopenia. Bundles of normal appearing and occasional extremely hypertrophic (asterisks) myofibers are intermingled with bundles of severely atrophied myofibers (white arrows with blue border). Normal, atrophic, and hypertrophic myofibers also have nuclei with a central location (small black arrows) indicating that these nuclei have recently been recruited to the myofiber from the satellite cell pool. This histological appearance is very similar to what can be seen following traumatic or accidental injury to the muscle nerve. Black arrow heads point to intramuscular branch profiles of the muscle nerve. Notice the low density of large axon profiles. The black arrow with white border indicates a muscle-spindle, a sensory organ that records the tension of the muscle (unpublished image by BU).

Changes in the peripheral innervation during aging and drop out or dying back of MNs

Skeletal muscles receive sensory innervation and both somatic and autonomic efferent innervation. Skeletal muscle function relies on a preserved innervation but degenerative changes in the peripheral nervous system are wide-spread and progressive during aging (reviewed in Cowen et al., 2005). These include loss of innervation, loss of peripheral sensory receptor organs, axonal atrophy and dystrophy, and aberrations to the myelin. In many respects, these changes are similar to those observed in the central nervous system albeit the environmental distinctions between these two compartments (Burek et al., 1976; Ramirez-Leon et al., 1999). Myelin de-and dys-myelination during aging seem to cause a tissue loading of non-disposed myelin breakdown products, which drives inflammation through interferon-γ/TNFα signaling (Kullberg et al., 1998; Edstrom et al., 2004; Safaiyan et al., 2016). Myelin aberrations may also impact axon impulse conduction velocity and the conduction safety factor making impulse traffic less predictable and well-timed (McNeil et al., 2005). Due to the structural alterations of the myelin sheaths and the axonal atrophy/dystrophy, it is challenging to assess losses of specific subsets of axons in the peripheral muscle nerve during aging. In addition, there is ample evidence that the sensory feedback is likewise affected (reviewed in Cowen et al., 2005) and studies on aging-associated changes in peripheral sensory/motor nerves and muscle force seem to be in agreement (Strotmeyer et al., 2009; Ward et al., 2015).

Due to all the technical difficulties associated with unbiased counting of MNs in the spinal cord (e.g., in humans, cadaver examination using only anatomy-based identifiers), the estimation of MN numbers lost during aging varies considerably between studies (<15% up to 30–50%) but it is likely on the modest side of the range and in-line with assessment of loss of MN axons in peripheral nerves (Kawamura et al., 1977; Tomlinson and Irving, 1977). Results obtained by using a retrograde transported tracer taken up by motor axons in aged small rodents with sarcopenia suggest that up to 30% of the MNs are lost, or have impaired axonal transport and, further, that this decrease is more accentuated in the lower than the upper limb (Hashizume and Kanda, 1995). This is consistent with observations that loss of strength and power is more conspicuous in muscles of the lower compared to the upper limbs. Evidence that MNs lose contact partially or in full, with the target myofibers and, in parallel, other MNs that engage in axonal sprouting to re-innervate the vacated myofibers have support in the phenotypic changes observed in the cell bodies of spinal cord MNs of small rodents during aging (Johnson et al., 1995).

The stronger susceptibility to aging of fast twitch over slow twitch MUs may relate to the difference in number of target myofibers (up to 20x higher). Thus, the extensive and elaborate terminal axon arborization of fast MUs may generate a correspondingly larger burden for maintenance. Furthermore, their infrequent use in everyday locomotor activities may add to this. Alluding to the burden of maintenance due to size and complexity, available evidence suggests that distal projecting MNs suffer more from aging than those that project proximally. It should be recognized that the long peripheral trajectory of an MN to distal limb MUs may well exceed 1 m, making it more vulnerable to mechanical wear and tear along its path (Thomas et al., 1980).

The neuromuscular junction (NMJ)

The NMJ is a synapse, perhaps one of the most well-studied (Katz and Miledi, 1965), and the components of the NMJ, its development, and normal function have been discussed in detail elsewhere (for recent reviews see Tintignac et al., 2015; Willadt et al., 2018). In short, the action potential (AP) of the motor axon is transmitted through the release of acetylcholine (ACh) quanta acting on post synaptic nicotinic ACh receptors (CHRNs) are pentamers assembled from four different types of subunits (α, β, δ, and ϵ), to generate an excitatory end plate potential (EPP) which will initiate the contraction process of the myofiber. The signal is disrupted by enzymatic cleavage of the transmitter and re-uptake by a transporter to the presynaptic ending. In between APs, there is a leakage of ACh quanta generating miniature end plate potentials (mEPP) (*idem*). The importance of the mEPPs in between APs was not understood until it was shown that these mEPPs as well as leakage of ACh quanta from disconnected axons serve the purpose of maintaining the integrity of the end plate and facilitate re-innervation of vacated myofibers (Vyskocil et al., 1995). In addition to ACh, motor axons secrete agrin and α-calcitonin-gene-related peptide (CGRP). Agrin instructs the myofiber by interaction with the muscle-derived LRP4/MuSK complex to assemble and concentrate CHRNs into the folded subsynaptic sarcolemma and to build the complex sub-sarcolemmal molecular structure of the end plate. The role of CGRP is still unclear but has been proposed to maintain the end plate in situations of denervation (Machado et al., 2019) and to increase ACh quantal size (Gaydukov et al., 2016); furthermore, CGRP seems to be preferentially expressed by the less frequently recruited MNs of fast MUs (Piehl et al., 1993) and is massively expressed by MNs disconnected from their target or engaged in collateral re-innervation (Johnson and Duberley, 1998). Although convincing evidence is missing, CGRP may have a function similar to the spontaneous quantal release of ACh, i.e., to preserve the end plate integrity during periods of disuse or denervation.

Conversely, the target muscle produces trophic factors (e.g., CNTF, GDNF, NGF, BDNF, and more, reviewed in Kawabuchi et al., 2011; Mrówczyński, 2019; Stanga et al., 2020) that are taken up and retrogradely transported to the MN cell body. These neurotrophins are non-redundant for MN survival during development (Hollyday and Hamburger, 1976) but are continuously expressed throughout life and, as suggested by experimental data, may then serve different functions such as potentiation of transmission and frequency of mEPPs (Gaydukov et al., 2019) (BDNF), maintenance and re-innervation (Stanga et al., 2020) (GDNF), and MN maintenance (BDNF, GDNF; *idem*). Assessment in aged MNs (and sensory neurons) of the expression of target-derived neurotrophic factor receptors of the NGF family and the expression level of these neurotrophic factors

in the target tissue points toward decreased trophic support in old age (Edström et al., 2007). In contrast, GDNF, which has also been implicated in maintenance and regeneration of the NMJ, is increased in aged muscles, and the cognate tyrosine kinase receptor c-ret is upregulated in the innervating MNs (idem). Myofiber and/or Schwann cell-derived GDNF also interacts with the GRF-1α receptor and NCAM in the myofiber, and NCAM has been proposed as an interaction partner in collateral re-innervation processes (Kawabuchi et al., 2011). Another player at the NMJ is the population of terminal Schwann cells (TSCs). Ablation of this cell population impairs innervation, collateral re-innervation, and removal of axon endings (Nishimune and Shigemoto, 2018). TSCs provide a leading edge that aid axon sprouts to reach the site of a vacated end plate and also appear to have a protective function on the NMJ (Love and Thompson, 1999; Kawabuchi et al., 2001; Kang et al., 2019). Some data indicate that aging impairs these functions (Love et al., 2003) and that the integrity of the NMJ depends on activity (Love and Thompson, 1999; Soendenbroe et al., 2020). When engaged in collateral re-innervation, both the growing sprout and the TSC express the growth-associated protein 43 (GAP43) by which they can be identified. MNs in the aged rodent spinal cord contain highly increased levels of both CGRP and GAP43, suggesting that a large fraction of the MNs are involved in the denervation and re-innervation processes of the target muscles (Johnson et al., 1995). Moreover, the concurrent regressive impact by aging on autonomic efferent innervation (Cowen et al., 2005) may also adversely impact NMJ transmission since catecholamines released by autonomic nerve endings via parasynaptic action can modulate both the EPP and the mEPP at the NMJ and is used as an adjuvant therapy in conditions of myasthenia gravis (Vanhaesebrouck et al., 2019).

With advancing age the NMJ displays multiple molecular and structural alterations (see above) including the removal of pre-terminal axons and disintegration of end plates (Kawabuchi et al., 2001; Rowan et al., 2012). There are conflicting opinions about what instigates the denervation process; is it due to impairment of the motor axon or the target myofiber? However, if we consider that many myofibers are successfully re-innervated by sprouting axons until they clinically manifest as sarcopenia, the natural history argues strongly against a primary myofiber origin. In sarcopenia, the motor axon ending is removed from the NMJ by TSC, however, the signal triggering this event remains unknown (Yin et al., 2004). Subsequently, the vacated end plates may become re-innervated by collateral sprouting from nearby intact axons through guidance by the TSC. While we know that this happens, we do not understand the non-redundant signals in-between the denervated fiber, the TSC, and the axon sprout. Unraveling the crosstalk that drives these events need to be prioritized because they all represent potential targets for intervention. Leads may be provided from the removal during development of poly-innervation where axonal expression of neuregulin1-III appears to be a sufficient signal for removal of the axons (Lee et al., 2016). At later stages, the re-innervation process becomes insufficient possibly owing to limitation to the enlargement of an MN's terminal field evident also in young adult subjects (Rochel and Robbins, 1988).

Contrary to the consistency between studies on structural and molecular changes engaging the components of the NMJ, recordings of the transmission at the NMJ show contradictory results, with substantial variation within and between muscles and between species (reviewed in Willadt et al., 2018). Overall, there is not coherent evidence for transmission aberrations at the NMJ during aging. To some extent this may be due to unavoidable bias in the sampling of the NMJ to record from and thus that NMJs intact enough to lend themselves to electrophysiological examination are not seriously altered even at advanced age.

From the above it may be concluded that a large body of evidence indicates that disrupted innervation and concurrent re-innervation are present at an early preclinical phase of sarcopenia and that this re-organization due to drop out of axonal branches primarily impacts the integrity of the large fast twitch MUs. These changes instigated by the removal of dysfunctional endings take place in parallel with aging-caused aberrations of the peripheral axons and their myelin sheaths. Axon dystrophy and atrophy represents the most prominent signs of normal neuronal aging in both the central and peripheral nervous system (see above). It is therefore of considerable interest that genetic ablation of the FOXO transcription factors in mice accelerated aging-induced axonal degeneration and was accompanied by increased mTOR activity while this acceleration could be impeded by rapamycin (mTOR inhibitor) (Hwang et al., 2018). Recent work suggests that sirtuins, mTOR, and FOXOs may have significant roles in neurodegenerative diseases as well (Maiese, 2021). It is clear that we still have considerable knowledge gaps of the denervation process of myofibers and the underlying aging-induced degeneration of the motor axons. Furthermore, mapping of age of onset and pace of progression of sarcopenia to genetic polymorphisms (as FOXO3a, see above) is also missing. While awaiting progress in this research, it is encouraging that exercise, i.e., frequent use of the MUs, seems to slow down the aging of the connectivity between MNs and myofibers (Love et al., 2003; Soendenbroe et al., 2020). One contributing factor may be the higher levels of neurotrophic factors expressed in exercised over sedentary aged skeletal muscles (Mrówczyński, 2019; Stanga et al., 2020).

THE SARCOPENIC SKELETAL MUSCLE

Changes that take place in the skeletal muscle during aging have received comparatively more attention than muscle innervation, especially in studies of humans. The process that instigates sarcopenia is still the subject of debate (Gutman and Hanzlikova, 1972), at more advanced stages a multitude of alterations are present in peripheral innervation as well as in the target tissue, which makes it quite challenging to determine cause and effect. Most of our understanding of molecular and cellular mechanisms in myofibers and the muscle scaffold during aging stem from work done on laboratory animals. For a detailed account on myogenic mechanisms in the sarcopenia processes, we refer to some recent reviews (Larsson et al., 2019; Christian and

Benian, 2020; Pascual-Fernández et al., 2020). Briefly, early studies on sarcopenia (previously referred to as old-age-muscle-atrophy or senile-muscle-atrophy) in laboratory small rodents and humans revealed that aging is accompanied by a loss of myofibers, atrophy preferentially affecting fast twitch (type II) myofibers, and a fiber type grouping—features that increase with advancing chronological age. Further, myofibers in aged subjects display irregularities of the end plate, increased content of lipid droplets and of secondary lysosomes, and an increasing number of fibers that show a myosin expression pattern intermediate between type I and type II (hybrid fibers) (Lexell et al., 1983; Bowen et al., 2015; Larsson et al., 2019). Concurrent alterations in the amount of fat and connective tissue, vasculature, and thickening of the capillary basement membrane have been described indicating that the muscle scaffold is impacted by the aging process (McGregor et al., 2014; Larsson et al., 2019). On most of these variables, there is a good agreement between observations made in humans and small rodents. For obvious reasons, a major obstacle in human studies is the limitation of performing longitudinal studies and hence most studies use a cross-sectional design. This design does not take into account the individual variation in factors that are known to have a huge impact on sarcopenia, such as genetic make-up, socio-economic changes, physical activity, and medical history (Mitchell et al., 2012).

CHANGES TO THE REMODELING CAPACITY OF SKELETAL MUSCLES DURING AGING

In addition to the well-established age-related structural changes, another hallmark of the aged human muscle is a diminished muscle remodeling capacity. This will impact the possibility of the skeletal muscle to respond to age-associated changes in the innervation. An attenuated remodeling capacity is evidenced by the dissipating capacity to recover from immobilization-induced muscle mass loss with increasing age (**Figure 3**; Wall et al., 2013) and is further demonstrated by a diminished adaptation to both resistance and aerobic exercise with age. For example, it was recently reported that 12 weeks of heavy resistance training in very old men and women (age 83–94) had no effects on type II fiber area, satellite cell content, or myonuclear density, supporting the observation of reduced plasticity at advanced ages (Karlsen et al., 2019).

An important factor in this context is anabolic resistance, which refers to blunted protein synthesis in response to anabolic stimuli such as exercise and nutrients. Anabolic resistance has been attributable to attenuated TORC1- dependent and TORC1- independent mechanisms (e.g., MAPK/ERK pathways) and includes effects on ribosomal biogenesis (Cuthbertson et al., 2005; Fry et al., 2011). Other mechanisms that may participate in the progressive attenuation of remodeling capacity with age is the accumulation of somatic mutations in satellite cells (SCs; myogenic stem cells) in human muscles (Franco et al., 2019). An accumulation rate of 13 somatic mutations per genome and year was reported to occur and these mutations were also detected in the adult myofiber (Franco et al., 2018). The latter observation suggests that, through SC fusion, mutations propagate into the mature multinucleated muscle fiber and impact skeletal muscle cell function. In addition, several groups have reported a reduced number of SC in aged muscle, especially around type II fibers, concurrent with a reduced proliferation and differentiation capacity of the SCs (Verdijk et al., 2014; Franco et al., 2019). A linear correlation between number of SCs and fiber size has been reported in aged individuals (Mackey et al., 2011; Franco et al., 2019), and a reduced number of SCs seems to be associated with diminished muscle remodeling capacity (Suetta et al., 2013). This is further underlined by evidence that depletion of SCs in animal models leads to an increase in the amount of fibrotic connective tissue and adipocyte infiltration, two hallmarks of the aged skeletal muscle (Franco et al., 2019). From a mechanistic point of view, a blunted activation of SCs in aged individuals is observed in parallel with an attenuation of Notch signaling, which is possible to reverse through an activation of this pathway (Carlson et al., 2009; Suetta et al., 2013). An increased expression of the cellular senescence marker p16 has also been detected in SCs in aged individuals compared to young individuals (Carlson et al., 2009; Suetta et al., 2013). Other well-established age-associated changes in the muscle scaffold include alterations of the cellular composition (infiltration of inflammatory cells, adipocytes, and fibroblasts), as well as increased amounts of connective tissue and intra-myocellular lipids. This affects the microenvironment and the crosstalk between cells (Latroche et al., 2017). Moreover, the intra-myocellular lipids and their derivatives have been shown to induce mitochondrial dysfunction and increase generation of reactive oxygen species as well as the development of the age-related chronic low-grade systemic inflammation (Kalinkovich and Livshits, 2017). Changes in the micro-milieu are plausible mechanisms in both the dissipation of muscle function and the decreased ability to regenerate in response to various stimuli (Hiona and Leeuwenburgh, 2008; Pillon et al., 2013). Moreover, as observed in SCs from older individuals, changes in the micro-milieu could also introduce chromatin alterations and DNA methylation changes, which may add to changes induced by somatic mutations (Bigot et al., 2015; Franco et al., 2019).

By using participants of the population-based Uppsala longitudinal study of adult men (ULSAM) cohort (Skoglund et al., 2020), changes in skeletal muscle expression in a set of candidate genes involved in muscle remodeling was analyzed longitudinally. At age 70, an activation of the pathways associated with UPS was observed but this response was significantly attenuated by age 88. The gene expression pattern at 70 years was defined as beneficial since the subjects maintained their muscle fiber histology and appendicular lean body mass until advanced age. This is consistent with observations made in small rodents, where it was proposed to be related to an initial increased remodeling due to denervation/re-innervation followed by incapacitation at advanced age resulting in an increased number of permanently denervated myofibers (Altun et al., 2012). A similar response has recently been demonstrated in human skeletal muscle (Piasecki et al., 2018).

HOW DO WE COUNTERACT SARCOPENIA AND EXTEND THE HEALTHSPAN?

The literature clearly shows that both genetic and environmental factors are closely intertwined and together will define the individual's phenotype at different ages. Both the onset and the progression of sarcopenia are highly variable between subjects owing to genetic and environmental/life-style factors (Degens and Korhonen, 2012; Bann et al., 2015; Khanal et al., 2020; **Figures 3A,B**). Muscle strength and the amount of muscle mass in adult life are to a large extent due to genetic factors; heritability estimates of >50% are not uncommon (Zempo et al., 2017). A number of genes such as ACTN3, ACE, and VDR have been associated with skeletal muscle phenotypes (Pratt et al., 2019; Singh and Gasman, 2020). GWAS studies have linked genetic polymorphisms to both muscle growth/lean body mass and [handgrip] strength. However, linkage to establish their importance to skeletal muscle-trait variation has generally been identified as small, especially with advancing age (Carmelli and Reed, 2000). Notably, while some of the so-far identified genes associated with the above metrics have a function in neuromuscular transmission and neuronal plasticity, most of the strongly associated genes have a hitherto unknown function in skeletal muscle (*idem*). A problem with these linkage/association studies is that they address metrics such as static handgrip strength and lean body mass, while the association between genes/genetic polymorphism and the age-of-onset and pace-of-progression of sarcopenia remains to be addressed.

Since population-based studies clearly demonstrate that environmental factors such as nutrition together with neuroendocrine changes are associated with loss of both muscle function and mass as well as the tissue integrity of skeletal muscle during aging, many intervention regimens have been targeted to correct or override these changes (Larsson et al., 2019). Physical activity in adult life will, in contrast to a sedentary life-style, preserve muscle function better at old age in both humans and rodents (Trappe et al., 1996; Bann et al., 2015; Drey et al., 2016; **Figure 3**). However, sarcopenia also affects top athletes (Trappe et al., 1996; Gava et al., 2015; Drey et al., 2016; Piasecki et al., 2016; Ganse et al., 2020) and analysis of data on track and field records (both cross-sectional and/or longitudinal records) of master athletes discloses that there is steady decline in power of about 1% per year between 30–70 years of age. At more advanced age, most datasets show an accelerated drop (1–2% per year) (Trappe et al., 1996; Gava et al., 2015; Ganse et al., 2020) and the slope of the decrease is steeper for ballistic power demanding sports than running sports (Gava et al., 2015). This re-emphasizes that recruitment/contraction speed and coordination may be more sensitive to aging than strength and mass and, further, that once sarcopenia becomes clinically overt the process has probably been ongoing for decades but clinically compensated for by intrinsic mechanisms. The significance of physical activity is, however, underscored by the fact that the loss of muscle mass and function is accelerated during periods of immobilization (Wall et al., 2013). This is further aggravated by the diminished muscle remodeling capacity of aged individuals in the recovery following mobilization-induced muscle loss (Suetta et al., 2013). Even though the response to exercise is to some extent blunted at older age, it is still the best documented intervention strategy (Bao et al., 2020; Grgic et al., 2020). There are conflicting opinions concerning the benefits of strength vs. endurance training or if a combination of both tailored to the individual is optimal (Trappe et al., 1996; Mitchell et al., 2012; Bowen et al., 2015; Drey et al., 2016; Karlsen et al., 2019). Resistance-type training has the largest impact on skeletal muscle mass but aerobic exercise also attenuates aging-induced changes and it preferentially stimulates metabolic pathways such as oxidative capacity including mitochondrial density, structure, and function. Importantly, exercise increases insulin sensitivity by increasing sarcolemmal glucose transporter (Glut 4) levels, augmenting metabolic flexibility, and reducing the risk of development of insulin resistance (type 2 diabetes) (Biensø et al., 2015; Bowen et al., 2015; Cartee et al., 2016). Exercise also decreases fat infiltration, increases SC number, and appears to facilitate the maintenance of the NMJ integrity at advanced age (Valdez et al., 2010; Power et al., 2016; Franco et al., 2019). Furthermore, increased physical activity has positive effects on age-related diseases linked to secondary sarcopenia such as chronic heart failure and obstructive lung diseases (Coats, 1996; Bernard et al., 1998).

It could be argued that exercise, and especially resistance exercise, activates mTOR pathways and one of key changes identified by CR/DR models is a reduced mTOR activity which seems to attenuate the aging process. An interesting observation in humans by Phillips et al. (2013) that individuals with the greatest increase in lean mass gain following resistance training had a suppressed mTOR signaling over the training period. Moreover, the overall effects of exercise are largely coherent with the findings in the CR models, with increased PGC-1 expression and activation of AMPK (Egan and Zierath, 2013; Bowen et al., 2015). More knowledge is needed to understand how the activities of these signal pathways contribute to the aging process in skeletal muscle and the effects of counteracting exercise regimes. Importantly not all individuals respond to the same extent to exercise (i.e., high versus low responders) and meta-analyses provide evidence that very old individuals remain responsive to resistance-type training with increases in strength that occurs without any changes in muscle mass (Beckwée et al., 2019; Karlsen et al., 2019). The latter suggests that exercise-induced changes in advanced age may be more related to neuromuscular function and/or intrinsic contractility machinery than increase in muscle mass (Biolo et al., 2014). This may have implications for the design of physical activity programs for the elderly population.

SUMMARY AND FUTURE PERSPECTIVE

Sarcopenia is a facultative trait of the aged human. The underlying process of this disorder which becomes clinically overt during the 5th–7th decades of life may start as early

as during the 3rd decade as judged by decreases in muscle strength and power. We argue that sarcopenia has a neurogenic origin while in the clinically manifest disorder the innervating MNs, target myofibers, and muscle scaffold are probably components of a viscous cycle were it is challenging to decide on what is cause or consequence. We have pointed to gaps in knowledge, such as the crosstalk between motor axons, terminal Schwann cells, and myofibers in the denervation/re-innervation processes that lead to a loss of MU and muscle weakness during aging. To capture the early events of sarcopenia, we will need to modify the operational definition and introduce the concept of the pre-clinical phase that should include more functional metrics and validated biomarkers for susceptibility to develop severe sarcopenia.

It is clear that the rapidly changing composition of the human population will impact incidence and prevalence of diseases and aging-induced disorders and that closing the gap between healthspan and lifespan should be our next priority. To address this we must fill knowledge gaps in our understanding of the biology of aging including sarcopenia. Linkage studies of genetic polymorphisms associated with age-of-onset and pace-of-progression of sarcopenia are missing but warranted to shed light on the large difference in impact between individuals and why some respond to interventions while others do not.

We argue that preventive measures to intercept with biological aging are lagging behind efforts to treat manifest clinical outcomes of aging. To accomplish compliance with prescription of preventive measures, we need to identify reliable predictors to convince individuals to enroll in intervention programs that may be life-long.

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Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Neurocognitive Study for the Aging: Longitudinal Analysis on the Contribution of Sex, Age, Education and *APOE* ε4 on Cognitive Performance

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Objective: The effects of normal cognitive aging on executive functions (EF), Verbal Episodic Memory (VEM) and the contribution of age, sex, education, and APOE ε4 in a group of old Greek Cypriots across a five-year period were investigated.

Design: NEUROAGE, the first project on cognitive aging in Cyprus, is a prospective longitudinal study with a rolling admission process. Participants are assessed at baseline and retested every 24–30 months.

Subjects: 170 participants completed all three testing cycles; 86 men and 84 women with ages ranging between 60 and 88 years (mean = 73.21, *SD* = 5.84); education, 2–20 years (mean = 9.07, *SD* = 4.27).

Results: A Repeated Measures Multivariate Analysis of Covariance was conducted with one between-subject factor: sex; two covariates: age and education, while Time (time 1, time 2, time 3) served as a within – subject factor. Time did not have an effect on mini mental status examination in Greek (MMSE), EF or VEM. Also, sex had no effect on MMSE, EF and VEM. There was no time by sex interaction. Age and Education significantly predicted the EF performance, $F(1, 168) = 11.23$, $p < 0.05$; $F(1, 158) = 90.03$, $p < 0.001$ and VEM performance, $F(1, 171) = 17.22$, $p < 0.001$; $F(1, 171) = 61.25$, $p < 0.001$. Furthermore, there was a significant interaction effect between time and education, for EF, $F(2, 167) = 7.02$, $p < 0.001$. Performance of the *APOE* ε4 carriers did not differ on any of the above measures as compared to performance of non-carriers in this older adult group.

Conclusion: Cognitively healthy adults maintained overall cognitive performance across the five-year period. Male and female participants performed similarly and the pattern of change over time was similar across the two sexes. Education was predictive of VEM and EF performance across time. Furthermore, those with higher education maintained higher levels of EF performance. *APOE* results did not differentiate performance at baseline. Implications of findings are discussed.

Keywords: cognitive aging, aging, episodic memory, executive functions, cognitive trajectories, cognitive stability, STROBE, cohort

INTRODUCTION

The 2017 global action plan on the public health response to dementia, estimates that 47 million people worldwide have dementia and the prevalence is expected to rise due to increases in life expectancy (World Health Organization, 2017). A significant number of studies have been focusing on the pathogenesis of dementia and potential protective factors. The present study is part of a systematic effort to describe and characterize cognitive changes associated with healthy aging using a longitudinal design.

Research indicates that the trajectory of cognitive change varies for different cognitive functions (Schaie, 2005). Changes in cognitive performance associated with aging are probably a result of neurobiological processes that occur during the lifespan. Prefrontal cortical areas, inferior temporal lobe areas and the hippocampus sustain the greatest diminution of neuronal loss, blood flow, and shrinkage (Kramer et al., 2007; Rajah et al., 2010). While some studies show a decline in cognitive functions such as episodic memory (Lundervold et al., 2014), verbal fluency (Kosmidis et al., 2004) and mental flexibility (Wecker et al., 2005; Thomas and Kunzmann, 2014), with increasing age, other studies report an age-related advantage in cognitive abilities such as general and semantic knowledge (Hedden and Gabrieli, 2004). The latter, rely on knowledge and information acquired over time to form the theoretical construct of crystallized intelligence which is resistive to the clinical manifestations of cognitive aging (Giogkaraki et al., 2013).

The present study is part of our systematic research program on the Neurocognitive Study for the Aging (NEUROAGE). NEUROAGE is the first longitudinal study on cognitive aging in Cyprus. It explores modifiable and unmodifiable factors that contribute to cognitive health in the Greek-Cypriot population of Cyprus with its unique social-cultural, linguistic and genetic characteristics (Constantinidou et al., 2012a, 2015; Giogkaraki et al., 2013; Demetriou and Constantinidou, 2018). The present study is the first report on the longitudinal data from the NEUROAGE study.

Specifically, we investigated the stability of performance across a five-year period on two neuropsychological domains – verbal episodic memory (VEM), and executive functions (EF), two-well studied cognitive domains known to be affected by cognitive aging (Lezak, 2012). Most importantly, we explored the potential contribution of important demographic and genetic factors previously reported in the literature, namely sex, age, education, and *APOE* in cognitive performance (Constantinidou et al., 2014; Caselli et al., 2015).

VEM and EF are not completely independent processes. Studies with cognitively healthy individuals indicate that deficits in EF have an indirect impact on memory performance (Spaan, 2015). Theoretical models of working memory (WM) posit a central executive, or a common attentional control mechanism similar to the central executive mode of WM proposed by Baddeley (2000). Executive functions could be viewed as a supervisory system that is involved in the coordination and control of goal-directed behavior, including WM (Constantinidou et al., 2012b; Constantinidou and Thomas, 2017).

Some studies provide evidence that VEM declines in a linear fashion as a function of age (Park et al., 2002; Lundervold et al., 2014) starting at the age of 30 (Constantinidou et al., 2014). In contrast, others suggest a later start, between 65 and 70 years (Rönnlund et al., 2005). Methodological factors such as sample size and statistical power issues, longitudinal vs. cross-sectional designs, and complexity of WM tasks may in part account for these differences.

EF is a multidimensional construct incorporating planning/initiation, execution, self-regulation/monitoring and effective performance (Lezak, 2012). EF activities dependent on speed of processing and strategic planning are more sensitive to aging (Treitz et al., 2007). Age-related changes in EF can have a direct impact on memory performance because of their important role in implementation of encoding and retrieval strategies (Constantinidou et al., 2012b; Lezak, 2012).

In addition to age, the present study explored the contribution of education to cognitive aging across time. Specifically, existing research suggests that years of formal education independently affect cognitive performance in both crystallized (i.e., vocabulary and semantic knowledge) and fluid intelligence tasks requiring online processing (i.e., visual construction, verbal fluency and WM; Meguro et al., 2001; Bosma et al., 2003; Stern, 2006). Higher educational level mitigates cognitive decline in older adults without dementia because it promotes efficient cognitive processing and better cognitive performance (Bosma et al., 2003).

While higher education may result in improved cognitive performance in cross-sectional studies, it is not clear whether it safeguards cognitive stability over time. Some longitudinal studies found no evidence to support the notion that years of education moderate the decline of WM and EF (Zahonde et al., 2011) as measured by verbal learning, long-term memory, set shifting, and semantic fluency tasks (Van Dijk et al., 2008; Cadar et al., 2017). These results support the passive hypothesis of cognitive reserve in which older people with higher education continue to perform at a higher level than people with lower education; however, the slope of change in performance, is not influenced by education (Ritchie et al., 2016).

The divergence between the reports on the exact contribution of education to cognitive decline, may be due to methodological differences in study design, and lack of sensitivity of the cognitive tests implemented. The present study addresses the above limitations by incorporating tests with demonstrated sensitivity to cognitive aging and a longitudinal design with three time points in order to minimize measurement error (Winkens et al., 2006).

In addition to age and years of education, other unmodifiable factors such as sex and genetic risk were taken into consideration in order to study cognitive changes across time. Research has shown that women have an advantage in VEM as compared to men, whereas potential male advantage in visuospatial memory is not well delineated (De Frias et al., 2006; Karlsson et al., 2015). Previous research has provided mixed evidence for sex differences in EF (Munro et al., 2012; McCarrey et al., 2016; Zaninotto et al., 2018). How sex differences interact with cognitive aging remains unclear. The present study explores

the contribution of sex as a biological construct in cognitive performance across time for both VEM and EF.

The APOE ε4 allele has been implicated as a primary genetic risk factor in pre-clinical and late-onset dementia and cognitive decline (Reas et al., 2019). The *APOE* gene expresses the protein apolipoprotein ε. There are three slightly different versions (alleles) of the *APOE* gene. The major alleles are called ε2, ε3, and ε4. The most common allele is ε3, which is found in more than half of the general population. Possession of the ε4 allele has been associated with poorer cognitive abilities and more rapid longitudinal decline in healthy older people, particularly in episodic memory (Shin et al., 2014; Marioni et al., 2016). The allele ε4 is considered a biological risk factor for cognitive decline.

Over the long history of life on this island, the Greek-Cypriot population has sustained several population effects that shaped the gene pool accordingly. Examples to this effect have been the many well documented founder effects pertaining to several diverse monogenic diseases, including neurological, endocrine and renal conditions (Pierides et al., 2009). Smaller or greater migration waves during the history of Cyprus as well as events relating to persecutions during wars, concerning a rather small population of just a few hundred thousands, favored genetic drifts, bottlenecks and founder phenomena, all having left behind their signs, either on the population island-wide, on selected geographic isolates, or even on selected religious minorities (i.e., Armenians, Maronites). The genetic characteristics of a relatively homogeneous population provide unique opportunities for health research. For example, the primary genetic risk factor for dementia, the APOE ε4 allele, is lower in Cyprus as compared to other European countries (Cariolou et al. 1995). Yet, dementia is the 7th leading cause of death and the primary cause of loss of health in Cypriots (World Health Organization, 2017).[1]

The present work implemented a prospective longitudinal design to examine the effects of normal cognitive aging on mental status, EF and VEM performance in a group of healthy old Greek Cypriots across a five-year period. The aim of the present study was also to examine sex differences in mental status and domain-specific cognitive performance (EF, VEM) in relation to both, levels of performance at baseline and across time. Finally, the present study aimed to determine the contribution of age, education and APOE ε4. The dependent measures implemented in this study were selected carefully in order to provide multiple data on VEM and EF, two neurocognitive domains that are sensitive to the effects of aging and brain pathology.

It was hypothesized that after in a five-year period, participants would present statistically significant signs of cognitive decline on VEM and EF. We also hypothesized that women would show an advantage in VEM performance, whereas no sex effects were expected for EF performance. Hypotheses regarding sex differences in patterns of change were less clear due to the lack of fully consistent findings in prior studies. We also hypothesized that age and education would influence the patterns of change across time. Finally, we hypothesized that the performance of individuals with the *APOE* ε4 allele would be significantly lower on VEM, EF and mini mental status examination in Greek (MMSE) as compared to non-carriers.

[1]http://www.who.int/countries/cyp/en/

MATERIALS AND METHODS

The NEUROAGE began in 2009 and is the first longitudinal project of cognitive aging in Cyprus (clinicaltrials.gov Identifier: NCT01481246). With a prospective design and a rolling admission process, the study recruits Greek Cypriot volunteers from community settings in compliance with the Helsinki Declaration and following approval by the National Bioethics Committee (ΕΕΒΚ/ΕΠ/2008/26). Participants are assessed at baseline and are followed up every 2 years (follow up time range = 24–30 months). The average time of follow up for Time 3 was 57 months.

Participants

Five hundred and six Greek Cypriot men and women were recruited from the NEUROAGE project who completed baseline evaluations. Thirty-two participants were excluded from the study due to a baseline diagnosis of MCI (n = 5), possible diagnosis of Alzheimer's dementia (n = 4), and a Mini Mental Score Examination under 24, without a clinical diagnosis (n = 23). Out of the 474 who met study inclusion criteria, 307 participants were retained for the second evaluation (Time 2) at 24–30 months after their baseline assessment; 185 participants completed the third evaluation (Time 3) at 24–30 months after Time 2. **Figure 1** is the STROBE diagram. A total of 170 study participants completed all three assessment cycles (Baseline, Time 2 and Time 3) and were retained in the analyses. Out of the 170 participants, 86 were men and 84 were women with ages ranging between 60 and 88 years (mean = 73.21, SD = 5.84). Education, 2–20 years (mean = 9.07, SD = 4.27). The demographic distribution (age, gender, and education) of the study was consistent with the Cyprus Government census data (Cyprus Statistical Service, 2011).

All participants were community dwellers residing independently at home. They were recruited from senior social clubs/centers, press-releases and outreach activities. The inclusion - exclusion criteria for all participants were the following: (1) Native Greek speakers, (2) males and females over the age of 60, (3) Good general health with no previous history of neurological disorder such as head trauma, stroke or neurodegenerative disorder, (4) No history of severe psychiatric or emotional disorder requiring hospitalization, (5) Baseline MMSE score of 24 or higher, and (6) Baseline Geriatric Depression Score of 6 or lower.

Procedures

Participants were administered a battery of neurocognitive tests (translated and adapted into Greek and previously used in other research studies), sensitive to detect age-associated cognitive

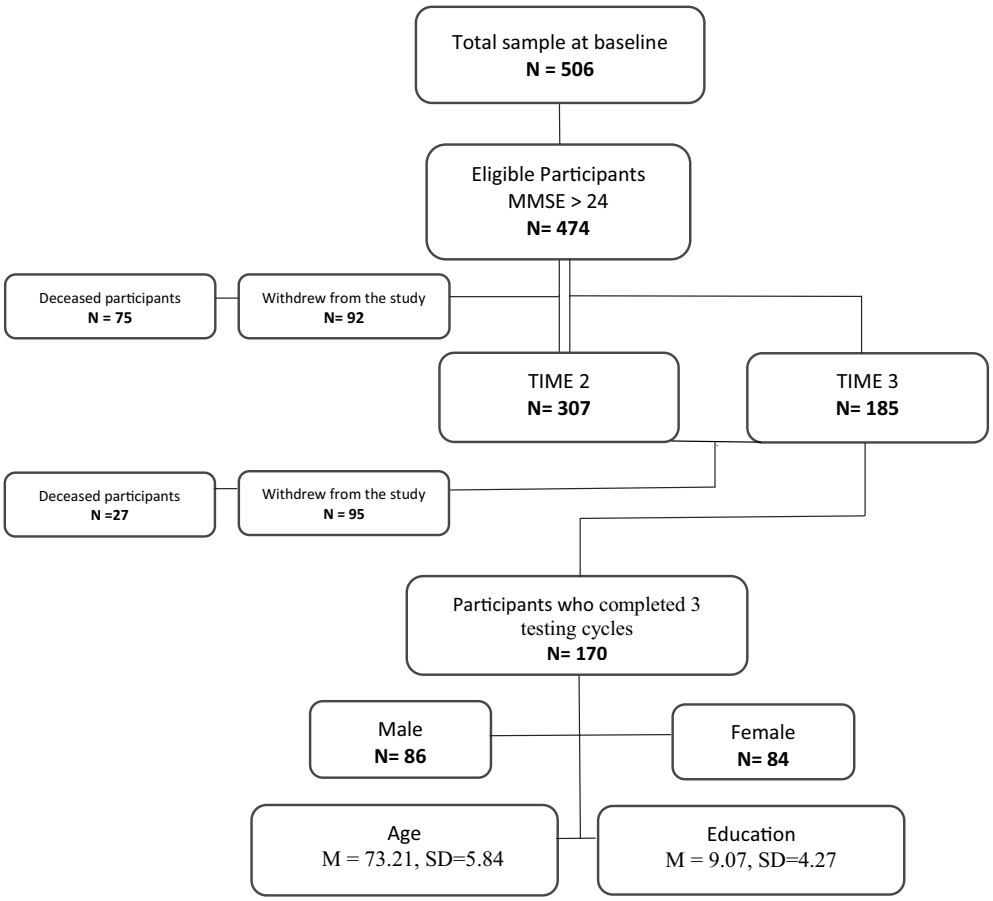

FIGURE 1 | STROBE flow diagram.

changes (Constantinidou et al., 2012a). Following are the measures included in the study.

General Cognitive Screening

- Mini Mental Status Examination in Greek was used a quick cognitive screening (Fountoulakis et al., 2000). A score under 24 (out of possible 30) was the cutoff for study inclusion to exclude participants with dementia, taking into account individuals with lower education (Constantinidou et al., 2012a).

Executive Functioning Tests

- Greek Version of the Trail Making Tests (TMT) A and B (Zalonis et al., 2008): The TMT provides information on visual search, scanning, graphomotor coordination, speed of processing, mental flexibility, and EF.
- Symbol Digits Modalities Test (Smith, 1982): It assesses complex scanning, visual tracking, and eye hand coordination within a time constraint. The total number of items correct in 90 s was used in the analysis.
- Greek Version of the Verbal Fluency. Two verbal fluency tasks: Animal recall and Words from the letter F were implemented, modified from the Controlled Oral Word Association Test (Kosmidis et al., 2004). The total correct items retrieved in 60 s for each test was used in the analyses.

Verbal Episodic Memory

- Hopkins Verbal Learning Test–Revised (Greek version; HVLT; Benedict et al., 1998); adapted in Greek by Constantinidou upon permission by the publisher (Constantinidou et al., 2015; Philippou et al., 2018). Learning trials (first trial and the total score of the three trials), delayed recall and delayed recall performance scores were used.
- Greek version of the Logical Memory Story A and B adapted from the Wechsler Memory Scale – Revised: Immediate and delayed recall administrations of short story material (Wechsler, 1997; Constantinidou and Ioannou, 2008).

APOE Genotyping

DNA was extracted from whole-blood samples from 308 study participants. The *APOE* molecular genotyping was carried out as described before in Cariolou et al. (1995). The genotypes of the ε2, ε3 and ε4 alleles were estimated by the gene counting method.

TABLE 1 | Factor loadings for each test to VEM and EF components.

	Verbal episodic memory	Executive functions
Items		
HVLT trial 1–3 total	**0.60**	−0.28
HVLT trial 4, Delayed recall	**0.66**	−0.21
Logical Memory – A, Immediate recall	**0.90**	0.07
Logical Memory – B, Immediate recall	**0.88**	0.04
Logical Memory – A, Delayed recall	**0.94**	0.06
Logical Memory – B, delayed recall	**0.85**	−0.01
Trail - A	0.08	**0.90**
Trail - B	0.00	**0.87**
Symbol digit modalities Test, correct	0.08	**−0.81**
Animals	0.32	**−0.47**
Words from F	0.00	**−0.63**

TABLE 2 | Genotypic and allelic frequencies of *APOE* polymorphism in the sample analyzed (N308).

***APOE* genotypes**	**Number of individuals (relative frequency)**
ε2 - ε2	2 (0.65)
ε2 - ε3	36 (11.69)
ε2 - ε4	5 (1.62)
ε3 - ε3	233 (75.65)
ε3 - ε4	30 (9.74)
ε4 - ε4	2 (0.65)
Allele	
ε2	45 (7.30)
ε3	532 (86.36)
ε4	39 (6.33)

The study population was in Hardy–Weinberg equilibrium (X^2 = 2.836, 3df; p = 0.418).

Data Scoring and Analyses

The primary interest of the current study was to determine the effects of time, on MMSE, EF and VEM abilities. Repeated Measures multivariate analysis of covariance was conducted, to determine statistical difference in MMSE, EF and VEM performance across Time (time 1, time 2, time 3). Sex was entered as a between-subject factor. Years of education and age, were entered as covariates in a continuous format. For a small effect size (0.30) and three repeated measures, 39 participants are required for an $\alpha = 0.05$ and power = 0.80 to detect significant effects. For the two group comparisons, 75 participants per group are required for an $a = 0.05$ and power = 0.80 to detect small group effects (0.25) and 35 participants per group for moderate group effects (0.60) across two variables (Stevens, 2009).

RESULTS

A principal component analysis (PCA) was run on the six memory and five EF tests in order to maximize reliability and generalizability, and minimize idiosyncratic aspects of single measures. The suitability of PCA was assessed prior to analysis. Inspection of the correlation matrix showed that all variables had at least one correlation coefficient greater than 0.3. The overall Kaiser-Meyer-Olkin measure was 0.89. Bartlett's test of sphericity was statistically significant ($p < 0.0001$), indicating that the data was likely factorizable. Principal component analysis revealed two components that had eigenvalues greater than one, explaining 56.5 and 11.9% of the total variance, respectively. Visual inspection of the scree plot indicated that the two components can be retained. An oblimin rotation was employed to aid interpretability. As seen in **Table 1**, two components were extracted, the first component was labeled VEM and the second component EF.

Following the PCA, composite scores were calculated for VEM and EF as they can be more powerful than their constituent parts for detecting change (Constantinidou et al., 2012a). Initially, scores were valenced so that a lower score indicated worst performance. Individual test scores obtained from each participant were transformed into z-scores (standard score) based on the mean of all the participants. Finally, the calculated standard scores from each test were averaged to derive a score for each construct.

APOE Gene Distribution

Table 2 is the genotype and allele relative frequencies in the sample analyzed. The distribution of *APOE* genotypes in the present study population was as follows: 76.5% were homozygotes for the ε3 allele, 11.6% were heterozygotes for the ε2 and ε3 alleles, 0.6% were homozygotes for the ε2 allele, and 9.74% were heterozygotes for the ε3 and ε4 alleles. Thirty-seven participants (12%) had at least one ε4 allele. For purpose of analysis, we categorized participants as *APOE* ε4 allele carriers and non-carriers.

Time, Sex, Age, Education and Mini Mental State Examination

The first analysis examined the effect of Time on MMSE while sex was conducted as between-subject factors and age and education included as covariates. Preliminary checks were conducted to ensure that there was no violation of the assumptions of normality, linearity, homogeneity of variances, and reliable measurement of the covariate. Time did not yield significant changes on MMSE performance, $F(2, 176) = 0.520$, $p = 0.59$, partial $\eta^2 = 0.006$, observed power = 0.13. None of the other main effects (sex; age; education) or multivariate interactions (time × age; time × education; time × sex) were significant. These finding indicate that participants exhibited no significant change on MMSE across the three testing periods and other important variables such as age, sex and education were not associated with changes on MMSE performance across time. The means and standard deviations displayed in **Table 3**, also indicate non-clinically significant changes across time for the individual tests and composites.

Relationship Between Age, Education, EF and VEM

Pearson correlation analyses were conducted to examine relationships between age, education, EF and VEM. Preliminary

TABLE 3 | Means and standard deviations on MMSE, Executive Functioning (EF) tasks and Verbal Memory tasks by Age and Educational Group by sex groups and time.

	Female			Male		
	TIME 1	**TIME 2**	**TIME 3**	**TIME 1**	**TIME 2**	**TIME 3**
MMSE	27.31	27.04	27.06	27.21	28.78	27.43
	(1.81)	(2.22)	(1.95)	(1.74)	(2.75)	(1.70)
EF	0.288	0.299	0.229	0.297	0.281	0.205
composite	(0.420)	(0.446)	(0.385)	(0.412)	(0.470)	(0.381)
TMT A	80.71	78.48	81.86	85.53	85.87	89.66
	(35.54)	(29.46)	(53.59)	(44.43)	(29.15)	(45.16)
TMT B	181.46	188.51	198.14	191.66	192.51	203.99
	(73.69)	(68.83)	(94.71)	(90.74)	(78.69)	(101.83)
SDMT	24.01	23.71	23.19	24.92	23.64	23.01
	(7.76)	(7.72)	(7.75)	(7.46)	(7.72)	(7.75)
Animals	10.50	10.95	10.47	11.05	10.43	10.63
	(2.67)	(3.18)	(3.12)	(2.96)	(3.11)	(3.49)
F Words	8.30	8.37	7.88	8.05	7.81	7.71
	(3.06)	(3.23)	(3.32)	(3.31)	(3.29)	(3.04)
VEM	0.184	0.230	0.210	0.171	0.087	0.078
composite	(0.648)	(0.691)	(0.738)	(0.667)	(0.656)	(0.623)
HVLT Trial	4.81	4.84	4.97	4.43	4.50	4.46
1	(1.58)	(1.59)	(1.37)	(1.66)	(1.73)	(1.77)
HVLT Trial	6.68	6.88	7.03	6.11	5.98	6.11
2	(1.81)	(1.93)	(1.34)	(1.81)	(1.93)	(1.97)
HVLT Trial	7.51	7.89	7.75	6.82	6.92	6.87
3	(2.10)	(1.98)	(2.24)	(2.06)	(2.23)	(2.10)
HVLT Trial	5.37	5.77	5.51	4.50	4.90	4.85
4	(2.84)	(2.56)	(2.72)	(2.82)	(2.91)	(2.62)
LMA	10.52	11.15	10.40	11.03	10.79	9.89
	(3.57)	(3.82)	(4.16)	(4.17)	(3.79)	(3.77)
LMB	9.00	9.21	8.89	9.01	9.23	8.93
	(3.41)	(3.64)	(3.80)	(3.97)	(3.59)	(3.18)
LM- A del	7.03	7.60	7.51	7.84	7.61	7.46
	(3.95)	(4.30)	(4.24)	(4.33)	(4.32)	(4.33)
LM- B del	6.54	6.94	6.77	6.70	7.10	6.34
	(3.82)	(4.04)	(3.93)	(4.68)	(4.39)	(3.79)

MMSE, Mini Mental State Examination; EF composite, Executive Functioning Composite consisted of TMT A and B, Trail Making A and B in seconds, SDMT, Symbol Digits Modalities, items correct in 90 s, Animals, Animals recall in 60 s, F Words, Letter F in 60 s; VEM composite, Verbal Memory Composite consisted of HVLT trials, Greek version of Hopkins Verbal Learning Test-Revised, trials 1–4, LM A&B, Logical Memory A&B, LM – A &B del.

analyses were performed to ensure no violation of the assumptions of normality, linearity and homoscedasticity. Results indicated that there were significant moderate-strong, negative correlations (Bonferroni $\alpha/k = 0.01$) between age, VEM and EF across the three time points, respectively. Additionally, education yielded in significant moderate-strong correlations with EF and VEM across the three time points. The association between education and VEM increased across time, whereas the association between education and EF decreased. **Table 4** depicts the correlations.

Time, Sex, Age, Education and Executive Functioning

The second analysis examined the effect of Time on EF while sex was conducted as between-subject factors and age and education included as covariates. No violations from preliminary checks were found (normality; linearity; homogeneity of variances; reliable measurement of the covariates). Time as main effect did not yield significant changes on EF performance, $F(2, 167) = 0.923$, $p = 0.39$, partial $\eta^2 = 0.01$, observed power = 0.2 indicating that participants exhibited no significant change on EF across the three testing periods. No main effect was found for sex, $F(1,168) = 1.26$, $p = 0.26$, partial $\eta^2 = 0.08$, observed power = 0.23, on EF after partialing out the effect that the covariates has on the outcome. Age and Education significantly predicted the EF performance $F(1, 168) = 11.23$, $p \leq 0.05$, partial $\eta^2 = 0.05$, observed power = 0.91; $F(1, 158) = 90.03$, $p < 0.001$, partial $\eta^2 = 0.36$, observed power = 1. There was a significant interaction effect between time and education, $F(2, 167) = 7.02$, $p < 0.001$, partial $\eta^2 = 0.08$, observed power = 0.9. As observed on **Table 4**, the relationship between education and EF, while significant, it decreased from Time 1 to Time 3. None of the other multivariate interactions (time × age; time × sex) were significant. These finding indicate that while the overall performance on EF tasks does not change significantly across time, education does have a positive influence on the patterns of change. The patterns of change did not differ between males and females.

Time, Age, Education, Sex and Verbal Episodic Memory

The third analysis examined the effect of Time on VEM. Sex was a between-subject factor and age and education were included as covariates. No violations from preliminary checks were found (normality; linearity; homogeneity of variances; reliable measurement of the covariates). Time as main effect again did not yield significant changes on VEM performance, $F(2, 170) = 0.680$, $p = 0.84$, partial $\eta^2 = 0.002$, observed power = 0.07 indicating that participants exhibited no significant change on VEM across the three testing periods. The main effect for sex was not significant, $F(1, 171) = 1.03$, $p = 0.31$, partial $\eta^2 = 0.003$, observed power = 0.16. Age and Education significantly predicted the VEM performance $F(1, 171) = 17.22$, $p = p < 0.001$, partial $\eta^2 = 0.09$, observed power = 1; $F(1, 171) = 61.25$, $p < 0.001$, partial $\eta^2 = 0.26$, observed power = 1. In contrast with EF, none of the multivariate interactions (time × age; time × sex; time × education) were significant. These finding indicate that VEM performance was similar across the time periods and across the two sexes. Furthermore, while age and education are significant predictors of VEM performance, the patterns of performance were not affected by age and years of education.

Effects of *APOE* ε4 on MMSE, EF, VEM, Baseline Performance

Three Mann–Whitney test were conducted to compare the MMSE, VEM and EF performance for *APOE* ε4 allele carriers and non-carriers. There was no significant difference between the two groups on the MMSE indicated that performance was not different for *APOE* ε4 carries and *APOE* ε4 non-carries, $U = 3{,}233$, $p = 0.25$. The distribution of EF and VEM was the same for *APOE* ε4 carries and *APOE* ε4 non-carries $p = 0.99$ and $p = 0.47$, respectively.

TABLE 4 | Correlations between age, education and cognitive domains.

S. No.		1	2	3	4	5	6	7	8
1	Age	1							
2	Education	−0.293**	1						
3	EF, TIME 1	−0.479**	0.565**	1					
4	EF, TIME 2	−0.336**	0.574**	0.776**	1				
5	EF, TIME 3	−0.422**	0.442**	0.645**	0.717**	1			
6	VEM, TIME 1	−0.484**	0.401**	0.606**	0.503**	0.455**	1		
7	VEM, TIME 2	−0.403**	0.412**	0.494**	0.506**	0.476**	0.776**	1	
8	VEM, TIME 3	−0.439**	0.530**	0.501**	0.452**	0.403**	0.721**	0.804**	1

$^{**}p < 0.01$.

DISCUSSION

The Neurocognitive study for the aging is the first longitudinal project on cognitive aging in Cyprus. It offers a unique opportunity to study modifiable and unmodifiable risk factors, including demographic, biological and genetic indices, in respect to brain health. Study participants were community dwellers living independently, who were born and raised in a small Mediterranean country with its unique geopolitical, social-cultural and genetic characteristics. As noted in previous publications stemming from NEUROAGE (Constantinidou et al., 2012a; Philippou et al., 2018), the project has been able to capture the last generation of Cypriots who have attended very little formal schooling. While the average education is 9 years, there was a wide variability among study participants, ranging from 2–20 years. It is stressed that this is the last generation of individuals with low education. Since, following the establishment of the Republic of Cyprus in 1960, public education has been free and mandatory through grade 9 or age 15. The present study is our first effort to examine the longitudinal performance of study participants and examine change at two additional time points over a 4–5 year period in three key cognitive domains: overall cognition, EF and VEM. The contribution of key demographic and genetic variables, namely sex, age, years of education, and *APOE* ε4 were also examined.

Demographic Factors of Age and Education and Cognitive Change

Older adults in our NEUROAGE cohort maintained overall cognitive performance across the five-year period as measured by the MMSE, the most widely used screening measure for global cognition. Age and education were not significant predictors of performance on the MMSE which adds to its versatility as a universal screening tool by a variety of health care professionals. Age systematically predicted performance on the VEM and EF measures, but was not associated with different patterns of cognitive change across the follow up period. The present findings are in contrast with longitudinal studies supporting significant changes in VEM and EF (Treitz et al., 2007; Lundervold et al., 2014) and in agreement with (Van Dijk et al., 2008) who used a similar experimental design. The difference between our study and studies demonstrating different findings might be due to methodological factors. For example, the average age of our cohort was a bit older (73 years) as compared to the Lundervold et al. (61 years), which may contribute to differences in findings. In addition, different measurements of verbal learning may influence study results. In the present study, the VEM composite consisted of both verbal learning tasks (HVLT-R Greek Version) and story recall tasks in order to obtain a more comprehensive representation of VEM. Future research will continue to explore the stability of VEM as more testing waves are recruited in the NEUROAGE project.

Another important finding of the present study was the stability of the EF performance across time. Several studies have provided evidence on the effects of aging on various aspects of EF. The literature is fairly consistent that EF abilities diminish as a result of biological age. However, this is the first study demonstrating that in cognitively and neurologically healthy older adults, EF abilities do not change significantly over a five-year period, as opposed to individuals with MCI or other neurodegenerative conditions.

In current literature, there has been a growing debate on the exact contribution of education in cognitive aging. In cross sectional studies, older adults with higher education perform better on VEM and EF measures. Education, is also the primary proxy measure included when estimating cognitive reserve, the mind's ability to implement strategies and compensate for the effects of aging in healthy individuals (Giogkaraki et al., 2013). However, the existing aging literature is not conclusive on the potential influence of education on the pattern of change across time in individuals without dementia. Research based on longitudinal data report inconsistent findings concerning the link between education and patterns of cognitive decline (e.g., Alley et al., 2007; Ritchie et al., 2016). The present study makes significant contributions towards characterizing the contribution of education in cognitive change across time.

The findings indicate that education systematically predicted EF and VEM performance across the five-year period. Of importance is the finding that while education was positively associated with VEM at each time point, and in fact increased the association at Time 3, education did not influence the pattern of change on the VEM composite score across time. In contrast, years of education influenced the pattern of change on the EF composite for our cohort; those with higher education had better performance. This significant interaction was observed

despite the fact that the association between education and EF, diminished across time. Previous research with our cohort analyzing baseline performance (Constantinidou et al., 2012a; Giogkaraki et al., 2013; Constantinidou et al., 2015; Philippou et al., 2018) demonstrated that younger participants and those with higher levels of education performed better as compared to older participants and those with lower levels of education on VEM and EF measures. Furthermore, cognitive reserve moderated the effects of biological age on VEM and EF, however, the moderating effect was higher for EF at baseline (Giogkaraki et al., 2013). Similar to our findings, Aschwanden et al. (2019) in their longitudinal analysis demonstrated that the highly educated sample showed better performance on memory in five-years, but the cognitive performance was rather stable over time despite of education. The present findings advance our current understanding on the beneficial effects of education in maintaining higher levels of EF performance across time. Future research should continue to characterize the slope of change across time across the key domains of VEM and EF.

Biological Factors of Sex and *APOE* ε4 and Cognitive Change

Previous research has provided mixed evidence on sex differences and potential interaction with cognitive performance in healthy aging (e.g., McCarrey et al., 2016; Zaninotto et al., 2018). In the present study, sex did not appear to have an effect on performance at baseline or to differentiate cognitive performance patterns over time. While the VEM scores of our male participants diminished slightly across time, and the scores of the female participants were stable, those differences were slight and not statistically significant. Both sexes showed a slight but not significant change on EF across the three time points. Our findings are in agreement with other longitudinal studies (De Frias et al., 2006; Karlsson et al., 2015). In those studies, both males and females demonstrated a similar pattern of change across time on episodic memory, verbal fluency, visuospatial functioning and logical reasoning measures. The present study does not support the notion of a potential sex advantage for women for general cognition, VEM or EF at baseline or across time when controlling for critical factors such as age and education.

Finally, the *APOE* ε4 allele has been implicated as a primary genetic risk factor in pre-clinical and late-onset dementia. The present findings did not yield differences in the baseline performance between carriers and non-carriers on the MMSE, VEM and EF composite. This is in contrast to other studies suggesting that ε4 is associated with memory decline, EF and general cognitive decline (Wisdom et al., 2011; Reas et al., 2019). It is important to note that in the present study, we selected individuals with a global cognitive score as measured with the MMSE of 24 and higher who completed all three cycles of the assessment. In a previous study examining the NEUROAGE cohort at baseline, we included individuals with lower MMSE scores. Carriers performed significantly lower than demographically matched non-carriers on the MMSE (Marsitopoulos and Constantinidou, 2015). Similar patterns were reported by Batterham et al. (2013). In their study, ε4 carriers scored significantly lower in initial memory performance and demonstrated greater decline in processing speed and word recognition than ε2 and ε3 carriers. However, after excluding 125 participants with low global cognition scores, all genotype effects became nonsignificant. As previously indicated, the frequency of the *APOE* allele ε4 in the Greek Cypriot population is among the lowest in Europe. Perhaps different patterns will emerge as we analyze future waves of the NEUROAGE cohort.

Clinical Implications, Limitations and Future Studies

This study contributes to the promising idea of health aging in which older adults can maintain good brain healthy and cognitive performance into older age (Livingston et al., 2017). The study implemented sensitive clinical/diagnostic tools to measure cognitive change at baseline and across the longitudinal assessment of our NEUROAGE cohort. Our findings characterize the role of important demographic, biological and genetic indicators in individuals who are cognitively healthy and how these indicators interact over time in general cognition, memory and EF. The findings make significant contributions to our understanding of anticipated changes within a five-year period and contribute to the growing evidence on the exact contribution of sex, age and education in cognitive performance. As future waves of testing are completed, and as more participants are included in the testing cycles that will increase study power, we will be able to determine if the patterns observed in the present study are stable across longer periods of time and to detect potential change points in performance.

The current cohort of an island population is characterized by a low frequency of *APOE* ε4. Therefore, the generalizability of the current findings is limited to cohorts with similar characteristics. The study included a fairly homogeneous sample focused on the Greek Cypriot population which is the majority population residing in the Republic of Cyprus. Future studies should explore other ethnic and cultural groups residing in Cyprus, including Armenians and Maronites. Furthermore, Turkish Cypriots should be included in future studies, once the Cyprus problem is resolved and populations currently residing in the occupied part of Cyprus would be accessible.

While the current study focused on individuals with intact global cognition, future studies should include individuals with lower MMSE scores in order to capture the full spectrum of cognitive abilities and investigate change across time. Every effort was made by our team to retain study participants and to follow them up across time. The MMSE is a gross cognitive tool, so it is possible that some individuals with a score of 24–25 might experience mild cognitive changes that were not captured by the MMSE, but should have been captured by the EF and VEF.

The results of the study can guide clinical intervention trials aiming to improve cognitive performance in older adults. While formal education is typically acquired in early life, it is a significant predictor of cognitive health in later life. Furthermore, the present findings indicate that education clearly contributed to baseline performance for key cognitive factors (e.g., memory and EF), and also impacted the rate of cognitive change in

healthy adult individuals. Since education is a key proxy for cognitive reserve, the present findings support the need to develop theory driven programs designed to improve cognitive functioning and facilitate EF and VEM. Research with the Categorization Program (CP), an intense neurocognitive rehabilitation program resulted in improved performance after a ten-week treatment period in healthy older adults who experience neurocognitive changes associated with the normal aging process (Constantinidou, 2019). Furthermore, gains generalized into novel tasks and were maintained after 4 months post training. Currently, we are exploring the utility of the CP in patients at risk for cognitive decline. In addition, we are exploring the effects of group interventions focusing on problem solving, EF and memory strategies combined with psychosocial training and emotion regulation strategies with our NEUROAGE cohort.

The present findings may provide some guidance on the patterns of cognitive testing required for cognitively healthy older adults, while also safeguarding valuable resources which could be diverted towards testing older populations with greater needs. While the study findings support cognitive stability for at least 4 years for our cohort, individual characteristics should be taken into consideration based on the current findings and from existing research for providing personalized advice in clinical practice. For example, those with lower education, those with higher cardiometabolic risk (Philippou et al., 2018), those with hearing and vision loss (Maharani et al., 2018) and those with depression and/or subjective cognitive decline (Constantinidou et al., 2015; Dimitriadou et al., 2018) should be closely monitored and tested more frequently. Future studies should incorporate contextual testing in the form of an informant report and direct patient observation because both EF and VEM play an integral part in effective participation during daily life activities (Constantinidou et al., 2015; Demetriou and Constantinidou, 2018). In a recent publication from the same cohort, healthy older adults reported changes in memory performance prior to their informants, suggestive of intact self-awareness (Dimitriadou et al., 2018).

Finally, given the complexity of biological aging, lifestyle habits, general health, metabolic and more genetic indicators could be important factors to be studied longitudinally in the context of cognitive/brain health, as they may play a mediating role for those with lower education. We are systematically investigating variables that affect aging such as cognitive reserve, psychosocial risk factors, hearing loss and cardiometabolic factors in order to develop multidimensional, theoretical and statistically valid prognostic models for successful cognitive aging (Maharani et al., 2018; Philippou et al., 2018). Currently, we are collecting data in order to explore the contribution of cognitive, social, and physical engagement to genetic and health factors to determine their potential contribution to adult brain health.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are not publicly available, but certain data could be made available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Bioethics Committee (ΕΕΒΚ/ΕΠ/2008/26). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC collected data, coordinated data collection, conducted the data analyses, and wrote the manuscript. FC is the principal investigator of the study. She conceptualized and designed the project, coordinated the data collection, analyzed data, and wrote the manuscript. MH conducted genetic data analyses and revised the manuscript. SP collected clinical data and revised the manuscript. All authors contributed to the article and approved the submitted version.

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