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Urine and Serum Biomarkers of Cardiovascular Status and Early Vascular Ageing in Children and Adults

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Abstract

Given that cardiovascular disease remains the leading cause of morbidity and mortality worldwide, there is a need to identify biomarkers that are accurate and reproducible to be able to identify which individuals are most at risk of early vascular ageing (EVA) to then allow for prioritisation of interventions to reduce this risk. To date, a myriad of different urine and blood biomarkers have been reported in studies looking at cardiovascular risk and EVA. These biomarkers primarily focus on oxidative stress, inflammation, haemostasis and thrombosis, metabolic markers, cardiovascular injury and epigenetic changes. As such, this review seeks to summarise the most common blood and urine markers reported in the literature and their current reported uses. Reference data in both adult and paediatric populations remain elusive for many of these biomarkers and may also be dependent on the assays used for analysis. It is possible that multi-marker risk scores may be of increased utility in the diagnosis of EVA. In addition, advances in technology may change the landscape of biomarker discovery in future years, with a need to prioritise research in the field of EVA to reduce the worldwide cardiovascular disease burden.

Keywords Vascular, Hypertension, Inflammation, Oxidative stress, Prevention, Risk stratification

1 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 38% of premature deaths below the age of 70 years [1]. Over the last 30 years, premature death due to CVD has been increasing, highlighting the ongoing importance of research in the field to understand how to determine cardiovascular status as well as the study of underlying pathophysiological mechanisms to improve outcomes through the development of prevention and treatment strategies [2].

Early vascular ageing (EVA) is defined as the presence of accelerated age-related changes in blood vessels, whereby the structure or function of the vessels resemble a more advanced biologic age than chronologic age, due to a series of characteristic changes (Table 1) [3]. EVA is a recognised independent risk factor for CVD and can be responsible for premature mortality with cardiovascular aetiology [4]. EVA can develop secondary to environmental and social exposures (air pollution, poverty, smoking, obesity, lack of exercise) as well as personal medical risk factors (hypertension, diabetes, genetic factors). Accumulation of subclinical vascular changes directs an individual towards a trajectory of EVA, with the potential to occur even in early fetal life, influencing future adult disease risk [5, 6]. Importantly, EVA changes can be reversible if interventions are implemented at an early stage, thus identifying people at risk and developing strategies for timely detection is crucial [7, 8].

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Table 1 Features which characterise early vascular ageing (EVA)

Type of change	Characteristic feature of EVA
Change in macrovascular structure	Reductions in networks of collateral vessels Decreased number of arterioles Decreased number of arteriolar anastomoses Shorter, more fragmented elastin fibres Increased collagen cross-linking Reduced collagen elasticity Loss of capillaries
Change in morphology	Increased wall thickness Hypertrophy particularly in the tunica media layer and in the tunica intima via infiltration of migrating vascular smooth muscle cells
Change in function	Increased vascular stiffness Reduced elasticity and compliance of the vessel wall Increased vascular resistance Reduced vascular contractility

The pathognomonic changes of EVA in the vasculature have been studied on an experimental basis in blood vessels, however, these specimens are obtained by invasive procedures and thus are not practical for routine use in clinical practice to determine those at risk of EVA and premature CVD. As such, it is clear that there is a need to determine which other biomarkers are useful in the initial detection of EVA. In particular, urine and serum biomarkers are relatively easy to obtain through minimally invasive procedures and can be used in clinical practice to identify individuals with EVA before manifestations of CVD, to focus on primary prevention in this patient group.

There is increasing interest in the field of EVA and large international groups of experts in the field of EVA have produced extensive reviews on the matter, for example from the Aging Biomarker Consortium [9] and the European Union Vascagenet [5, 10, 11] and some have now been endorsed in the clinical guideline for the management of CVD [12]. This field, however, is rapidly evolving, with a need to continually synthesise the most up-to-date literature. In addition, there remains little published information regarding current applications in both paediatric and adult populations. Whilst these reviews also consider other clinical biomarkers of EVA, the current review will focus on providing a concise introduction to urine and serum biomarkers of EVA only and will aim to highlight where these have been used in paediatric and adult populations and whether any sex differences have previously been reported to date. A large number of biomarkers have been described in the literature and it is out with the scope of this review to make a comprehensive list of all available and researched biomarkers. Rather we aim to describe the biomarkers of oxidative stress, inflammation, haemostasis and thrombosis, and metabolic and epigenetic markers associated with EVA and CVD that are currently most used.

2 Methods

Literature searches were conducted by 3 independent authors between October 2024 and December 2024 using Pubmed, Science Direct and Google Scholar with no date limitations on the searches. Search terms included 'biomarker', 'vascular', 'early vascular ageing' and 'premature ageing'. Reference lists of included articles were also included. Biomarkers were included where they had been measured in blood or urine in human subjects with the aim to identify changes in vascular structure or function associated with EVA. For inclusion, biomarkers had to have been described in more than one study including more than one patient and with a clear description of methodology to allow replication. Table 2 summarises the biomarkers discussed in this review.

2.1 What is a Biomarker?

A biomarker is any objective biological measure that can be used as an indicator of the body's physiological state, pathological processes, or its response to therapeutic interventions [13]. For a biomarker to be clinically useful, it must possess certain additional characteristics which are summarised in Fig. 1. Although the term often refers to substances circulating in the body's tissues or fluids, it also encompasses measurements of anatomical structures and functions. The difficulty with the use of these clinical biomarkers is that reference data remain sparse particularly in paediatric populations; they are operator-dependent and require cooperation from the patient as well as specialist expertise and equipment. As such their routine use to detect EVA remains limited. Urine and serum are commonly used for biomarker studies due to

Table 2 Summary of biomarkers

Category	Name	Primary sample type*	Methods of measurement	Paediatric data available?	Reported sex differences	Current regularly reported clinical use
Oxidative stress	8-OHdG	Urine blood	ELISA Liquid chromatography/mass spectrometry	Yes [6, 34]	Higher in women than men [36–40]	No
	F2-isoprostanes	Urine blood	Gas or liquid chromatography/mass spectrometry	Yes [48–50]	Higher in women than men [36–38]	No
	MDA	Urine blood	Thiobarbituric Acid Reactive Substances (TBARs) Assay High performance liquid chromatography with Fluorescence Detection	Yes [6]	Variable data [36–38, 51–53]	No
	MPO	Blood	ELISA Colorimetric or Fluorometric Enzyme Activity Assays	Yes [51]	Variable data [36–38, 51–53]	No
	Total antioxidant capacity	Urine blood	ELISA	Yes [56]	Higher in men than women [57]	No
	Total ROS	Urine blood	Superoxide.-lucigenin enhanced chemiluminescence, electron paramagnetic resonance (EPR) Peroxide – amplex red	Yes [6, 62]	Higher in men than women [63, 64]	No
Inflammation	Hs-CRP	Blood	Nephelometry Turbidometry	Yes [72, 73]	Higher in men [73]	Yes
	IL-6	Blood	ELISA Multiplex immunoassay Chemiluminescence immunoassay	Yes (82–83]	Variable data [84–86]	No consensus for clinical use in CVD
	TNF-alpha	Urine blood	ELISA Chemiluminescence immunoassay Radioimmunoassay Multiplex immunoassay Bioassay	Yes [79–81)	Higher in men than women [76]	No consensus for clinical use in CVD
Thrombosis/haemostasis	D-dimer	Blood	ELISA Latex agglutination assay	Yes [93, 94]	Higher in females [92, 95]	Yes
	Fibrinogen	Blood	Clauss Fibrinogen Assay	Yes [99, 100]	Raised fibrinogen is a higher risk to women than men [101]	Yes
	PAI-1	Blood	ELISA Immunoassay Latex agglutination	Yes [105, 106]	Men higher levels than pre-menopausal women [101, 107]	Yes
	vWF	Blood	Quantification: VWF antigen Function: Ristocetin cofactor Functional assay Collagen binding assay	Yes [111, 112]	Variable data [113, 114]	Yes

Table 2 (continued)

Category	Name	Primary sample type*	Methods of measurement	Paediatric data available?	Reported sex differences	Current regularly reported clinical use
Epigenetic	Chromatin	Blood	Chromatin immunoprecipitation (ChIP)	No	Variable data [115, 116]	No
	DNA methylation	Blood	Methylation assays (luminometric, Infinium) Immunoprecipitation Methyl capture using binding proteins Next generation sequencing	Yes [120, 121]	Lower methylation of CpG regions in boys [122, 123]	Yes
	Histone modification	Blood	ELISA	Yes [124]	Variable data [124]	No
	Micro-RNA	Blood	RNA sequencing Northern blot qRT-PCR	Yes [131]	Yes- dependent on miR [132–134]	No
	Telomere length	Blood	qPCR Southern blot	Yes [137, 138]	Longer in females at age of four years [137]	No
Cardiovascular injury	Cell adhesion molecules ICAM-1, ICAM-3, VCAM-1	Blood	ELISA	Yes [151]	VCAM-1 more elevated in boys than girls [151]	No
	NT-proBNP	Blood	ELISA Electrochemiluminescence bioassay	Yes [156]	Higher in females than males [59]	Yes
	Troponin (cTNT, cTNI)	Blood	Chemiluminescent immunoassays ELISA	Yes [163–175]	Higher in boys than girls [159]	Yes
Other	Albumin	Blood urine	ELISA	Yes [178, 179]		
	Alpha-Klotho	Blood	ELISA	Yes [183, 184]	Variable data [181, 185]	No
	Endothelin-1	Blood	ELISA	Yes [188]	Higher in men than women [189, 190])	No
	IGF-1	Blood	Immunoassay Liquid chromatography tandem mass spectrometry	Yes [197, 198]	Earlier IGF-1 peak in girls than boys [199]	Not for CVD
	MMP-2 and MMP-9	Blood	ELISA Immunoassay Gel zymography PCR	Yes [205, 206]	MMP-9 higher in males [207, 208]	No
	Lactate	Blood	Immunoassay ELISA	Yes [213]	No	Yes
	Technologies	Urine blood	Proteomics Metabolomics Peptidomics Genomics	Variable Variable	Variable	No

Clinical use reported as of date of publication

8-OHdG 8-hydroxyguanosine, *cTNI*, cardiac troponin I, *cTNT* cardiac troponin T, *CVD* cardiovascular disease, *ELISA* enzyme-linked immunosorbent assay, *Hs-CRP* high sensitivity C-reactive protein, *ICAM-1* intercellular adhesion molecule-1, *ICAM-3* intercellular adhesion molecule-3, *IGF-1*:insulin like growth factor-1, *IL-6* interleukin-6, *MDA* malondialdehyde, *MMP-2* matrix metalloproteinase-2, *MMP-9* matrix metalloproteinase-9, *MPO* myeloperoxidase, *NMR* nuclear magnetic resonance spectroscopy, *NADPH* nicotinamide adenine dinucleotide phosphate, *NT-proBNP* B-type natriuretic peptide, *oxLDL* oxidised low-density lipoprotein, *PAI-1* plasminogen activator inhibitor-1, *PCR* polymerase chain reaction, *RNA* ribonucleic acid, *TBARs* Thiobarbituric Acid Reactive Substances, *TNF-alpha* tumour necrosis factor alpha, *VCAM-1* vascular cell adhesion molecule 1, vWF von Willebrand factor

* May be possible to measure in other bodily fluids but primarily reported in blood or urine

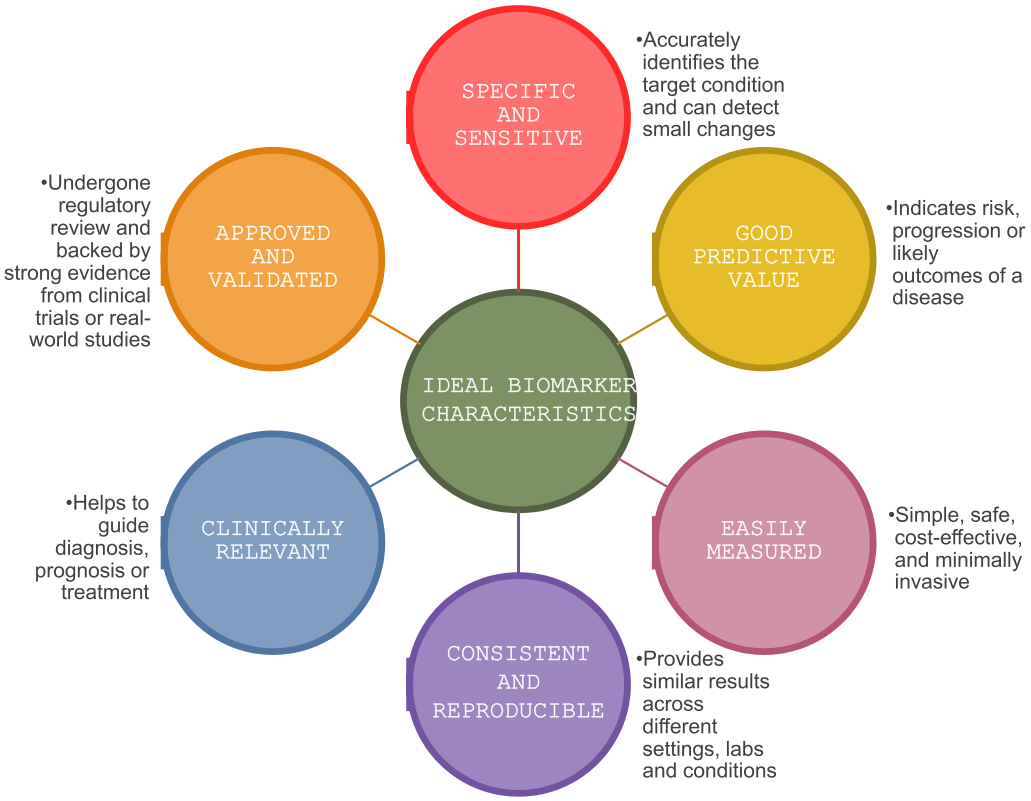

Fig. 1 Key characteristics of an ideal biomarker

their relatively convenient accessibility and cost-effectiveness, and as such, this review will focus solely on these [14].

Urine samples can be collected non-invasively in large volumes, are relatively uncomplex in terms of proteins and cellular components allowing for simplified analysis, and contain certain metabolites in higher concentrations than blood, making them easier to detect [15]. Urine is, however, limited in its biomarker range as large proteins or cells may be absent, and its composition is dependent on factors such as hydration and diet, potentially affecting biomarker stability and reliability [16].

Serum, on the other hand, contains a broad array of proteins, hormones, enzymes and metabolites allowing for comprehensive analysis, and its composition is much more consistent making it potentially more reliable for longitudinal studies [16]. However, the serum collection process, while routine in clinical settings, is invasive, albeit minimally, and samples may be affected by haemolysis which can allow for biomarker measurement errors [17].

2.2 Biomarker Validation

A further potential challenge to the effective use of biomarkers is biomarker validation. This multi-step process assesses a biomarker's measurement performance characteristics and evidence of the test results' clinical significance, thereby ensuring the biomarker is reliable, accurate, and meaningful for its intended use [18].

2.2.1 Analytical Validation

The process begins with analytical validation, which assesses the biomarker measurement's accuracy and reproducibility across different settings and methodologies [13]. Biomarker accuracy is generally expressed via its sensitivity (ability to detect disease when the disease is present e.g. true positives) and specificity (ability to recognise the true absence of disease e.g. true negatives). Receiver-operating curves are often used to visualise and study the relationship between sensitivity and specificity against the clinical gold standard [19], although other statistical models may be appropriate depending on the analyses in question. Changes associated with EVA are often subtle and subclinical, so any assay used to measure or monitor vascular status is likely to need to be measurable at small quantities, adding to the complexity of validation.

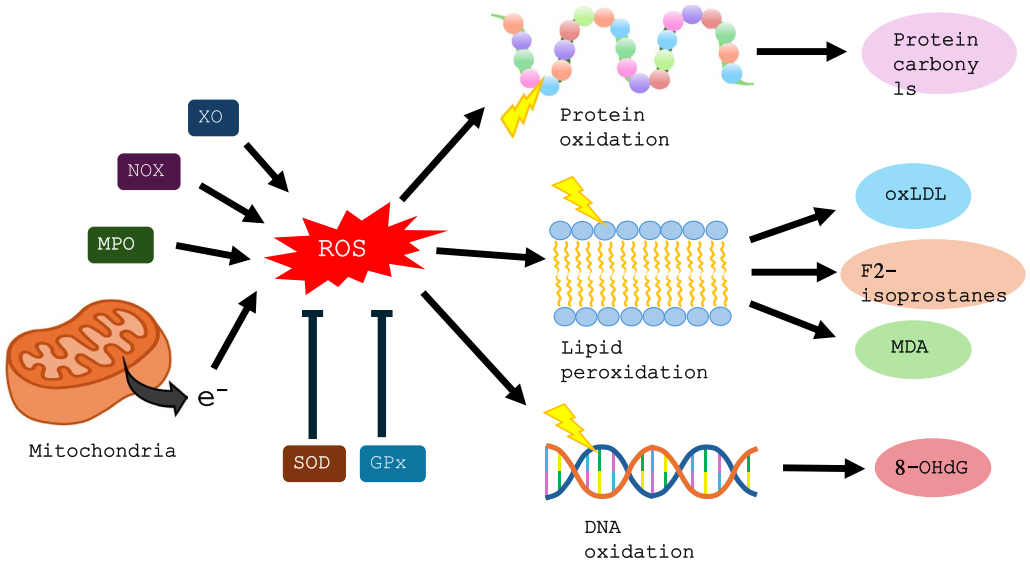

Fig. 2 Pathways of oxidative stress and the biomarkers produced. Reactive oxygen species (ROS) are produced from mitochondrial respiration, myeloperoxidases (MPO), NADPH oxidases (NOX), and xanthine oxidases (OX), and are removed by superoxide dismutases (SOD) and glutathione peroxidases (GPx). ROS oxidise protein, lipids and DNA resulting in protein carbonyls, oxidised low-density lipoprotein (oxLDL), F2-isoprostanes, malondialdehyde (MDA), and 8-Hydroxy-2'-deoxyguanosine (8-OHdG) being released into the blood and subsequently urine

2.2.2 Clinical Validation

Clinical validation then establishes how the biomarker correlates with a clinical outcome—its ability to correctly identify individuals with a disease and predict disease risk or progression. This can be done by retrospective use of clinical trial data or via prospective population sampling [20]. Afterwards, the clinical utility of the biomarker must be validated by establishing whether the use of the biomarker in clinical care results in improved patient outcomes via the improved diagnosis, risk stratification or treatment decisions [20]. This requires the availability of long-term longitudinal data and may, therefore, take many years to generate.

2.2.3 Regulatory Validation

Regulatory validation ensures compliance with standards for safety and efficacy, allowing it to be used routinely in clinical practice [21]. Different countries have different regulatory authorities, with their own individual conditions for final approval and validation. Each regulatory board, however, will seek to confirm the data reviewed in earlier stages and to determine the safety and efficacy of the biomarker to be studied.

2.3 Biomarkers of Oxidative Stress

Biomarkers of oxidative stress are strongly associated with cardiovascular diseases and EVA [22]. Whether in urine or serum, they are each consistently elevated in those diagnosed with CVDs compared to healthy controls, evidencing their potential as diagnostic tools [6, 23–25]. Reactive oxygen species (ROS) have been implicated in the pathophysiology of EVA and CVD [10]. ROS are highly reactive oxygen-derived molecules with one or more unbound electrons and an excess of ROS results in oxidative stress, which may develop secondary to excessive ROS production, depleted supply of antioxidants, inactivation or impaired production of antioxidant enzymes or a combination of these. Due to their short half-lives, it can be difficult to accurately assess ROS levels directly, therefore the most frequently employed urine and/or serum markers measure post-translational modifications induced by ROS including indicators of oxidative DNA damage (e.g. 8-Hydroxy-2'-deoxyguanosine (8-OHdG)), markers of lipid peroxidation (F2-Isoprostanes and malondialdehyde (MDA)); ROS-producing enzymes (e.g. myeloperoxidase [MPO]); antioxidant capacity (e.g. total antioxidant capacity (TAOC), and total ROS [26] (Fig. 2), each of which will be discussed in this section (Table 2). Several other markers are available and have been reviewed elsewhere [27–30].

2.3.1 8-OHdG

8-OHdG is commonly measured in blood and urine as a marker of oxidative stress and DNA damage and its associations to cardiovascular diseases have been recently reviewed elsewhere [31, 32]. It can be measured in spot urine samples, with the best results with first-passed urine [33]. Higher levels have been associated with a number of CV diseases and there is evidence it can be used to prognosticate and risk stratify individuals with

conditions such as heart failure [34]. Data are available in different cohorts of children, including in preterm neonates [35]. The significance of sex differences remains unclear with studies suggesting conflicting results as to whether men or women have higher levels of MDA, 8-OHdG, and F2-isoprostane levels [36–40]. In male children with early onset hypogonadism, levels are higher than in those with normal sex steroid levels [6].

2.3.2 F2-isoprostanes

F2-isoprostane levels are higher in women [36–38]. Additionally, these may aid in evaluating cardiovascular therapies, with lowered urinary F2-isoprostanes in men receiving anti-hypertensive treatment [41]. They are also valuable for risk stratification as elevated F2-isoprostanes predict cIMT and the risk of atherosclerosis, coronary artery disease, and cardiovascular events, including myocardial infarction and ischemic stroke [42–45], as a positive relationship has been reported between F2-isoprostane levels and the number of diseased vessels [46]. In addition, F2-isoprostanes seem to independently predict CVD mortality [44, 45, 47] and have been measured in children to monitor vascular status [48–50].

2.3.3 MDA and MPO

MDA and MPO can be measured in serum or urine with the Thiobarbituric Acid Reactive Substances (TBARs) assay most commonly used to measure MDA and ELISA used for MPO. MDA, and MPO independently predict CVD mortality [44, 45, 47] and have been reported in paediatric studies [6, 51]. Again, sex-aggregated data are conflicting with higher levels of both MDA and MPO being reported in men compared to women, in some studies [52–54] and the opposite in others [36–38].

2.3.4 Total Antioxidant Capacity (TAOC)

TAOC is inversely associated with the incidence of cardiovascular events and can be measured in blood, saliva or urine via ELISA [55]. These have been measured in paediatric populations, including in trials to determine whether antioxidants can improve vascular status in children at risk of EVA [56]. Women have been reported to have lower levels than men [57].

2.3.5 Total ROS

Total ROS can be analysed via measurement of superoxide, hydrogen peroxide or overall ROS levels. Guidelines exist regarding optimal methods of analysis including the use of electron paramagnetic resonance (EPR) for specific measurement of superoxide levels [58, 59]. Total ROS levels have been measured using a variety of probes after the isolation of cells from blood samples (mostly leukocytes and neutrophils) derived from adults [60]. Reactive oxygen metabolites (d-ROMs) have also been measured in the serum of adults and linked to CVD mortality [61]. ROS and their association with CVD have been described in different paediatric populations as reviewed elsewhere [62]. Overall men are reported to have increased levels of ROS compared to women [63]. This may be in part because SIRT1 is an inhibitor of ROS-producing pathways within the vasculature and is necessary for modulating oestrogen receptor signalling [64].

2.4 Biomarkers of Inflammation

Inflammation has long been hypothesised to be a feature of the ageing process, and studies of in vitro and in vivo models have shown its involvement in EVA [28, 65, 66]. In particular, the inflammatory pathways involved in vascular ageing have been shown to be mediated by several cytokines, and these are usually measured in human serum using immunoassays [67, 68]. To date, several biomarkers of inflammation in vascular ageing have been described and shown to correlate with cardiovascular risk. This section will consider 3 of the most commonly reported biomarkers of inflammation: high-sensitivity C-reactive protein (hs-CRP); interleukin (IL)-6 and tumour necrosis factor-alpha (TNF-alpha) (Table 2, Fig. 2).

2.4.1 hs-CRP

High sensitivity C-reactive protein (hs-CRP) is a biomarker used in clinical practice, measured in serum by nephelometry or turbidimetry [69], and has been shown to correlate with increased risk of cardiovascular events including stroke, acute coronary syndrome, and peripheral arterial disease [70]. In a cohort of 3,166 adults, more adverse cardiovascular events occurred in patients with elevated hs-CRP [71]. A further study by Guran et al. 2007 showed that hs-CRP was elevated in children at risk of CVD compared to healthy children with no risk factors [72]. Another study found that hs-CRP levels were significantly higher in male compared to female patients in their cohort of 6060 healthy people and correlated with lower levels of high-density lipoprotein [73].

2.4.2 TNF-alpha

TNF-alpha is usually measured via immunoassay. It has been found to be more abundantly expressed in veins of more advanced age, and it was shown by in vitro models to accelerate endothelial cell death [74, 75]. Bernardi et al. report a higher level of TNF-alpha in healthy men compared to women [76]. Yuan et al. 2020 found that single nucleotide polymorphisms that are associated with increased TNF levels led to an increase in coronary artery disease and ischaemic stroke [77]. A study by Liberale et al. 2021 showed that a higher TNF-alpha level was

associated with more advanced age and worse outcomes in ischaemic stroke [78]. A study in children with obesity also demonstrated raised TNF-alpha, which correlated with levels of endothelin 1, a marker of endothelial dysfunction [79]. Other studies have investigated TNF-alpha in children and found this to be raised in other cohorts at risk of EVA [80, 81].

2.4.3 IL-6

Interleukin (IL)-6 is another pro-inflammatory cytokine with a role in stimulating the production of fibrinogen. It has been found to be more expressed in the vascular smooth muscle cells (VSMCs) of ageing blood vessels in an ex-vivo mouse model, and IL-6 levels correlate with long-term poorer prognosis due to cardiovascular events [82, 83]. The reference range of IL-6 levels has been studied and reported in a meta-analysis [84]. We could not identify any papers linking IL-6 to vascular ageing in children, although one study showed that IL-6 could predict the presence of pulmonary arterial hypertension (PAH) [85]. Different studies reported different effects of sex on IL-6 levels, with two groups reporting a correlation of IL-6 complexes with cardiovascular events in men but not women, while Bermudez and colleagues reported a relationship between IL-6 levels in women and the presence of hypertension, high cholesterol and diabetes [86–88].

2.5 Biomarkers of Haemostasis and Thrombosis

Ageing has long been associated with increased rates of both venous and arterial thrombotic events [89, 90]. The following biomarkers will be discussed: D-dimer; fibrinogen; plasminogen activator inhibitor-1 (PAI-1) and Von Willebrand factor (vWF) (Table 2, Fig. 3).

2.5.1 D-dimer

D-dimers represent clot degradation products, and have also been shown to rise with increasing age [91]. The LIPID study investigated baseline D-dimer levels in 7,863 individuals, and showed a positive correlation between D-dimer level and age, female sex, hs-CRP, and was a predictor of cardiovascular events and mortality [92]. D-dimer is used clinically and reference data are available for adults and children according to local laboratory assays [93, 94]. A study by Reagh et al. found that women had higher D-dimer levels than men in the absence of thromboembolic events [95] (see Fig. 4).

2.5.2 Fibrinogen

Fibrinogen levels have been shown to rise with age and in association with ischaemic heart disease [96]. Fibrinogen is considered an acute-phase protein as it increases during acute inflammation [97], and gene expression has been shown to be mediated by higher IL-6 [98]. Again, fibrinogen is often used for haematological investigations

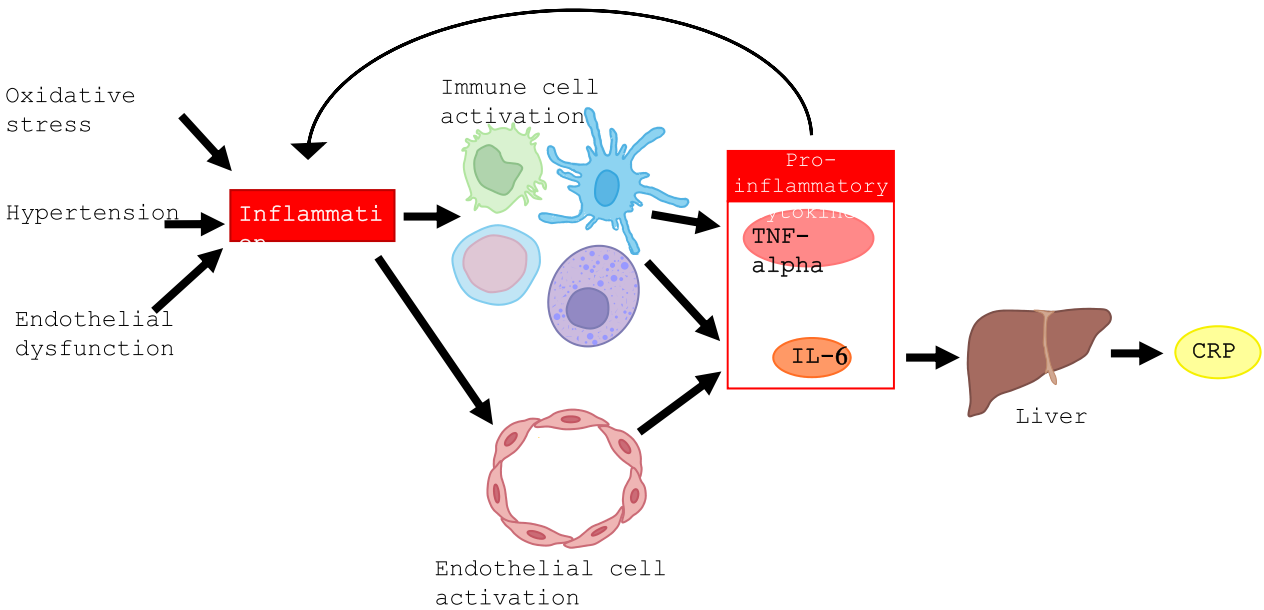

Fig. 3 Pathways of inflammation and the biomarkers involved. Triggers such as oxidative stress, hypertension and endothelial dysfunction initiate inflammation resulting in the activation of immune cells and endothelial cells. These cells, particularly macrophages and monocytes, release the pro-inflammatory cytokines interleukin-6 (IL-6) and tissue necrosis factor-alpha (TNF-alpha). IL-6 stimulates hepatocytes in the liver to produce C-reactive protein (CRP), which is then released into circulation. Positive feedback loops occur where TNF-alpha and IL-6 further stimulate their own production

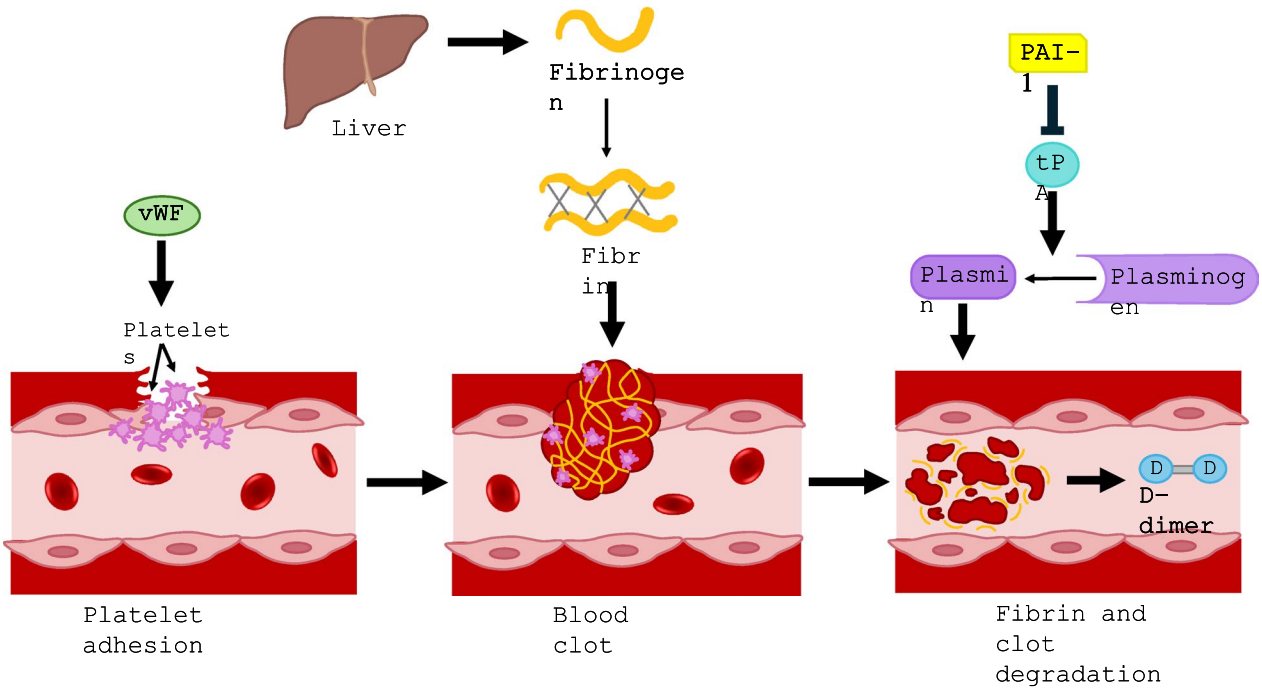

Fig. 4 Pathways of haemostasis/thrombosis and the biomarkers involved. Von Willebrand factor (vWF) is produced by endothelial cells and mediates platelet aggregation and adhesion to form a platelet plug after vascular injury. Fibrinogen is produced by the liver and released into the circulation where the coagulation cascade results in its conversion to fibrin. Fibrin forms a mesh to stabilise the forming blood clot. The enzyme plasmin degrades fibrin, producing D-dimers as clot breakdown products. Plasmin is converted from its inactive precursor by tissue plasminogen activator (tPA), which is inhibited by plasminogen activator inhibitor-1 (PAI-1), thereby inhibiting clot breakdown

clinically with assay-specific reference data available for adults and children [99, 100]. Fibrinogen has been reported as higher in women and to be of increased risk to women when raised [101].

2.5.3 PAI-1

Concentrations of PAI-1 increase and plasminogen activator activity is known to decrease with age [102]. PAI-1 is overexpressed in senescent human umbilical vein endothelial cells, while expression of sirtuin-1 (SIRT-1) leads to epigenetic modifications that led to downregulation of PAI-1 and reduced senescence [103]. In patients, rising PAI-1, measurable by immunoassay of blood specimens, was associated with acute myocardial infarction and with recurrence of coronary events [104]. PAI-1 has been measured in children and adolescents at risk of EVA [105, 106]. Data are conflicting as to whether PAI-1 is higher in males or females [101, 107].

2.5.4 vWF

Von Willebrand factor is a protein associated with platelet aggregation and vessel wall adherence and as a carrier protein to factor VIII to the area of clot formation. On immunohistochemistry in vitro, vWF has been shown to be increased in endothelial cells of more advanced age [108]. In a genome-wide association study, mendelian randomisation demonstrated that plasma vWF levels were associated with an increased ischaemic stroke risk, and of a causative effect of factor VIII on venous thrombosis and coronary artery disease [109]. A further case–control study further investigated markers of thrombosis and haemostasis in patients with kidney failure and found that prothrombin I and II, D-dimer and vWF were downregulated, while antithrombotic factors such as protein C were downregulated [110]. Reference data for vWF are available and this assay has been measured clinically in different paediatric populations [111, 112]. Levels of vWF are generally higher in females but also differ according to ethnicity [113]. That said, the ristocetin assay, which can be used to measure vWF function reports higher activity in males compared to females [114].

2.6 Epigenetic Markers

With increasing age, cells are more prone to mutations and loss of genomic stability and altered epigenetic signatures, which can be quantified in a number of different ways. This section will consider: chromatin; DNA methylation; histone modification; micro-RNA and telomere length (Table 2).

2.6.1 Chromatin

Chromatin measurements have been associated with atherogenic changes in vascular smooth muscle cells [115]. Chromatin can be measured in blood or urine where there is sufficient genetic material. Single-cell analysis of chromatin in the human heart has shown sexual dimorphism and it is clear there are sex differences associated with epigenetics within the cardiovascular system [116].

2.6.2 DNA Methylation

DNA methylation is an epigenetic change that happens usually on CpG regions (cytosines preceding guanine), and interferes with gene transcription, downregulating expression of coded regions. Increasing DNA methylation is associated with increased atherosclerotic cardiovascular disease [117]. DNA methylation can affect the transcription of genes coding for proteins involved in other pathways of vascular ageing, for example fibrinogen and Klotho gene [118]. DNA methylation is best measured using serum due to increased quantities of DNA although urine can be used. A great deal of recent research has focused on DNA methylation and its association with CVD, with a DNA methylation map of atherosclerosis being published in 2015 [119]. DNA methylation has been measured in individuals from newborn age throughout the lifespan with sex differences noted at all ages. For example, a meta-analysis of newborn blood identified lower methylation levels at CpG regions in boys than girls [120] and multiple differences have been noted between the sexes from samples from placenta, cord blood and newborn blood [121]. DNA methylation differences in males and females for CVDs have been reviewed extensively elsewhere [122, 123].

2.6.3 Histone Modification

Histone modifications are epigenetic changes that underpin gene regulation and chromatin structure in eukaryotic nuclei. The significance of modifications have been reviewed elsewhere and can be found in those with evidence of EVA as well as congenital cardiovascular disease with some sex differences noted [124].

2.6.4 Micro-RNA

Micro-RNAs are non-coding RNA sequences that have a regulatory role in gene expression. There has been increasing interest in the field of micro-RNA research, and there is a rapid growth of the list of identified significant micro-RNAs in human physiological processes, with continuing expansion in their role as biomarkers in disease. There are several methods for quantification of micro-RNAs, including RNA sequencing and Northern blot, quantitative real-time reverse transcriptase PCR (qRT-PCR), and microarray. Serum or plasma samples can be used with qRT-PCR in clinical practice, and several studies have been carried out to determine intra- and pre-analytic standardisation and normalisation of results, including for use in cardiovascular events. Specifically, miR-133a/b, miR-122 miR-124, miR-34a and miR-134 were found to be significantly increased in patients with coronary artery disease [125, 126], while miR-145 and miR-155 were inversely related to development of coronary artery disease [127, 128]. miR-208b and miR-133a were associated with increased mortality [129]. miR-1 has also been implicated in vascular ageing: rising levels of miR-1 correlate with lower mean cardio-ankle vascular index and positively correlated with carotid artery stiffness [130]. Multiple studies have looked at different micro-RNAs in paediatric populations as recently reviewed by Paslawska and colleagues [131]. Sex influences levels of micro-RNAs in different ways depending on the miR type [132–134].

2.6.5 Telomere Length

A hallmark of cellular ageing is a reduction in telomere length [135]. Telomere length can be measured by quantitative polymerase chain reaction (PCR) or by Southern blot [136], and has been found to be longer in females during early childhood [137, 138]. Telomere length has also been demonstrated to be associated with advanced and not early arteriosclerosis [139], but preceding atherosclerosis [140]. Mean telomere length is also related to the clinical parameters of systolic and diastolic blood pressure and with odds of hypertension [141]. In a cohort of 308 adult patients requiring haemodialysis, a longer telomere length was more frequently observed in patients with a vascular cause of nephropathy and end stage renal failure, and importantly was associated with an increased risk of adverse cardiovascular outcomes [142].

2.7 Biomarkers of Cardiovascular Injury

Various biomarkers of CV injury have been studied to date. This section will consider: cell adhesion molecules (ICAM-1, ICAM-3, VCAM-1); N-terminal fragment of pro-brain (B-type) natriuretic peptide (NT-proBNP) and troponin (Table 2).

2.7.1 Cell Adhesion Molecules

Intracellular adhesion molecules (ICAM) 1 and 3 are glycoproteins that have potential as biomarkers associated with vascular injury. ICAM-3 has been associated with increased stroke risk [143], while soluble ICAM-1 has been found to be associated with vascular cognitive impairment in an elderly population [144], carotid plaque formation, and risk of stroke and stroke recurrence [145, 146]. Vascular cell adhesion molecule 1 (VCAM-1) is another glycoprotein that has been suggested as a

biomarker of vascular injury, having been shown to associate with early atherosclerosis in a mouse model [147]. ICAM and VCAM can be measured by ELISA immunoassay, and both ICAM-1 and VCAM-1 can be used to predict the risk of major adverse cardiovascular events in patients who have had ST-elevation myocardial infarctions [148] and in haemodialysis patients [149]. Circulating VCAM-1 levels are also independently associated with more advanced chronological age [150]. In paediatric patients with metabolic syndrome, VCAM-1 has been found to be more elevated in boys than girls [151].

2.7.2 NT-proBNP

NT-proBNP is measured in blood samples using immunoassays [152]. It can be used to prognosticate mortality in heart failure and ischaemic heart disease [153–155]. In children with congenital heart disease, NT-proBNP levels that have been adjusted for age can be predictive of major adverse cardiovascular events [156]. Ethnic differences have been noted for NT-proBNP [157] but reference data are available, even for specific populations such as pregnant women [158]. Overall women have higher levels than men [159].

2.7.3 Troponin

Troponin I (cTnI) and troponin T (cTnT) are serum tests with high sensitivity to myocardial injury, routinely used for the detection of acute coronary syndromes and myocardial injury in clinical practice. High-sensitivity cTnI can be used as a predictive biomarker for future cardiovascular events in the non-acute setting [160]. Trajectory analysis of hs-cTnI showed that a trend of rising troponin occurs over decades, and years before cardiovascular events manifest [161]. Other applications for cTnI as a biomarker show that it can be used to predict the risk of death following trauma and admission to intensive care [162]. In paediatric populations, the level is highest in healthy infants and neonates and then reduces with progression to childhood and adolescence, and boys have higher baseline troponin levels than girls [163–175]. In children, hs-cTnI does not show an elevation on serial measurements as in adults, however, if the baseline result is elevated, it may point towards a cardiac aetiology [176]. Women have lower troponin levels compared to men [159].

2.8 Additional Biomarkers

Additional biomarkers that have been studied and will be discussed in this section are: albumin; alpha-klotho protein; endothelin-1; insulin-like growth factor 1 (IGF-1); matrix metalloproteinases; lactate; and omics technologies.

2.8.1 Albumin

The simple measurement of albumin in the urine (microalbuminuria) has been demonstrated consistently to be associated with all-cause mortality and cardiovascular mortality independent of glomerular filtration rate via data analysis of large population datasets including the Hunt-II study and the Dutch Prevention of renal and Vascular Endstage Disease (PREVEND) study [177] among others. As such, the measurement of microalbuminuria has been postulated as a useful tool to track the development of EVA in paediatric populations at risk [178, 179]. Sex differences have been noted with stronger associations between albumin and blood pressure identified in women [180]. Currently, albumin is one of the few clinically validated tools that can be utilised, however, the use of 24 h urine samples to do so is costly and can be considered inconvenient and difficult to comply with in some patients.

2.8.2 Alpha-klotho Protein

Alpha-Klotho is a protein that has been associated with anti-ageing effects and has been suggested as another potential biomarker of CVD and EVA. Suppressed levels of alpha-Klotho are associated with an increased risk of congestive heart failure and stroke [181]. In a mouse model with Klotho deficiency, which demonstrates features of hypertension, obesity, hypertriglyceridaemia, a viral delivery system of klotho gene delivery improved endothelial dysfunction and remodelling, and reduced hypertension [182]. Cohort studies in populations of children and adolescents at risk of EVA have shown detectable levels, which correlate to their disease group [183, 184]. Males have been reported as having lower alpha-klotho levels [181], with a U-shaped association between klotho levels and all-cause mortality in females, but no significant pattern in males [185].

2.8.3 Endothelin-1

Endothelin-1 (ET-1) and relating peptides have been detected in the circulating plasma and have been widely studied as potential biomarkers of CVD, with associations reported between endothelin-1 and blood pressure and age-related cardiac remodelling and cardiac fibrosis [186]. In addition, studies have investigated its use as a prognostic marker of coronary artery disease, myocardial infarction and heart failure [187]. ET-1 can be measured in children although data remain conflicting regarding whether levels increase in individuals at risk of EVA, such as those with hypertension and more research in this area is therefore required [188]. Sex differences in the vascular endothelium are well described, with young women having greater endothelium-dependent dilation compared to men, with age-related declines in endothelial function

being delayed by approximately 10 years in women compared to men [189]. Plasma ET-1 levels are higher in men and increase with age [190].

2.8.4 IGF-1

Insulin-like growth factor 1 (IGF-1) is another potential serum marker of vascular ageing. Studies in individuals with hypopituitarism and in older adults with falling levels of IGF-1 suggest that cardiovascular aetiology for mortality increases in individuals with lower IGF-1 levels, and life expectancy decreases [191–196]. Studies in Laron syndrome (growth hormone resistance leading to IGF-1 deficiency) demonstrate impairments in left ventricular mass, stroke volume and cardiac output [197], and improvement in ventricular mass following initiation of treatment [198]. IGF-1 has been used clinically for a long time in the assessment of growth in children, with reference data available and no differences between the sexes, although IGF-1 peaks earlier in girls than boys [199]. Of note, a recent UK Biobank study identified a U-shaped association between serum IGF-1 and incident CVD, CVD mortality, myocardial infarction and heart failure, suggesting that monitoring of circulating IGF-1 status may be important for CV screening [200].

2.8.5 MMPs

Matrix metalloproteinases (MMPs) are a group of enzymes that are involved in the degradation and remodelling of the extracellular matrix. Declining levels of MMP following an ischaemic stroke in patients was predictive of a better outcome [201]. Individuals with obesity and smokers who are also at increased risk of cardiovascular events, were found to have increased MMP-9 levels [202, 203]. In a study by Cancemi et al., elderly individuals found a direct relationship between cholesterol and low-density lipoprotein concentrations and MMP-9 levels, and a negative correlation between MMP-2 and CRP [204]. MMPs have been successfully measured in paediatric populations assessing vascular function [205, 206].

The role of sex and gender remain conflicting. Zhong et al. showed an increased level of MMP-9 in males with ischaemic stroke, which was associated with worse prognosis and higher rates of long-term disability and mortality [207], however, Tegeler et al. found no association between sex and MMP levels [208].

2.8.6 Lactate

Lactate is a metabolic product of glycolysis that is commonly measured in the blood in acute clinical practice, including by point of care quantification. It is a non-specific marker elevated in multiple conditions. In the context of cardiovascular disease and vascular ageing, lactate has a role in multiple conditions including pulmonary arterial hypertension and heart failure [209]. Aside from its function as a metabolite and substrate in myocardial tissue, lactate is also a signalling molecule, promoting proliferation and migration of VSMCs [210], promoting angiogenesis [211], electrophysiological changes and vasodilation of vessels leading to vascular resistance [212]. Lactate has been used clinically in the assessment of sepsis and other conditions for decades, with reference data available in both child and adult populations at local biochemical laboratories [213] and no significant sex differences in lactate ranges have been noted.

2.8.7 Omics Technologies

One difficulty with biomarker development for CVD is that its development is multifactorial, with multiple different mechanisms predisposing an individual to pathology. As such, a multidisciplinary approach to CVD biomarker discovery may be required, which the advent of emerging technologies may be best placed to study. Plasma and urine metabolomics, peptidomics, proteomics and even genomics have shown promise in the detection of EVA, with evidence of alterations in individuals with ageing syndromes such as progeria [214] and good correlation to clinical vascular phenotype in those with hypertension [215–217]. These technologies offer potential for new biomarker discovery with the aim of precision medicine and treatments catered to the individual patient for maximum benefit [218]. Currently, however, omic analysis is expensive and results in the production of large volumes of data, of which much is currently of unknown clinical significance.

3 Future Directions

3.1 New Biomarker Discovery

Table 2 summarises the markers discussed in this review. As advances in technologies such as proteomics, metabolomics and genomics develop, more urine and blood biomarkers are likely to be reported in the coming years, although the clinical significance of these will require investigation. Given the significant number of potentially measurable targets, it is possible that multi-marker risk scores, combining the results of multiple different markers may be of increased utility in the diagnosis of EVA. Large-scale population data studies incorporating machine learning will be required to determine which are most useful and future studies should focus not only on the reporting of novel biomarkers but also on consideration regarding which are most accurate and accessible to researchers and clinicians alike.

3.2 Studies in More Diverse Populations

Particular research gaps have been noted within this review. Firstly, there is a paucity of data on the use of

biomarkers associated with EVA in children. This is disappointing given the fact that where EVA is detected early, there is potential for vascular plasticity to reverse the process of biological ageing and reduce future cardiovascular disease risk [4, 180]. As previously discussed, sex and gender clearly influence CVD pathophysiology but many of the reported biomarkers do not segregate data and may not consider that sex and gender have different definitions. In addition, the majority of studies do not report whether ethnicity alters biomarker results, an area which has become increasingly apparent as racial disparities in CVD have been reported. Finally, could any of these biomarkers be of clinical significance in other important at-risk populations, such as during pregnancy or the post-partum period to detect individuals who would benefit from increased lifestyle or other intervention to improve future CV health, or indeed the CV health of the offspring? To address these considerations, panels of markers should be measured in the urine and serum of large groups of healthy individuals from different populations and compared on an individual basis, and also in different combinations. Reporting of these studies requires that data be segregated to determine if different populations have different normative values. Comparisons should be made with clinical data such as BP, PWV and population data such as hospital admissions or primary care diagnoses to determine which are most likely to be clinically reliable.

3.3 Reference Data and Clinical Applicability

The challenge of interpreting biomarker results also remains. Many studies do report reference data, but as above, these need to be considered in the context of the population included in the study and the assay used. In addition, comparison with clinical biomarkers such as BP, PWV and BMI is required as well as longitudinal studies to determine the clinical significance of elevated biomarkers. Where biomarkers become endorsed in clinical practice guidelines, consideration also needs to be given regarding service delivery of CVD prevention strategies to ensure equitable access to biomarker detection and risk stratification, particularly given the preponderance of CVD in low-middle income countries.

4 Conclusions

A large number of different urine and blood markers of EVA have been reported in the literature to date, with a particular focus on markers associated with oxidative stress, inflammation, haemostasis and thrombosis, metabolic markers, cardiovascular injury and epigenetic changes. In conclusion, there remains a significant need for future research into biomarkers for CVD to confirm which are most useful in different clinical settings, all with a view to reduce the current burden of CVD throughout the world.

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