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Review Article

Diagnostic circulating biomarkers to detect vision-threatening diabetic retinopathy: Potential screening tool of the future?

Karen Frudd,[1] Sobha Sivaprasad,[1,2] Rajiv Raman,[3] Subramanian Krishnakumar,[3] Yeddula Rebecca Revathy[3] and Patric Turowski[1]

[1]Institute of Ophthalmology, University College London, London, UK
[2]NIHR Moorfields Biomedical Research Centre, Moorfields Eye Hospital, London, UK
[3]Vision Research Foundation, Sankara Nethralaya, Chennai, Tamil Nadu, India

ABSTRACT.

With the increasing prevalence of diabetes in developing and developed countries, the socio-economic burden of diabetic retinopathy (DR), the leading complication of diabetes, is growing. Diabetic retinopathy (DR) is currently one of the leading causes of blindness in working-age adults worldwide. Robust methodologies exist to detect and monitor DR; however, these rely on specialist imaging techniques and qualified practitioners. This makes detecting and monitoring DR expensive and time-consuming, which is particularly problematic in developing countries where many patients will be remote and have little contact with specialist medical centres. Diabetic retinopathy (DR) is largely asymptomatic until late in the pathology. Therefore, early identification and stratification of vision-threatening DR (VTDR) is highly desirable and will ameliorate the global impact of this disease. A simple, reliable and more cost-effective test would greatly assist in decreasing the burden of DR around the world. Here, we evaluate and review data on circulating protein biomarkers, which have been verified in the context of DR. We also discuss the challenges and developments necessary to translate these promising data into clinically useful assays, to detect VTDR, and their potential integration into simple point-of-care testing devices.

Key words: biomarker – diabetic retinopathy – serum – plasma – point-of-care testing

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Introduction

Diabetes mellitus (DM) is a complex group of diseases characterized by high blood glucose levels due to either an inability to produce insulin or an insensitivity to insulin. The number of adults with any form of diabetes worldwide is estimated to have quadrupled from 108 million in 1980 to 463 million in 2019 (International Diabetes Federation, 2019). This equates to a doubling of % incidence across the population (Zhou et al., 2016). Hyperglycaemia, caused by diabetes, is a major risk factor for microvascular complications of diabetes. Diabetic retinopathy (DR) is a highly prevalent complication of diabetes throughout the world, with around 35% of people with diabetes thought to also have DR. The worst outcome of DR is blindness; 10% of people with diabetes have VTDR, and, as such, DR is a leading cause of acquired blindness in the adult population (Cheung et al., 2010; Yau et al., 2012).

Diabetes is a global disease and has not spared any nation. Many low- and middle-income countries (LMICs) such as India and high-income countries such as China are now facing a high public health burden due to diabetes (in China, the prevalence of diabetes rose from 0.67% to 9.7% between 1980 and 2008 (Yang et al., 2010)) and consequently increasing levels of DR. Whilst early detection and tight control of risk factors have decreased DR prevalence in some western countries (Wong et al., 2009; Liew et al., 2014; Liew et al., 2017; Claessen et al., 2018), this is not the case in LMICs where all forms of DR continue to be on the rise (Leasher et al., 2016; Flaxman et al., 2017). As the disease is largely asymptomatic in its early stages and thus not detected by ophthalmic examination, there are no recommended treatments other than control of risk factors, until more advanced pathology is identified. Diabetic retinopathy (DR) is currently diagnosed through imaging of the retina, revealing changes consequent to damage of the retinal vasculature. This requires specialist equipment and

trained practitioners to both operate cameras and grade the images and is highly effective and efficient to detect referable cases of DR, as documented in countries with comprehensive healthcare systems. However, the ever-increasing number of patients with diabetes precludes the sustainable use of retinal imaging for universal and routine screening (Vujosevic et al., 2020). For many countries, the cost of DR screening and treatments runs into the tens of millions and likely into the hundreds of millions when taking into account absence from work (Aspelund et al., 2011; Andersson et al., 2020). In the case of many LMICs, health care provision is disparate and often privately funded, and so costly routine screening for DR by current examination pathways is simply not a viable option. Simpler and more cost-effective tests for DR are actively sought as they would benefit both high- and low-income countries. Indeed, new risk stratification pathways and novel technologies, including more portable imaging equipment, the automation of grading and the data integration into telemedicine pathways are currently trialled and validated (Natarajan et al., 2019; Karakaya & Hacisoftaoglu, 2020). Emerging technologies and cost assessments for new retinal screening modalities have recently been extensively discussed elsewhere (Vujosevic et al., 2020). Here, we will instead focus on reviewing promising blood-based protein markers that have potential to detect DR. Whilst many have withstood solid verification studies, caution is advised. Many markers have shown promise and specificity in detecting DR but may also be linked to the diabetic state generally, to inflammation or to parallel vascular morbidity. Thus, only large-scale clinical validation will reveal if simple and cheap blood tests, accessible to all people with diabetes, can be considered as an effective option in the arsenal of DR screening pathways.

Clinical features and current methods for diagnosis and monitoring of DR

DR is a progressive disease, which visibly affects the retinal vasculature. The initial instability of the vasculature eventually leads to microaneurysms and haemorrhages, and the consequent hypoxia triggers neovascularisation and, with breakdown of the delicate neuroretinal homeostasis, loss of visual acuity. Diabetic retinopathy (DR) is classified by observable clinical features of varying severities. In the early stages, the disease is asymptomatic and monitored for worsening but not treated. It is only in the later stages where there is a risk of vision loss that therapeutic intervention is applicable. It is widely recognized that effective screening and prompt intervention at the stage of VTDR limit losses in visual acuity (Jampol et al., 2020; Mansour et al., 2020). Indeed, clinical trials demonstrate that timely treatment for DR can reduce the risk of severe visual loss (ETDRS, 1991) (Wells et al., 2016).

The early stages of DR are referred to as non-proliferative diabetic retinopathy (NPDR) and are characterized by the emergence of initial damage to the retinal vasculature. Observable microaneurysms are usually the first sign of NPDR, and the disease is classified as mild if these are the only retinal lesions observed. Individuals with moderate NPDR have more microaneurysms and may also have evidence of intraretinal haemorrhage, venous beading and other microvascular abnormalities. If large numbers of these abnormalities are present throughout the retina but there is no evidence of neovascularisation, then severe NPDR will be diagnosed. Once evidence of neovascularisation is seen, proliferative DR (PDR) is diagnosed, which can lead to loss of vision and will require intervention (Wilkinson et al., 2003; Core NDESP team, 2012). A further complication from DR is the development of diabetic macular oedema (DMO). DMO is characterized by the presence of hard exudates, thought to be leaked lipids which appear as yellow or whitish deposits with either sharp or diffuse margins in fundus images. It is also accompanied by thickening of the retina, generally revealed by optical coherence tomography (OCT). DMO is categorized as mild, moderate or severe depending on the extent of hallmark alterations of the retina. Typically, if these are located at the centre of the macular, the disease is severe and results in moderate visual loss if left untreated.

DR is currently effectively diagnosed and graded by imaging (Goh et al., 2016). Colour fundus photography of the retina in seven overlapping fields has been the gold standard for almost 30 years; however, this type of test is time-consuming and can result in reduced patient compliance (ETDRS, 1991; Williams et al., 2004). In practice, up to three fields of fundus photography can provide adequate diagnostic power (Aptel et al., 2008; Vujosevic et al., 2009). One of the main downsides of colour fundus imaging is the difficulty in detecting DMO in 2D images. Optical coherence tomography (OCT), on the other hand, allows 2D and 3D analysis of the retina, showing changes in retinal architecture and thickness (Drexler & Fujimoto, 2008). Overall, this form of diagnosis is most accurate but also more costly. Not all people with diabetes, particularly in LMICs, can be subjected to annual retinal photographic screening due to the complexity and cost of this screening pathway and need for trained human resources (Vujosevic et al., 2020). To alleviate this problem, hand-held cameras for fundus photography are being trialled, but it will still not enable universal coverage and frequent systematic retinal evaluation. Therefore, there is an unmet need to identify those at risk of blindness from the population with diabetes so that they can be triaged to confirmatory retinal screening test.

In most countries, where this screening is used, people with diabetes are divided into risk categories, which forms the basis for the regularity of their check-ups, which again relies on retinal imaging. As only 8 to 10% of people with diabetes ever develop VTDR, isolating this group early will save much time and money. The availability of large datasets on individuals with DR has allowed some groups to devise algorithms, stratifying the risk of disease progression. Such methods, based on factors including duration of diabetes, HbA1c, systolic blood pressure, gender, and retinopathy grade, allow for more flexible screening intervals for those at lower risk and thus can reduce annual costs significantly (Aspelund et al., 2011; Broadbent et al., 2021). Whilst potentially highly effective for countries with defined DR treatment pathways, this type of monitoring is currently impractical and unachievable for LMICs where many of these factors are not routinely measured (Sivaprasad et al., 2020). Indeed, one study from India found that many

individuals only seek help once their vision has begun to deteriorate (Shukla et al., 2016).

More recently, it has been recognized that DR also involves retinal neurodegeneration, which can develop in the absence of clinically diagnosed microvascular disease (Sohn et al., 2016; Simó et al., 2018). Diabetic retinal neuropathy structurally affects a wide variety of non-vascular retinal cells and leads to measurable functional deficits (e.g. by electroretinogram). Whilst an extension of the currently used classification schemes such as the ETDRS has been proposed to incorporate novel technological advances and insights into DR pathogenesis including the comprehensive use of multimodal biomarkers (Abramoff et al., 2018), it should be noted that non-invasive technologies to detect neuropathy are even more resource-consuming than the imaging described above. Thus, additional focus on circulating biomarker may be of significant value to detect diabetic retinal neuropathy cost-effectively. Indeed, and in analogy to cerebral neurodegeneration (Ashton et al., 2020), the degenerating retina may give rise to tractable biochemical and molecular changes in the circulation, albeit possibly to a smaller extent due to its much smaller size.

Taken together, a unique blood profile that identifies individuals, who will develop VTDR, would revolutionize DR screening in all countries.

Pathogenesis of DR

Hyperglycaemia triggers multiple biochemical reactions, which contribute to the development and pathogenesis of DR (Brownlee, 2001). Oxidative stress, inflammation, accumulation of advanced glycation end products (AGEs), activation of protein kinase C (PKC), and dysregulation of the polyol and renin–angiotensin pathways can all contribute to vascular endothelial dysfunction leading to increased vascular permeability and/or neovascularisation, with no single process predominating (Cheung et al., 2010; Pusparajah et al., 2016; Wu et al., 2018; Antonetti et al., 2021).

Increased levels of circulating glucose during hyperglycaemia lead to surges in non-enzymatic glycosylation of proteins such as haemoglobin and basement membrane proteins. During persistent hyperglycaemia, as in DM, this initially reversible glycosylation becomes irreversible and leads to the formation of AGEs (Brownlee et al., 1988; Stitt, 2010; Xu et al., 2018). Accumulation of AGEs in the retina induces pericyte apoptosis, increased production of endothelial growth factors and subsequent neovascularisation, and increased inflammation, all prevalent hallmarks of DR. Increased flux through the hexosamine (fructose-6-phosphate to UDP-GlcNAc) pathway also leads to increased modification of proteins by o-linked glycosylation, further exacerbating a hyperglycaemic state (Brownlee, 2001).

High glucose concentrations also dysregulate glucose metabolism and in particular the polyol pathway, which converts glucose to sorbitol and then fructose (Safi et al., 2014). Enzymes of this pathway utilize both NADPH and NAD+, and during glucose-induced overload, large amounts of fructose will be produced at the expense of NADPH (Gabbay, 1973). This, in turn, results in an increased ratio of oxidized to reduced glutathione, and oxidative stress (Lorenzi, 2007).

Hyperglycaemia, through an excess of glycolytic intermediates, also leads to *de novo* synthesis of diacylglycerol (DAG), an activator of protein kinase C (PKC) (Koya & King, 1998; Guzik et al., 2002). In cultured endothelial cells, PKC activation causes permeability (Lynch et al., 1990). PKC activation also reduces endothelial vasodilation by dysregulation of endothelial nitric oxide synthase (eNOS) and upregulation of vasoconstrictors. In non-endothelial vascular cells such as smooth muscle cells and pericytes, PKC activation causes further vascular dysregulation. Due to the wide range of detrimental effects from PKC activation during DR, many studies have tested inhibitors for different isoforms of PKC *in vitro* and *in vivo* with mixed results (Davis et al., 2009; Geraldes & King, 2010; Wu et al., 2018).

Many of these hyperglycaemia-induced alterations of the vasculature and underlying neuronal-glia networks also result in non-specific inflammatory and oxidative stress responses, with increases in inflammatory mediators, such as IL-1β, IL-6, IL-8 and MCP-1 reported in plasma, serum and the vitreous and aqueous humour of DR patients (Youngblood et al., 2019). Naturally, DR shares many pathogenic mechanisms with DM, but also diabetic nephropathy (DN), another microvascular complication of DM. In both DR and DN, vessel stability and integrity are compromised, resulting in loss of function of the eye and kidney, respectively. Importantly, both of these microvascular complications of DM are risk factors for each other.

Persistent hyperglycaemia is considered a strong risk factor for the progression of DR. The Diabetes Control and Complications Trial (DCCT) reports that aggressive glycaemic control, along with control of blood pressure and circulating lipids, reduces DR progression in those with type-1 DM (Hainsworth et al., 2019). In a recent data-driven environment-wide association study, HbA1c has also been recognized as the strongest risk factor among over 400 laboratory parameters (Blighe et al., 2020) (see also below).

Biomarkers as tools for clinical assessment

A blood-based biomarker test for DR could provide a rapid, cost-effective and patient-friendly means of screening at the population level to identify those at risk of VTDR, broadening access to care globally. Biomarkers can identify disease and even subclinical disease, but are also used to monitor clinical response to treatments (Lyons & Basu, 2012). Therefore, biomarkers can be diagnostic, prognostic and predictive, and their purpose needs to be defined early. The best biomarkers are specific and easily monitored by non-invasive or minimally invasive methods, such as a blood test. For DR, many studies have focused on components in ocular fluids (Ma et al., 1996; Garcia-Ramirez et al., 2007; Kim et al., 2007; Gao et al., 2008; Simo et al., 2008; McAuley et al., 2014). As delicate surgical procedures are required to obtain them, they are clearly not practical for high-throughput screens at a population level. Nevertheless, many such studies have led to important insight into the pathogenesis of DR, e.g. the involvement of the kallikrein–kinin system (Liu & Feener, 2013), or formed the basis for subset selection in blood-based verification studies (Kim et al., 2007; Jin et al., 2016).

Typically, biomarker development needs to progress through several stages

before a clinically useful end-point is reached. These stages are often referred to differently but, broadly speaking, involve the following: a discovery step, whereby distinct control and target samples are tested in an unbiased way for any differences; a qualification step, where feasibility of identified markers is assessed in relation to the human disease of investigation; a verification step, where the specificity of markers is tested in a wider population-based sample set; and finally if a marker has passed all of these stages, it will have to be validated in target patient groups using an optimized clinical assay (Fig. 1A). Discovery and qualification are usually focused on demonstrating sensitivity, whereas verification and validation are concerned with specificity (see also below). The required sample number will generally increase through biomarker development, while the number of targets assessed will decrease. Importantly, whilst verification of protein biomarkers is often still done using medium-to-high-throughput methods such as MS, validation requires the development of a clinically robust (usually antibody-based) assay for each marker under investigation. An overwhelming majority of preclinical biomarker candidates never make it to clinical use and some of those that do are ineffective due to failures in either the analysis or experimental design of the above stages. This may well be due to biomarker development being led by specialists of the disease rather than of biomarker development. Indeed, many specialized articles describe in detail all stages of biomarker development, highlighting associated pitfalls and the importance of consistency throughout the process (Rifai et al., 2006; Ioannidis & Bossuyt, 2017).

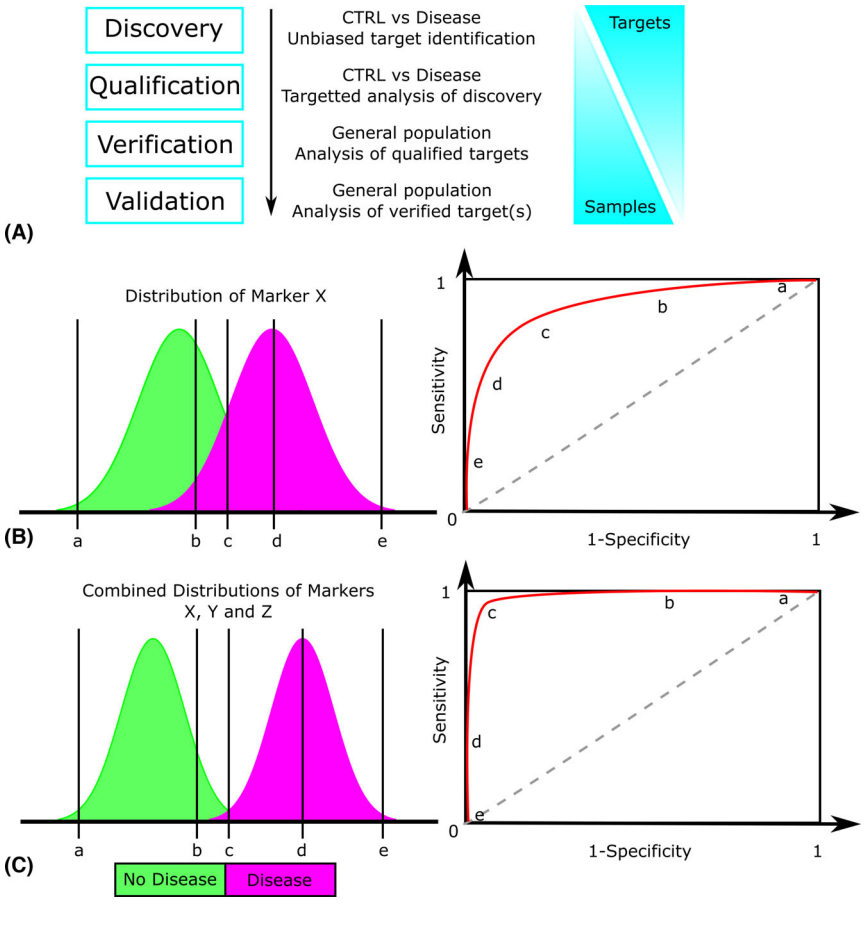

Fig. 1. The stages of biomarker development. A, Typical stages of biomarker research indicating the types of sample required and the relative number of targets and patient samples used at each stage. B, Potential distribution of a marker (X) in disease and control. Points a–e demonstrate how different thresholds relate to the sensitivity and specificity of this marker using ROC curve analysis. C, The contribution of multiple markers could be used to improve a tests ability to distinguish between disease and no disease. In this case, data from multiple markers can be combined into one value by principle component analysis (or similar), which may then result in two distinct populations. This results in an AUC closer to 1 with increased sensitivity and specificity.

Importantly, biomarker data must be carefully computed to reveal their desired diagnostic, prognostic and predictive value. Distribution plots for disease and non-disease groups reveal the overlap between the two. Theoretically, if all cases have reliably different values than all non-cases – with no overlap – then a perfectly accurate prediction is possible. In practice, considerable overlap exists and models of discrimination are used to assess how well a given biomarker separates the target groups. In biomarker research, the discrimination is most often measured using receiver operating characteristic (ROC) curves, or *c* statistics, with the area under the curve (AUC) used to distinguish the discriminating power of different models. For ROC curves, sensitivity (the ability to detect true positives) is plotted against 1-specificity (the ability to detect true negatives) across a range of thresholds creating a curve of increasing sensitivity with decreasing specificity (Fig. 1B, C). The area under the curve (AUC) gives a general measure of the accuracy of the test, with 1 indicating perfect prediction and 0.5 an equal likelihood of predicting disease or no disease, regardless of the biomarker value (Hoo et al., 2017). Points along the curve can be used to assess the relative specificity and sensitivity under those conditions and thus determine suitable inclusion or exclusion thresholds depending on the test and relative impact of misdiagnosis. Relying solely on *c* statistics and AUC has been criticized, in particular, when used for risk prediction (Cook, 2007). When the study cohort is representative of the general population, a large majority of cases will be non-disease with similar measures, whereas the disease cases will be much fewer but with greater variation in measure. Thus, a biomarker with a clinically significant odds ratio may show little discriminative power by *c* statistics; many risk factors used for cardiovascular risk prediction today would not be considered on the basis of AUC discrimination. Additional tests such as likelihood ratios, and further stratification of the disease cases may be required to ascertain the contribution of the measured parameter to the severity of disease.

Generally, individual molecules are used as biomarkers. While single biomarkers are simple to test for and analyse, they can only provide limited diagnostic information. Currently, only a few biomolecules have the required sensitivity and specificity to be used as reliable markers. Individual molecules, particularly those related to inflammatory pathways, could also be indicative of more than one disease, potentially leading to an incorrect diagnosis. A multi-marker panel could provide a more detailed diagnosis for complex diseases such as DR (Blighe et al., 2020). Multiple risk factors and pathways impact the development and progression of DR; thus, selecting successful classifiers that work across the population is challenging. In particular, shared pathological mechanisms of DR with other diabetic complications such as DN, but also other eye diseases, such as age-related macular degeneration, can be confounders. Multiple component biomarker panels have the potential for greater sensitivity, specificity and improved stratification of disease groups (Rusling et al., 2010).

Two verification studies from the Kim group suggest that the combination of multiple markers could enhance sensitivity and specificity of using blood-borne proteins in the detecting DR (Kim et al., 2013; Jin et al., 2016). These two studies identify early-stage DR biomarkers in plasma, through use of multi-marker panels, to enable accurate identification of individuals with VTDR. Candidate DR markers were analysed by multiple reaction monitoring mass spectrometry in plasma samples from people with all stages of NPDR (mild, moderate and severe) and people with diabetes without retinopathy (No DR) as a control. Twenty-eight and 15 candidate proteins, from each study, respectively, were found differentially expressed across the four disease groups. These markers were reduced by logistic regression analysis, in different combinations, to improve predictive power. Importantly, in each study, the combination of four markers stratifies mild NPDR and both mild and moderate NPDR cases, respectively, against No-DR cases much better than any single protein marker in isolation. If such four-marker panels withstood validation in a large clinical cohort, combined with inexpensive, high-throughput techniques, a new method to screen for DR could be rapidly developed.

Integrating biomarkers with sensors

Clinical biomarker testing traditionally requires samples to be taken by a medical professional, and then sent to a laboratory for analysis. Results can take several days, at which point the patient may need to visit their health care provider again to discuss the results and their options. Point-of-care testing (POCT) enables immediate readouts of results and, in some cases, enables patients to monitor their disease state themselves. Currently, this type of testing is used in a small number of instances, such as blood glucose monitoring and pregnancy tests. However, advancements in biomarker identification could make it possible to diagnose many more diseases at the point of care (POC). Biosensors can be employed as portable POC devices, designed to detect and quantify target biomarkers. They are powerful analytical tools in medical diagnostics and provide an attractive platform for monitoring highly prevalent diseases, such as diabetes and its complications. A biosensor can detect biological molecules such as proteins and nucleic acids or monitor antigen–antibody interaction, for example. To generate an easily interpretable readout, a biosensor requires an element that can detect the biomolecule of interest, which is connected to a suitable transducer, capable of converting the biochemical signal into a quantifiable readout (Sethi, 1994; Vo-Dinh, 2008; Devi et al., 2020). POC biosensors can be used in the clinical setting or in the field to give instantaneous results, reducing the need for travel and delays in diagnosis. To be maximally effective, electrochemical biosensors need to be easy-to-use, minimally invasive, sensitive, and inexpensive. This will enable rapid identification of at-risk patients with reduced dependence on centralized medical and laboratory facilities.

Integrated biosensors also often come with further practical benefits including low sample volume requirements, easy-to-use interfaces, portability and low energy requirements. These mean that, although some training may be required, highly skilled operators may not be necessary, and in some cases, patients may self-administer their tests. Overall, the lower costs and portability associated with this style of testing are ideal for LMICs where patients are likely to be spread across remote areas.

As sample processing is minimal or completely absent, POC biosensors must have high sensitivity and specificity for their target analyte in their target sample type (blood, urine or tear fluid, for example). Furthermore, as discussed above, complex diseases, such as DR, may require a combination of markers for accurate diagnosis. Thus, multiplex detection of biomarkers is necessary to ensure precise diagnostics and reduce costs in comparison with performing multiple single tests. Optimisation will, therefore, be required to enable the measurement of differentially expressed biomarkers in complex biological fluids, without interference from other highly abundant proteins.

The biosensor field continues to grow at pace, and the focus is not only on new target molecules but also new materials with enhanced capabilities in detection and signal transduction (Dinesh et al., 2019). Novel techniques, which do not rely on antibody-based detection, may also be developed (Devi et al., 2020; Shalini Devi et al., 2020). There is significant interest in developing single-step detection of pathogenic RNA and DNA in blood samples, which could be extended to circulating non-coding RNA. Rapid detection of targets in unprocessed sample fluids is a major problem in this field and requires reactions to be both highly specific and highly sensitive to identify low-abundance species in complex mixtures. Portability and compatibility with current technologies such as those in smart phones are also crucial to the success of novel biosensors (Banik et al., 2021). Miniaturisation of electronic transducers and microfluidics technology will hopefully maximize portability and allow multiplexing capacity in small devices. Ultimately, new diagnostic tests need to be an improvement on central laboratory testing that is the current norm. In some cases, cost per test may be increased, but this may result in improved management of chronic diseases, which will reduce overall health care costs. In the case of DR, even replacing a small percentage of imaging

required for diagnosis would result in a huge saving in both time and money.

Markers

As referred to previously, circulating biomarkers could be useful, not only for identifying VTDR but also for monitoring and stratifying patients based on their responses to treatments. Other reviews have covered promising biomarkers for DR and cover different molecule types as well as sample source and potential quantification or qualification (Jenkins et al., 2015; Raffort et al., 2015; Pusparajah et al., 2016; Ting et al., 2016; Nath et al., 2017; Safi et al., 2018). Here, we only focus on the most advanced circulating, protein biomarker candidates, for which verification data is already available, and for which clinical validation appears feasible using existing antibody-based platforms.

Glycated Haemoglobin (HbA1c)

Biological Role

HbA1c is formed by the non-enzymatic glycation of haemoglobin in the blood and, in healthy adults, accounts for 1–4% of total Hb (Rahbar, 2005). HbA1c reflects the average blood glucose concentration over the preceding 120 days due to the irreversible nature of the glycosylation and the circulating life of erythrocytes. It has been adopted as a measure for the presence of diabetes, alongside other blood glucose measurements (Goldstein et al., 2004). The long-lived nature of Hb glycosylation means such modifications can progress to AGEs which, as discussed above, can contribute to the pathogenesis of diabetic complications, including DR. Chronic hyperglycaemia, associated with diabetes, is thought to be the key driver of pathological changes indicative of DR and is measured by elevated HbA1c values (Cheung et al., 2010). The EURODIAB prospective complications study found that HbA1c level was significantly correlated with inflammatory markers measured in diabetic individuals, suggesting a link between persistent hyperglycaemia and endothelial inflammation (Schram et al., 2003).

Evidence

Many studies have identified the link between elevated HbA1c and increased risk of developing DR and several focus on maintaining strict control of HbA1c to mitigate this risk. For example, the DCCT study demonstrated that intensive control of HbA1c (<6.05%) in insulin-dependent diabetic patients reduced the incidence of DR significantly compared to less stringent control (DCCT, 1995). However, in this study, HbA1c and diabetes duration only explained ~10% of the difference in retinopathy risk, suggesting a significant contribution of other factors (Hirsch & Brownlee, 2010). More recently, Lind et al. reported that such strict control of HbA1c (<6.5%) does not confer a significant reduction in DR risk and increases the risk of hypoglycaemia in type-1 diabetics. They identify a range of 6.5–6.9% as ideal to prevent the development of serious complications. The risk of major and mild complications was significant for individuals with HbA1c >8.6% and >7.0%, respectively (Lind et al., 2019). Similarly, a 2012 meta-analysis of global data on DR prevalence identified an increase in prevalence of any DR from 18% to 51.2% when comparing HbA1c of <7 and >9 (Yau et al., 2012).

Not all studies find that Hba1c levels are statistically predictive of DR progression or severity however, likely due to differences in patient cohort and study design. For example, the Veterans Affairs Diabetes Trial found no benefit to strict glycaemic control with regard to DR risk; however, this group was mainly male, had a mean age of 60 years and around 40% had already experienced an adverse cardiac event (Duckworth et al., 2009).

More recently, Hba1c variability rather than its static value has been posed as a better predictor of DR risk. High variability between HbA1c measurements on successive clinic visits is predictive of new-onset DR but not directly predictive of progression to worsening forms of DR. Interestingly, abrupt decreases in HbA1c, as well as increases, contribute to this (Kilpatrick et al., 2008; Kim et al., 2021). Furthermore, in a Cox regression model predicting DR risk, the addition of Hba1c variability improved predictive power (Hermann et al., 2014). It is important to note that HbA1c variability can be calculated in different ways and not all give the same results with respect to DR prediction (Foo et al., 2017). HbA1c is inextricably linked to diabetes and the risk of developing further complications, although it is clearly not the only factor or marker. Taken together, HbA1c levels are a good predictor of DR risk and could become a useful clinical marker, especially when combined with other clinical readouts (Blighe et al., 2020).

Enzyme inhibitors

Alpha-2-macroglobulin (A2MG)

Biological Role. Alpha-2-macroglobulin (A2MG) is a major blood glycoprotein that functions as a proteinase inhibitor by physically entrapping a broad range of proteases including trypsin, thrombin and collagenase and delivering them to an endocytotic clearance pathway (Idiris et al., 2003; Wang et al., 2011). A2MG has also been implicated in immunomodulation and extracellular proteostasis (Borth, 1992; Armstrong et al., 1999; French et al., 2008). Alpha-2-macroglobulin (A2MG) is known to bind to growth factors and cytokines, including transforming growth factor-β, tumour necrosis factor-α (TNFα), interleukin 1β, interleukin 8 and vascular endothelial growth factor (LaMarre et al., 1991; Feige et al., 1996). Binding can result in degradation of the complex or stabilize circulating factors, depending on the form of A2MG, modulating immune and inflammatory responses.

Evidence. There has been a long-standing link between A2MG levels and DM, and a correlation between levels of A2MG and HbA1c has been noted (James et al., 1980; Turecký et al., 1999). Characterisation of the salivary proteome in individuals with type 2 diabetes mellitus (T2DM) indicated that A2MG was incrementally increased in the saliva of those in a prediabetic state and further increased in those with diagnosed T2DM (Rao et al., 2009). It has long been known that circulating A2MG is elevated in people with diabetes compared to healthy controls and even that elevated A2MG is associated with the presence of DR (James et al., 1980; Gray et al., 1982; Takada et al., 2013; Yoshino et al., 2019). Indeed, A2MG has been identified as a marker for DR in several sample types including saliva (Rao et al., 2009), vitreous (Kim et al., 2007) and, importantly for this review, in plasma (Kim et al., 2013). The latter work shows that A2MG is increased in plasma of patients with mild NPDR compared to no DR and is useful, in

combination with other markers, to identify patients with mild NPDR.

Cystatin C

Biological Role. Cystatin C (CysC) belongs to the evolutionarily well-conserved cystatin type 2 superfamily of cysteine protease inhibitors (Barrett, 1986). Originally identified in cerebrospinal fluid in humans, CysC has since been identified in all mammalian body fluids and tissues where it regulates endogenous proteinases including multiple cathepsins and papain (Bobek & Levine, 1992; Turk et al., 2008). CysC is particularly abundant in brain tissue (Hakansson et al., 1996) where it is expressed by neurons, astrocytes, endothelial and microglial cells (Yasuhara et al., 1993; Palm et al., 1995; Miyake et al., 1996). Cystatin C (CysC) is also used as a marker of glomerular filtration rate as it is completely removed from the circulation in the kidney and then almost fully resorbed by proximal tubular cells for degradation. Circulating levels of CysC remain fairly constant, and some studies refer to it as a housekeeping protein; however, changes in expression have been associated with diseases such as cancer, neurodegenerative disorders, DN and cardiovascular disease (Mussap & Plebani, 2004; Jeon et al., 2011; Kim et al., 2018).

Evidence. Recent studies have reported a positive correlation between serum CysC levels and DR in T2DM patients (He et al., 2013; Wong et al., 2015; Kim et al., 2018). Importantly, He et al., (2013) observed that circulating CysC levels are linked to the severity of DR and could be a predictor of VTDR. The authors showed that, along with the duration of DM and HbA1c, CysC is a risk factor for DR. The risk of VTDR was increased 11-fold in patients with serum cystatin C levels over 1.25 mg/L (He et al., 2013; Wong et al., 2015) and revealed that serum CysC in T2DM patients correlated positively with moderate DR, suggesting that CysC may play a role in the pathogenesis of DR, although the mechanisms are unclear. In the eye, CysC is secreted by the retinal pigment epithelium and could contribute to the progression of macular degeneration (Zurdel et al., 2002; Paraoan et al., 2010), thus explaining the correlation between serum CysC and DR. Notably, CysC has been analysed in serum using an electrochemical immunosensor with high sensitivity (Devi & Krishnan, 2020). Using 1.2 mg/L as a cut-off value the device showed 85% accuracy in predicting DR in a small ($n = 10$) cohort of clinical samples.

Plasma protein transport regulators

Apolipoproteins

Biological Role. Apolipoproteins are lipid-binding proteins, which help to transport triglycerides, phospholipids and cholesterol in blood, CSF and lymph. Apolipoproteins solubilize circulating lipids by forming lipoproteins, which are vehicles for the transport of lipids in the intra- and extravascular space. Apolipoproteins belong to several groups (ApoA–ApoH); individual forms are differentially expressed and associated with different types of circulating lipoprotein particle including chylomicrons, HDL, LDL and VLDL. Most apolipoproteins can move between lipoprotein particles as they are remodelled and circulate in the plasma, a feature that is referred to as exchangeable. ApoAI, the major protein component of HDL (constituting ~70%), is produced primarily in the liver and small intestine and is crucial to the regulation of cholesterol homeostasis. Furthermore, it possesses antioxidant, anti-inflammatory and atheroprotective properties and is involved in the anti-clotting process (Yui et al., 1988). ApoCIII is a component of very-low-density lipoprotein (VLDL), constituting ~40% of its protein mass, and HDL (Sundaram & Yao, 2012). It regulates the secretion and clearance of VLDL and inhibits the activities of several fat-metabolising enzymes (Mendivil et al., 2010). ApoAIV is secreted from intestinal enterocytes and is mainly associated with chylomicrons and is potentially involved in their assembly (Green et al., 1980). ApoB is a non-exchangeable apolipoprotein, remaining with the same lipoprotein from synthesis to cellular uptake and degradation. ApoB is mainly associated with VLDL, with one of its forms constituting the ligand for the LDL receptor (Boren et al., 1998). ApoB is strongly associated with the risk of developing coronary artery disease.

Evidence. Elevated serum levels of ApoAI and ApoCIII are associated with T2DM risk (Onat et al., 2009; Brahimaj et al., 2017), and analyses of vitreous fluid demonstrate a positive correlation between ApoA1 levels and PDR (Simo et al., 2008). By contrast, circulating ApoAI levels are inversely associated with DR, according to severity, in several studies of people with diabetes (Sasongko et al., 2011; Hu et al., 2012; Moosaie et al., 2020). A recent study by Zhang et al., (2018) further confirmed the association between circulating ApoAI and risk of DR but also found a positive relationship for ApoCIII levels. Elevated ApoA1 levels could be a protective factor against DR, where a baseline serum level of ApoAI $\geq$ 7.4 μmol/L was associated with a decreased risk of DR. In contrast, baseline levels of ApoCIII $\geq$ 6.3 μmol/L and ApoCIII-to-ApoAI ratio $\geq$0.9 correlated with an increased risk of DR (Zhang et al., 2018). Further studies by Chung et al. and Moosaie et al. also report an inverse relationship between ApoA1 and DR severity and a positive correlation of ApoB/ApoAI ratio to DR severity (Chung et al., 2019; Moosaie et al., 2020). Patients with type-1 diabetes in the DCCT/EDIC cohort with severe retinopathy had significantly higher circulating ApoCIII concentration, compared to those with moderate or mild retinopathy (Klein et al., 2005). Kim et al., (2013) describe strong correlations between apolipoproteins in plasma with differing severity of DR. ApoCIII, ApoAII and ApoAIV are reduced in mild and moderate NPDR, compared to controls without retinopathy. ApoAI and ApoC1 are elevated in mild NPDR, but ApoC1 only in moderate NPDR (Kim et al., 2013).

Afamin (AFM)

Biological Role. Afamin, an albumin superfamily member, shares 55% amino acid similarity with albumin. It is primarily expressed in the liver and secreted into the bloodstream (Lichenstein et al., 1994) and noted for its high degree of glycosylation (Lichenstein et al., 1994; Araki et al., 1998). It is highly abundant in plasma but can also be found in follicle, seminal and cerebrospinal fluid (Voegele et al., 2002). Its role in vitamin E-binding has been reported by several groups, and its ability to transport vitamin E across the blood–brain barrier has implications for neuroprotection (Heiser et al.,

2002; Jerkovic et al., 2005; Kratzer et al., 2009).

Evidence. Strong correlations exist between serum afamin and the development of metabolic syndrome and high BMI (Kronenberg et al., 2014). A population-based study on T2DM, including more than 20 000 individuals, also showed increased afamin concentrations in individuals with T2DM (Kollerits et al., 2017). Proteomics analysis showed decreased afamin expression in plasma from DR patients compared to no DR (Lu et al., 2013). Kim et al., (2013) point to its usefulness as a marker for DR since afamin, in combination with some of the other target proteins mentioned above, improves specificity in distinguishing moderate NPDR from T2DM patients with no DR.

Retinol binding protein 4 (RBP4)

Biological role. Circulating RBP4 binds and transports retinol (vitamin A), taking it from the liver to its target peripheral tissues. Retinol binding protein 4 (RBP4) solubilizes retinol, limiting the free amount in the circulation, which would otherwise be toxic. Many studies have identified links between RBP4, retinol, retinoic acid and obesity and its related conditions (Graham et al., 2006). These interactions and their implications have been reviewed comprehensively (Zabetian-Targhi et al., 2015; Olsen & Blomhoff, 2020).

Evidence. Several studies have reported positive correlations between circulating RBP4 levels and T2DM or DR. Takebayashi et al. found serum RBP4 to be elevated in patients with diabetes compared to healthy controls and to be correlated positively with other markers of T2DM. Furthermore, RBP4 is elevated in patients with PDR compared to DR and non-DR (Takebayashi et al., 2007). A similar trend was seen in another study where a significant positive correlation was also reported between serum RBP4 and urine albumin excretion rate (Li et al., 2010). More recently, in a cohort of 287 T2DM patients and 150 healthy controls, Li et al., (2018) found RBP4 to be significantly elevated in T2DM patients with DR or VTDR; the AUC was found to be 0.79 and 0.9 for DR and VTDR, respectively. However, other studies have reported a decrease in serum RBP4 with DM or simply no difference in circulating RBP4 in patients with DR (Akbay et al., 2010; Zhang et al., 2019a, 2019b). Due to the apparent links between RBP4 and metabolic disorders, correcting for BMI, fat deposition and urine albumin excretion may be key to elucidating genuine links between RBP4 levels and disease status.

Coagulation cascade mediators

Complement cascade proteins

Biological role. The complement cascade is a key component of the innate immune system, which modulates various immune and inflammatory responses (Walport, 2001a, 2001b). The complement system is always active at a basal level, but its activity is monitored by complement regulators. It is now recognized that chronic, low-grade inflammation and innate immune system over-activation are features and influencers of T2DM (McLaughlin et al., 2017; Saltiel & Olefsky, 2017). Recently, circulating exosomes have been postulated as potential activators of complement in diabetic models (Huang et al., 2018). Elevated, circulating complement factor B (CFB) increases the risk of endothelial dysfunction (Hertle et al., 2016), which may lead to coronary heart disease (Donahue et al., 2006). Complement factor B (CFB) binds component C3 forming C3B, contributing to the formation of the membrane attack complex (Ricklin et al., 2010). Therefore, CFB is essential for pathogen clearance and host cell apoptosis. CFH is a soluble serum glycoprotein that regulates the function of the alternative complement pathway in blood and on cellular surfaces.

Evidence. The complement system has been implicated in the pathogenesis of DR and related conditions. Increased circulating CFB has been found in south Asian populations at risk of developing T2DM (Somani et al., 2012), and expression of CFB in adipose tissue has a strong correlation with fasting glucose and circulating lipids (Moreno-Navarrete et al., 2010). Several studies have identified increased expression of CFB in the vitreous of DR patients, which led to further investigation of these proteins as potential specific markers for DR (Garcia-Ramirez et al., 2007; Gao et al., 2008). Wang et al. (2013a) showed that polymorphisms in CFH and CFB genes are associated with the development of DR and that the combined effect of CFH rs80029 and CFB rs1048709 results in a significantly increased risk of DR. Additional polymorphisms in the *CFH* and *CFB* genes are also correlated with a higher risk of developing age-related macular degeneration (Gold et al., 2006; Liu et al., 2010), a disorder that shares many pathophysiological features with DR. Based on differential expression of complement proteins in the vitreous of DR patients (Kim et al., 2007), Kim et al., (2013) identified CFB, CFH and complement component C3 as potential circulating markers for DR. In this case, plasma levels were decreased in mild and moderate NPDR patients compared to non-DR controls. An additional paper from the same group also identifies complement component C7 as a circulating marker for DR, with ROC curve analysis showing the highest AUC (0.85) of any single marker analysed (Jin et al., 2016). Upon analysis of vitreous and serum samples from PDR, NPDR and healthy controls, Shahulhameed et al. identified a decrease of CFB and an increase in CFH in the vitreous of PDR patients. In contrast, CFH levels were downregulated in serum of these patients (Shahulhameed et al., 2020). Therefore, CFB and CFH could be accurate markers of DR, but the sample type appears crucial.

Factor 2 (F2, Thrombin)

Biological role. Blood coagulation crucially prevents blood loss and thrombin, a serine protease, plays a central role in the coagulation cascade. In a first step, its inactive precursor, prothrombin is cleaved to form active thrombin (Jeon et al., 2011). Thrombin then cleaves and solubilizes fibrinogen into strands of fibrin, an important step in the formation of clots. It also plays a key role in platelet activation and the catalysis of other coagulation-related reactions. Further to its role in clot formation, thrombin is also a potent activator of angiogenesis and permeability during inflammatory responses (Maragoudakis et al., 2002; Mullins et al., 2009; Rathnakumar et al., 2016). Most mice lacking expression of thrombin die *in utero* due to defects in yolk sac vasculature, while those that are born succumb to haemorrhage on the first postnatal day (Sun et al., 1998). Mutations in the prothrombin gene, F2, lead to various forms of

thrombosis and dysprothrombinaemia (Girolami et al., 2018).

Evidence. Kim et al., (2013) point to the usefulness of F2 (in combination with other markers) to detect and stratify DR. Additionally, proteomic analysis, as well as targeted ELISA analysis, showed increased prothrombin in vitreous samples from individuals with PDR compared to individuals with no diabetes (Gao et al., 2008; Abu El-Asrar et al., 2016). Thrombin–antithrombin III complex (TAT) is a parameter of coagulation and could act as a proxy for thrombin levels. Plasma and vitreous TAT levels have been shown to be significantly higher in patients with retinopathy (Asakawa et al., 2000) and have been shown to positively correlate with the severity of DR (Dan-Brezis et al. 2020; Fujiwara et al., 1998). Given the many associations and activation steps thrombin is involved in, care must be taken in the comparison of studies.

Kallistatin (SerpinA4)

Biological role. Kallistatin (SERPINA4) is a serine protease inhibitor. Kallistatin binds to and inhibits the activity of tissue kallikreins which cleave kininogens to generate bioactive, pro-inflammatory kinins (Chao et al., 1990; Zhou et al., 1992). Bradykinin has been implicated in the pathogenesis of DMO and DR due to its pro-inflammatory and permeability-inducing effects (Liu & Feener, 2013). Kallistatin activity triggers multifactorial effects, including vasodilation and inhibition of oxidative stress, inflammation, fibrosis and apoptosis, primarily by increasing NO formation via eNOS levels (Chao et al., 1990; Chao et al., 2006; Shen et al., 2008; Shen et al., 2010; Yin et al., 2010; Li et al., 2014).

Evidence. Kallistatin levels have been shown to be significantly reduced in the vitreous fluids of patients with PDR and the retinas of streptozotocin-induced diabetic rats (Ma et al., 1996; Hatcher et al., 1997). Furthermore, Liu et al. showed that overexpression of kallistatin in an *in vivo* model ameliorates diabetes-induced retinal leukostasis and vascular leakage, by inhibiting diabetes-induced Wnt/β-catenin signalling pathway activation (Liu et al., 2013). Interestingly, Kim et al. (2013) showed a stepwise fold-increase in plasma kallistatin between control and mild NPDR subjects and also between mild NPDR and moderate NPDR subjects (Kim et al., 2013). This is in accord with other studies showing circulating kallistatin to be elevated in patients with diabetic vascular complications compared to control and subjects with diabetes with no vascular complications (Jenkins et al., 2010; McBride et al., 2014; El-Asrar et al., 2015). Kallistatin may, therefore, be a more generalized marker for diabetes but does appear to be further increased in patients with additional complications such as DR and thus be a valuable marker in combination with others.

Inflammatory markers

Lipoprotein-associated phospholipase A2 (Lp-PLA2)

Biological role. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a circulating phospholipase that binds to LDL-cholesterol (LDL-c) and HDL in the plasma. As an A2-type phospholipase, Lp-PLA_2 hydrolyses modified polyunsaturated fatty acids within oxidized low-density lipoprotein (oxLDL) releasing lysophosphatidylcholine (LPC) and oxidized non-esterified fatty acids, which can elicit a range of pro-inflammatory and pro-apoptotic effects (Silva et al., 2011). Elevated Lp-PLA_2 has been proposed as a predictive biomarker for several vascular diseases including stroke (Oei et al., 2005), atherosclerosis (Katan et al., 2014) and coronary heart disease (Thompson et al., 2010). Macrophages and other pro-inflammatory cells are a primary source of Lp-PLA_2 in the systemic circulation (Stafforini et al., 1990), although many ot her cells including endothelial cells also express this enzyme (Doublier et al., 2007).

Evidence. Lp-PLA_2 activity releases pro-inflammatory lipids, which have been implicated in endothelial damage leading to disruption of the inner blood-retinal barrier, observed in DR and DME. In vitro and in vivo studies with Lp-PLA_2 antagonists, darapladib and SB435495 (GlaxoSmithKline), have shown favourable responses in rats (Canning et al., 2016) and pigs (Acharya et al., 2017), improving visual loss by reducing retinal vascular leakage. Crucially, Lp-PLA2 inhibition has demonstrated efficacy as a treatment for DMO, improving visual loss and reducing retinal thickness (Staurenghi et al., 2015). Interestingly, Siddiqui et al. showed that, in an adult Caucasian population, increased serum Lp-PLA_2 activity is not only associated with increased risk of development of DR but also with a transition to more advanced forms of DR (Siddiqui et al., 2018).

Leucine-rich alpha-2-glycoprotein (LRG1)

Biological role. Leucine-rich alpha-2-glycoprotein (LRG1) is a highly conserved member of the leucine-rich repeat family of proteins (Andersen et al., 2010), which is involved in cell adhesion (Kobe & Kajava, 2001), granulocytic differentiation (O'Donnell et al., 2002), cell migration (Saito et al., 2002), signalling (Li et al., 2007), cell survival and apoptosis (Ai et al., 2008; Weivoda et al., 2008). Leucine-rich alpha-2-glycoprotein (LRG1) has already been identified as a marker for various chronic inflammatory diseases, including rheumatoid arthritis and asthma (Fujimoto et al., 2015; Honda et al., 2016). Additionally, it can act as a mitogen for endothelial cells in tumour neovascularisation and, importantly, retinal neovascularisation (Wang et al., 2013b; Zhang et al., 2016). Leucine-rich alpha-2-glycoprotein (LRG1) modulates endothelial transforming growth factor-β (TGF-β) signalling to promote angiogenesis (Wang et al., 2013b).

Evidence. Leucine-rich alpha-2-glycoprotein (LRG1) exclusively localizes with the vasculature of various human tissues including the eye. Interestingly, its expression increases in response to the murine model of oxygen-induced ischemic retinopathy (Wang et al., 2013b), which mimics neovascularisation seen in PDR. Plasma and intravitreal LRG1 has been described to be significantly increased in PDR patients, suggesting that LRG1 levels increase with DR progression (Chen et al., 2019; Hase et al., 2017; Zhang et al., 2019). This increase appears to be particularly detectable in more severe DR such as PDR but modest increases in milder forms of the disease could contribute to early detection.

Interleukin-6

Biological role. Interleukin 6 (IL-6) is a pleiotropic pro-inflammatory cytokine that is mainly secreted by monocytes (Navarro et al., 1989) and binds to its specific receptor (IL-6R) on the surface of cells. Also, IL-6 can bind to soluble

IL-6R and thus directly activate cells. Interleukin 6 (IL-6) promotes B-cell maturation and T-cell differentiation, while at the same time synergizing with TNFα and IL-1 to promote a systemic inflammatory response (Romano et al., 1997; Skelly et al., 2013). IL-6 production is rapidly upregulated in response to infections and tissue injuries; however, this is transient. As such, it is a key contributor to host defence through the stimulation of acute-phase responses, haematopoiesis and immune reactions. The expression of Il-6 is tightly controlled both transcriptionally and post-transcriptionally. However, dysregulation of these mechanisms can lead to continual synthesis, which affects the pathology of chronic inflammation.

Evidence. IL-6 has been implicated in the pathogenesis of DR because it is elevated in the vitreous fluid and blood of patients with DR (Schram et al., 2005; Kaviarasan et al., 2015; Feng et al., 2018; Yao et al., 2019). In the EURODIAB prospective complications study, circulating IL-6, in combination with C-reactive protein and TNFα, was able to stratify T1DM patients with no retinopathy, NPDR and PDR (Schram et al., 2005). Interleukin 6 (IL-6) may be a key early indicator of DR as higher circulating levels were detected in children with DR, who, crucially, will have diabetes and DR for much shorter times than adults (Zorena et al., 2007). Furthermore, IL-6 concentration in serum also positively correlates to the severity of DMO (Shimizu et al., 2002).

TNFα

Biological role. Tumour necrosis factor-α (TNFα) is a primary cytokine linked to many cellular processes. Crucially, it can promote the production of reactive oxygen species, promote leukostasis and induce blood-retinal barrier breakdown (Woo et al., 2000; Derevjanik et al., 2002; Chandrasekharan et al., 2007; Bradley, 2008). Tumour necrosis factor-α (TNFα) has two receptors, TNFR1 and TNFR2, through which it signals and regulates cellular functions including proliferation, survival, differentiation and apoptosis. Tumour necrosis factor-α (TNFα) is produced and secreted by macrophages and plays a pivotal role in inducing the cytokine cascade in many inflammatory diseases and is therefore under investigation as a therapeutic target for several diseases.

Evidence. As mentioned above, TNFα, in combination with other inflammatory markers, is an indicator of DR in T1DM patients (Schram et al., 2005). Circulating TNFα levels have been associated with retinopathy in several studies on patients with both T1DM and T2DM. In children with T1DM, this correlation was found to be a predictor of NPDR and was completely absent in healthy non-diabetics (Zorena et al., 2007). Elevated TNFα level was found to be associated with severe retinopathy in T1DM patients with kidney disease; however, at a 15-year follow-up, this correlation was no longer observed (Klein et al., 2009). In African-Americans with T1DM, it has been reported that baseline circulating TNFα is a predictor of PDR incidence as well as DME (Roy et al., 2013). Additionally, the TNFα level in tears is highly correlated with DR severity (Costagliola et al., 2013). Notably, moving towards alternative screening methods, a nanoparticle-based sensor has been described in a proof-of-concept study to detect TNFα in tear fluid (Chuang et al., 2018).

Basement membrane and extracellular matrix turnover markers

Collagen IV

Biological role. Collagen IV is an essential component of the basement membrane. It forms a mesh-like network, surrounding epithelial and endothelial cells, supporting cellular adhesion, migration and wound healing (Boudko et al., 2018). Due to its integral role in the basement membrane, collagen IV acts as a scaffold for many different binding partners. It is degraded by specialist proteases, releasing subdomains important for signalling. Increased urinary collagen IV is a biomarker for diabetic nephropathy and microangiopathy (Haiyashi et al., 1992; Lee et al., 1994; Yagame et al., 1997).

Evidence. Collagen IV concentration has been evaluated in the serum, urine and vitreous of patients with diabetes and its associated microvascular complications. Elevated collagen IV in each of these fluids is associated with retinopathy or other diabetic complications, such as nephropathy or microalbuminuria, in both adults and children (Haiyashi et al., 1992; Yagame et al., 1997; Nicoloff et al., 2001. Plasma collagen IV levels were identified as indicative of severity of DN and DR (Lee et al., 1994). Kotajima et al., 2001 also found that collagen IV was elevated in the serum and also vitreous fluid of patients with DR. In the vitreous, this increase also correlated with disease duration.

Matrix metalloproteinases (MMPs)

Biological role. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, which degrade and remodel all types of extracellular matrix, apart from polyglycan. Humans have at least 23 MMPs, out of a total of 28 found in vertebrates, which can be broadly subdivided based on their target proteins. Their functions involve tissue remodelling, wound healing, bone remodelling and cell migration, and, as such, MMPs can play roles in cancer metastasis and invasiveness as well as other diseases. In retinopathy, MMPs can degrade junction proteins, increase vascular permeability, exacerbate inflammatory responses, initiate cell death and promote neovascularisation (Kowluru & Mishra, 2017). In addition to this primary role, MMPs are also able to influence cell signalling behaviours through activation or inhibition of cell surface receptors. To prevent erroneous degradation, MMPs are produced as inactive pro-enzymes that need to be proteolytically activated. The activity of MMPs is closely regulated by different factors including tissue inhibitors of metalloproteinases (TIMPs), which serve as their endogenous inhibitors. The balance between MMPs and TIMPs is crucial to their function in tissue homeostasis.

Evidence. MMP14 was found at significantly higher levels in vitreous from patients with PDR compared to non-diabetic controls as well as in the retinae of diabetic rats (Abu El-Asrar et al., 2018). Additionally, MMP14 was also higher in patients with active neovascularisation compared to those with stable PDR. MMP1 has been found in the vitreous of patients with PDR (around 40% of patients) but is not present in those without DM. Furthermore, a correlation was also seen between those expressing MMP1 and those with the highest levels of VEGF (Kwon et al., 2016). Circulating MMP1, MMP7 and MMP9 have also

been found to be elevated in patients with diabetes, alongside the MMP/TIMP1 ratio, and these circulating factors are further increased in patients with DR (Jacqueminet et al., 2006) (Maxwell et al., 2001) (Abu El-Asrar et al., 2014). In the EURODIAB study MMP2, MMP3, MMP10 and TIMP1 were higher in PDR patients with adjustment for age, sex, duration of DM and HbA1c; however, when these results were further corrected for CVD and albuminuria, only the MMP2 changes remained significant (Peeters et al., 2015). As CVD is a common complication of DM, these MMPs alone are unlikely to be suitable markers for DR or PDR.

Other circulating factors

Advanced glycation end products

Biological role. As described above, AGE formation in response to hyperglycaemia is part of the DR pathogenesis (Brownlee et al., 1985). AGEs can perturb cellular function and also disrupt cell structure by accumulating in the vessel wall. In addition, they also act through specific receptors (RAGE) on endothelial cells, Muller glia, pericytes and retinal pigment epithelial cells by which they contribute further to vascular complications of diabetes. AGEs disrupt cellular homeostasis by modifying the extracellular matrix (ECM) but also by impacting on the action of hormones, cytokines and free radicals and the function of intracellular proteins (Brownlee et al., 1988).

Evidence. Two AGEs, in particular, have been proposed as biomarkers for DR. N-Epsilon-carboxymethyl lysine (N-ε-CML) is the most common circulating AGE and has been found at elevated levels in the serum of patients with diabetes and to an even higher extent in those with DR and other microvascular complications (Wautier et al., 2003; Boehm et al., 2004; Hirata & Kubo, 2004). Choudhuri et al., (2013) found that subjects with both PDR and NPDR had significantly increased total serum AGEs compared to no DR; however, the NPDR group had significantly higher levels of N-ε-CML than the PDR group. Kerkeni et al., (2012) showed that serum levels of AGEs were elevated in DR patients and also reported an increase in pentosidine, another AGE related to DR, in DR patients compared to controls. However, this may not be a highly specific marker for DR as, in the EURODIAB study, the association between pentosidine and DR was attributed to the duration of diabetes (Schram et al., 2005). To further complicate the presence of pentosidine in DR, Salman et al., (2009) found elevated levels in the early and moderate stages of NPDR, but this was lost once patients had developed PDR, as seen by some groups with N-ε-CML. Kidney disease is often a further complicating factor in the pathogenesis of DM. This may be key to levels of circulating AGEs in DM patients as AGE levels tend to increase with loss in kidney function (Hirata & Kubo, 2004).

Vascular endothelial growth factor (VEGF)

Biological role. Vascular endothelial growth factors (VEGFs) are a family of endothelial-specific cytokines which have functions in both physiological and pathological angiogenesis of different vessel types throughout the body. VEGFA is the prototypical form, often just referred to as VEGF, responsible for endothelial homeostasis but also vascular permeability. Dysregulated levels of VEGF can lead to aberrant leakage and vessel growth and have been directly implicated in the pathogenesis of DR. VEGF also drives early events of DR pathogenesis by inducing ICAM-1 expression, leading to leukocyte adhesion and blood-retinal barrier breakdown (Joussen et al., 2002). Due to the roles of VEGFs in DR, anti-VEGFs are increasingly used to treat advanced retinopathies; however, they are not effective in all patients (Ford et al., 2013). In this regard, it is interesting to note that, in at least one study, VEGF was not detectable in the ocular fluids of some patients with DR, which may explain why not all DR patients respond to anti-VEGF treatment (Aiello et al., 1994).

Evidence. Many studies have described links between circulating VEGF levels and DR; however, these studies often do not agree on the degree of correlation or ability to predict disease severity. Increased serum VEGF levels have been linked to DR and raised HbA1c values (Celebiler Cavusoglu et al., 2007), which have also been associated with an increased risk of DM complications (Nordwall et al., 2015). Furthermore, several studies have shown that VEGF levels in serum are increased with the severity of DR (Celebiler Cavusoglu et al., 2007; Du et al., 2014). Levels of serum VEGF correlate positively with disruption of the external limiting membrane and the inner-segment–outer-segment junction, suggesting that increased serum VEGF is associated with severity of DR (Jain et al., 2013). Ozturk et al., (2009) reported a significant correlation between serum VEGF and severity of DR although there was no statistically significant difference between NPDR and PDR. A further study reported that although VEGF significantly increases in DM compared to controls, it is lower in PDR compared to NPDR (Suguro et al., 2008). Other studies, such as the one from Chaturvedi et al, found only a weak correlation between VEGF and the severity of DR, this time in plasma (Chaturvedi et al., 2001). A recent meta-analysis of 29 different studies found that these showed overall that serum but not plasma VEGF levels were increased in DR patients compared to controls, with increases also correlating with severity of disease (Zhou et al., 2019).

Limitations of circulating protein biomarkers

Diseased tissues generally display molecular signatures related to their pathology and pathogenesis, and these can sometimes be utilized through tissue biopsies. However, serum and plasma are preferred for biomarker-based tests: they can be considered a circulating representation of all body tissues, also reflecting disease-specific molecular signatures. Discovery of proteomic signatures is often hampered due to the complexity and dynamic range of serum and plasma (often requiring predepletion of highly abundant constituents). In addition, with pathologies that are restricted to a relatively small proportion of the body, many specific biomarker changes cannot be detected reliably. This is undoubtedly an important factor for biomarkers of DR, as the retinal blood volume constitutes a small proportion of the total circulation. For instance, increased intraocular VEGF has been measured in the vitreous of all forms of DR, but changes in circulating levels

do not reflect this robustly enough to justify its use as a blood-based biomarker. In the case of pigment epithelium-derived factor (PEDF), circulating levels are increased in patients with PDR, compared to those without, yet in ocular tissue, PEDF levels are lower in patients with late-stage DR than in those without retinopathy (Jenkins et al., 2007; Li et al., 2012; McAuley et al., 2014). This may not be an issue, in theory, provided the results for circulating levels are consistent and reliable. However, it does pose questions regarding why this is the case and what altered levels of PEDF are indicative of. In this case, targeted basic science studies can show how biomarker levels correlate to pathogenesis (Elahy et al., 2014) and, in combination with more longitudinal studies on patients, could help to develop a more nuanced classification of disease. Lastly, as DR constitutes a complication of a complex systemic disorder, one should be wary of changes that may in fact not be specific for DR but possibly of generalized inflammation or vascular disease.

Biomarker validation is highly dependent on preanalytical specimen handling, which needs to be standardized to minimize technical variance between studies (also reviewed by (Rifai et al., 2006)). Several studies have demonstrated significant changes in the levels of analytes following different processing protocols. Additionally, protein biomarkers are not very robust analytes, which could make them disadvantageous in an outpatient setting, where the period between sampling and sample analysis can sometimes vary due to unforeseen delays. Other extreme conditions such as repeated freeze and thaw cycles can compromise protein stability in serum. Additionally, classical immunoassays, such as ELISA, are highly sensitive, but labour-intensive and challenging to implement for multiplexing detection.

Biomarkers must be validated on large cohorts to determine usefulness across the general population. However, changes that are only significant in large cohorts may not provide sufficient specificity and sensitivity in individual patients. New biomarkers also need to be tested on diverse populations in case they have altered specificities based on gender, age, ethnicity or type of diabetes. Indeed, it has recently been suggested that people with diabetes can be more usefully subdivided into five groups, based on clinical characteristics, rather than the two more commonly used to date. These cohorts allow better stratification of disease outcomes and could provide an early indication of complications (Ahlqvist et al., 2018). None of the DR biomarker verification studies have correlated marker levels to these more advanced clinical subgroups of diabetes. In addition, DR classification often differs considerably between studies, thus making direct comparison difficult. In line with this, pre-existing comorbidities, medications and other environmental factors could also alter biomarker levels and their relationship with DR. Consideration of such comorbidities is not always included in study design and analysis, which could explain, in part, some differences between reports. Nephropathy is a closely related microvascular complication to retinopathy and many studies describe a greater risk of retinopathy in patients with nephropathy and cardiovascular disease risk is also elevated in patients with existing diabetes complications (Hahr & Molitch, 2010; Son et al., 2011; Grunwald et al., 2012; Rajalakshmi et al., 2020). This is perhaps unsurprising as both diseases affect microvascular beds, dense with capillaries, and share many of the same risk factors including high HbA1c, duration of diabetes, hypertension and poor lipid control (Romero et al., 2007; Lee et al., 2014). Therefore, biomarkers may in fact stratify the high-risk group of people with diabetes that should be triaged for at risk of complications. In addition, validation in multiple cohorts needs to be done before clinical pathways can be redesigned to include biomarkers and biosensors.

Future trends

Circulating biomarkers will continue to evolve with increased identification of markers, ongoing improvements in detection limits, and reduction of the operating cost and time. Furthermore, new technologies, including proteomics, metabolomics and genomics, will enable exploration of previously unavailable target molecules and will potentially lead to identification of novel biomarkers.

As discussed in this review, electrochemical biosensors have emerged as advantageous molecular sensing devices with the potential to benefit POC diagnostics (Shalini Devi et al., 2020). Furthermore, the emergence of nanotechnologies is providing new materials and methodologies for POC devices, reducing sample volumes and improving portability (Pirzada & Altintas, 2019). It is also becoming increasingly possible to couple devices to smartphones, allowing for at-home testing and increasing the possibility to monitor complex conditions with regularity (Kou et al., 2020). Traditional tests for many conditions, such as diabetes, use antibody-mediated detection to confirm the presence or quantity of a target analyte. Miniaturisation of this process must take into consideration the stability of the biological components, ease of sample preparation, as well as the cost and reliability of the device (Chen et al., 2020). Devices and reactions need to be particularly robust if patients are to self-administer as there will be variations in compliance and environment.

The use of blood-based biomarkers is ubiquitous throughout current medical practice and many types of molecule can be detected, including proteins, lipids and sugars. However, in recent decades the research community has been exploring additional metrics such as circulating RNAs and metabolic by-products. There is a wealth of published data on the use of microRNA as biomarkers for DR as well as more novel omics approaches such as metabolomics (Raffort et al., 2015; Gong & Su, 2017; Zhang et al., 2017; Martinez & Peplow, 2019; Zhu et al., 2019); however, this is beyond the scope of this review. Additionally, proteomics techniques are being applied to different sample types to identify more specialized markers. As discussed in this review, and others, aqueous and vitreous humour are only obtained with invasive surgery and so not suitable for screening; however, tear fluid could be a non-invasive sample source for detecting diseases of the eye (Csősz et al., 2017).

It is anticipated that any novel biomarkers will be embedded in current and future care and diagnostic pathways, and undoubtedly current screening methods and pathways will evolve at pace as well. Thus, for DR

and many other retinal disorders, automated image analysis and cloud technologies are being harnessed to reduce the need for manual retinopathy grading (Trucco et al., 2013; Tufail et al., 2017). Recent work on the use of machine learning has shown that with a large amount of data, an algorithm can be trained to detect DR from fundus images (Takahashi et al., 2017; Ting et al., 2017). In addition, the use of smartphone cameras is being explored to improve the accessibility of imaging analyses. Progress is also being made in using non-mydriatic cameras, thus further easing burden of intervention (Nderitu et al., 2021). Machine learning techniques promise to detect early changes in vasculature, which may be beyond the capabilities of any trained ophthalmologist, and thus will form a key part of future telemedicine. Nevertheless, they may continue to require high-quality images, which cannot be easily obtained for the most remote patients, and data storage, processing and administration will continue be associated with considerable cost and requirement of expert input. Overall, this leaves a clearly defined role for a routine and cheap biomarker test, should it become available.

Whilst current biomarker development focuses on detecting and stratifying ongoing retinopathy, future studies should also explore if predictive molecular signatures can be identified. In addition, markers that predict the effectiveness of current interventions for individuals with DR could reduce costs and streamline clinical pathways considerably. For instance, even with aggressive anti-VEGF treatment around 50% of patients have persistent macular oedema and moderate to no improvement in their visual acuity (Ford et al., 2013), suggesting a different treatment plan could have been more beneficial. However, developing predictive markers will require much more extensive longitudinal cohort studies fuelled by clearly defined preclinical candidates.

Conclusion

Efficient, cost-effective methods for monitoring DR and specifically for identifying early-stage VTDR will be a game-changer in the management of this disease, particularly in LMIC. Circulating biomarkers could be complementary to existing pathways, not only for identifying these patients but, also, for stratifying patients according to their treatment responses and monitoring their progress. Indeed, a more holistic approach to diagnosis and care of all microvascular complications of diabetes may be the most appropriate model, and circulating parameters are the best surrogate for such disease phenotypes. Effective collaboration between specialists would undoubtedly improve the risk stratification of individuals with diabetes. However, a cheaper screening marker may help stratify the population with diabetes better, so that the group at risk of complications can be triaged for more detailed screening of complications using gold standard tests. For example, DR is a costly disease in all countries, either through cost of treatment and monitoring or through the burden of blindness. Therefore, all available tools should be exploited to suit the means and requirements in each region or country.

At present, a selective marker for early-stage DR remains elusive. In reality, it may be most achievable to identify people with diabetes most at risk of developing any form of microvascular complication and then further triage these people to the most appropriate specialists. For either of these outcomes, large, comprehensive studies are required comparing markers for different microvascular complications of DM.

If a blood-based test or sensor can be developed, this could easily be incorporated into existing clinical settings or laboratories for onward referral to specialist care centres. Streamlining this diabetes care pathway will have significant immediate impact, especially in LMIC, where patients tend to self-refer themselves when complications are already advanced and symptomatic. Nevertheless, the complexity of integrating a blood-based test into some existing clinical practice should not be underestimated. Even regular HbA1c measurements are not accessible to many people with diabetes.

Many small studies have identified and verified potential circulating biomarkers for DR; however, none of these have been validated in large multi-centre studies. Multiple potential confounders need to be addressed in the search for screening markers, including geographic, ethnic and genetic variations in the study populations as well as the varying phenotypes of DR. Therefore, large-scale, collaborative, multi-centre studies will be needed to conclusively validate and determine the reliability of the various biomarkers of DR.

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Correspondence:
Patric Turowski, PhD
Professor of Vascular Signalling
UCL Institute of Ophthalmology
University College London
11-43 Bath Street
London EC1V 9EL
Tel: +44 (0) 20 7608 6970
Fax: +44 (0) 20 7608 6810
Email: p.turowski@ucl.ac.uk

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