

Circulating Biomarkers of Inflammation and Endothelial Activation in Diabetic Retinopathy

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Inflammation and endothelial activation play a pivotal role in development and progression of diabetic retinopathy (DR), a vision-threatening complication of diabetes mellitus (DM) and the leading cause of blindness in the working age population. Easily accessible and validated biomarkers for DR early diagnosis and progression are required for use in clinical trials: here, we reviewed the available literature to understand the association of circulating levels of selected markers of inflammation and endothelial activation with the presence of nonproliferative and proliferative DR (NPDR and PDR) and investigate the relationship between their systemic and ocular levels. We additionally provide data synthesis and perform statistical analysis for interpretation of the collected evidence. CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1 circulating levels were increased in subjects with DM compared to healthy individuals. Moreover, TNF α and sVCAM1 showed increasing systemic levels with DR presence and severity; circulating CRP increased with the transition from no DR to NPDR, whereas IL-6 was increased in PDR compared to NPDR stages. The relationship between ocular and systemic concentrations of these proteins remained unclear due to the low number of studies with matched sampling. In conclusion, the available data supports the use of systemic biomarkers of inflammation and endothelial activation to identify DM status and DR severity. These systemic biomarkers are likely reflecting an overall state of inflammation and vascular activation in different tissues of the body, including the eyes. Prospective, longitudinal datasets are required to validate these biomarkers as predictors of early DR presence, of DR progression, or for disease monitoring.

Introduction

Diabetes mellitus (DM) is a systemic metabolic disease affecting 1 in 11 adults worldwide, for a total of approximately 463 million people, a number that is expected to rise in the next decades.¹ Hyperglycemia (i.e. elevated blood glucose levels) is the main clinical characteristic of DM, which can occur in two forms: type 1 DM is an early onset form of DM due to autoimmune-mediated insulin deficiency; type 2 DM accounts for approximately 90% of the total cases and is caused by insulin resistance and progres-

sive pancreatic β cell failure. In both cases, diabetic complications can arise as a consequence of long-term hyperglycemia, metabolic disturbances, including dyslipidemia, oxidative stress, hemodynamic changes, and low-grade inflammation, and affect the entire body and its tissues. The traditional concept of diabetic complications as purely mediated by vascular disease has evolved in recent times and the direct involvement of the whole tissue has been recognized: for example, growing evidence indicate that neurodegeneration plays an important role in diabetic retinopathy (DR)² and the same is true for podocyte drop out in diabetic nephropathy.³ Vascular complications

of DM can be categorized into microvascular and macrovascular depending on the caliber of the involved blood vessels.⁴ Damage to the small blood vessels in the retina is a hallmark of DR, a microvascular complication which affects one in three subjects with DM and is the leading cause of blindness in the working-age population.¹ There are various stages of DR, which are classically described according to the vascular phenotype. DR starts as a nonproliferative form (NPDR) diagnosed based on visible microvascular abnormalities, such as microaneurysms, retinal hemorrhages, and hard exudate. Early NPDR often remains undiagnosed due to undetected visual impairment and can progress to the proliferative DR form (PDR), a serious sight-threatening condition characterized by neovascularization of the retina. Diabetic macular edema (DME) is also a vision-threatening complication that can occur at any DR stage and is characterized by exudation and swelling of the macula, the central part of the retina.⁵ In 1991, the Early Treatment Diabetic Retinopathy Study (ETDRS) group developed and reported a DR severity scale (DRSS) based on fundus photography that is still widely used to diagnose, classify, and monitor progression or improvement of DR, ranging from the lowest score of 10 (absence of DR) to a maximum of 85 (advanced PDR) and including mild, moderate, and severe NPDR and PDR stages in between.⁶ Whereas clinical diagnosis and staging of DR based on such fundus images is well established in the clinics and clinical trials, molecular biomarkers that could help identifying patients who are prone to develop ocular complications before they are detectable in fundus pictures (early NPDR diagnosis) are not available at the moment. In addition, biomarkers of DR progression that could help identifying groups of patients with higher risk of developing vision-threatening forms of DR are needed to improve the performance of currently available systemic metabolic markers (see below). A combination of such biomarkers could enable their use in clinical trials or even contribute to the appropriate treatment selection for individual patients in the future.

Although the exact mechanisms are unknown, evidence supports involvement of inflammation in development and progression of DR (reviewed in Refs. 7–12). Briefly, the levels of several inflammatory mediators, such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF α), are increased in vitreous or aqueous humor (VH and AH) of subjects with DR. Their contribution to DR lesion development, like breakdown of the retinal-blood barrier, vessel leakage, and microglia activation, has been shown in animal models of diabetes. Along the same line, anti-

inflammatory agents can be effective in slowing down disease progression in DR experimental models and affected individuals. A key process linked to inflammation in DR pathogenesis is endothelial activation (i.e. expression of adhesion molecules on retinal vessels and circulating leukocytes), and consequent leukostasis and immune cell infiltration into the surrounding tissue.^{13,14} This represents an early process in the immune / inflammatory response to retinal vasculopathy, including basement membrane thickening, loss of pericytes, and ischemia, during manifestation of DR in the eyes.¹⁵ Overall, these inflammatory and vascular changes are thought to contribute to tissue damage and neurodegeneration in the diabetic retina ultimately leading to the development of sight-threatening events, including DME and / or PDR in 6.81% and 6.96% of patients, respectively.¹⁶ In line with this, the risk of progression from NPDR to DME and PDR over a 3- or 5-year period has been reported to be higher in patients who were more severely affected at baseline.^{17,18}

Complications of diabetes, including DR, arise as a consequence of a systemic dysfunction, as indicated by the fact that increased disease duration, poor glycemic control (elevated HbA1c levels), dyslipidemia, and high blood pressure are systemic risk factors for DR development and, in case of HbA1c and blood pressure, progression toward vision-threatening disease.^{12,19,20} There is, however, controversial clinical evidence that dyslipidemia is associated with DR^{21,22} and that blood pressure control prevents the incidence and progression of DR.^{23,24} Besides these metabolic factors, several studies explored the levels of inflammatory and endothelial activation mediators in the plasma or serum of subjects with DM / DR and their association with presence of the disease (cross-sectional studies) or risk of developing the disease (prospective, longitudinal studies). These individual analyses resulted in controversial data about the relationship of such markers and DR. Systematic reviews and meta-analyses have been conducted to address this issue and generally concluded that circulating markers of inflammation and endothelial activation are increased in the DM population when compared to healthy controls and in subjects with DM with complications compared to subjects without complications.^{25–32} The prospective risk of developing the disease or its complications is also associated with higher levels of these markers in the blood.^{33,34} Most of the published reviews and meta-analyses, however, did not focus on different DR stages or took only one type of DM into consideration. Therefore, no clear association between circulating levels of inflammatory and endothelial activation biomarkers and the different DR stages has been

established so far. This association is the basis for identification of potential biomarkers of early diagnosis and / or disease progression.

Based on literature evidences, data availability, and translational potential for use as biomarkers in clinical trials, we selected four markers of inflammation (CRP, IL-1 β , IL-6, and TNF α) and two markers of endothelial activation (sICAM1 and sVCAM1) either known to be linked to DM or previously suggested, but not proven, to be associate to DR. In particular, C-reactive protein (CRP) is produced by the liver and considered a biomarker of systemic inflammation, type 2 DM, and risk of cardiovascular disease.^{35,36} IL-6 and TNF α are potential biomarkers of DR^{31,37} and, together with interleukin 1 β (IL-1 β), may contribute to the pathological mechanism of DR (reviewed in Ref. 10). Intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1) are upregulated on the surface of activated endothelium in inflammatory conditions, including DR; ICAM1 is additionally found and upregulated on the membrane of activated leukocytes.³⁸ ICAM1 and VCAM1 soluble versions (sICAM1 and sVCAM1) can be detected in circulation as biomarkers of endothelial activation.^{39,40} Because the evidence for leukostasis involvement in DR mainly comes from preclinical models,^{13,14} we selected these two proteins to explore the available clinical data on endothelial activation markers.

Here, we aimed to assess the association between systemic levels of the selected proteins and DR presence and severity by conducting a review of the available published data with the goal of identifying candidate systemic disease stage biomarkers and thereby contributing to the understanding of the pathological mechanism of DR with focus on inflammation and endothelial activation. Our goal is also to investigate the value of understanding the interplay between systemic and local biomarkers for disease diagnosis and progression and how this could be enabled by systematic sampling strategies in future clinical research. For interpretation of the collected evidence, we additionally provide data synthesis and perform statistical analyses using some of the tools used for meta-analyses, including evaluation of heterogeneity and subgroup analysis. Disease stage markers, if validated in longitudinal cohorts, could be used as easily accessible biomarkers of early diagnosis, progression of ocular complications, prediction, and / or monitoring of disease and response to therapy in clinical trials. Moreover, we reviewed the selected studies on the relationship between circulating and ocular (VH) levels of the same biomarkers in the subset of studies providing this additional information

in order to better understand the source of changes relevant for disease progression.

Methods

Search Methods for Identifying Studies

EMBASE was used as primary source for a literature search up to end of 2019 using the following terms: (“c reactive protein” OR “interleukin 1beta” OR “interleukin 6” OR “tumor necrosis factor” OR “intercellular adhesion molecule 1” OR “vascular cell adhesion molecule 1”) AND (“blood levels”) AND (“diabetes mellitus” OR “diabetic retinopathy”). The terms were chosen following recommendations of the Emtree vocabulary for biomedicine. Search results were filtered by study type “human” and publication type “article.” Additional records were identified through separate searches using the Clarivate Integrity Biomarker module database⁴¹ and MEDLINE with the following terms: “systemic biomarkers” AND (“diabetes mellitus” OR “diabetic retinopathy” OR “non-proliferative diabetic retinopathy” OR “proliferative diabetic retinopathy”).

Eligibility Criteria for Considering Studies for This Review and Study Selection

Studies meeting the following criteria were included in this review:

- The study was case-control and cross-sectional (for longitudinal or prospective studies: cross-sectional data available).
- The study included participants of any age, healthy or affected by DM, type 1, or type 2 with and without DR, at any time from diagnosis, regardless of previous and ongoing treatment, and giving consent for participation in the study; severity of DR was classified into absence of disease (no DR), non-proliferative DR (NPDR) and proliferative DR (PDR), regardless of the status within each individual group (mild, moderate, or severe disease) - which would roughly correspond to DRSS 10 (no DR), 20 to 53 (NPDR), and 61 to 85 (PDR) - and regardless of presence of DME.
- Blood (serum or plasma) concentration of at least one of the proteins of interest (CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1) were reported and expressed as mean and standard deviation (SD) or standard error of the mean (SEM) or 95% confidence interval (CI); available

data included sample size (N) and measurement unit (e.g. pg/mL).

- The study was reported in English or Spanish and published as an accessible journal article, conference abstract, or as part of a previous meta-analysis providing the data listed above.

Eligible full-texts or conference abstracts were subjected to a second screening round and 144 studies were finally included in this review.

Data Collection and Risk of Bias Assessment

For synthesis and statistical analysis, the following data were extracted from abstracts or full-texts of the selected records: mean, SD or SEM or 95% CI and measurement unit of the blood concentration of CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1, number of subjects included, DM type, and DR status of study population. In addition, study design, sample matrix (plasma or serum), and method (e.g. enzyme-linked immunoassay [ELISA]) used to determine protein concentration were recorded together with matching vitreous concentration of the markers, if available. All data were converted to the same measurement unit (ng/mL for sICAM1 and sVCAM1; pg/mL for IL-1 β , IL-6, and TNF α ; and mg/L for CRP). If SEM was given, the SD was calculated using the formula: $SD = SEM * \sqrt{N}$. If 95% CI was given, the SD was calculated using the formula: $SD = (CI\ upper\ limit - CI\ lower\ limit) * \sqrt{N}/3.92$. If overall data for the DM group were not available, pooled estimates were derived combining the data of the single DM subgroups (no DR and / or NPDR and / or PDR); number of the single subgroups were summed to get the overall number of the DM group. If study design was not explicitly stated in the published record, the authors inferred this information from the description of the study design.

One author (F.S.) extracted data from the publications and recorded them into tables, which were used as input for statistical analysis after review from the same author (Supplementary Table S1). Evaluation of obvious bias was performed at this stage: studies reporting questionable data (e.g. order of magnitude or measurement unit, incompatible format of summary data, and datasets incompatible with comparisons of interest) were excluded. An additional check, also including plausibility of the data, was performed by an independent author (J.P. or M.A.) at the time of analysis: potential conflicts, outliers or imprecisions in data extraction were discussed and resolved by three authors (F.S., M.A., and U.L.). Possible factors intro-

ducing bias in the analysis (DM type, region, sample matrix, and study size) were noted down and used for subgroup analysis (see below).

Data Synthesis and Statistical Analysis

The same statistical approach as for meta-analysis was applied to the collected data using the R package “meta” (<https://cran.r-project.org/web/packages/meta/meta.pdf>) on means and SDs in each group and computing standardized mean differences (SMDs) and 95% CIs using the random-effects model. The I^2 statistic provided a formal test of heterogeneity. SMDs were chosen for our analysis as it has been commonly used in meta-analyses performed on similar data.^{25,29,30,33} SMDs are standardized effect sizes allowing to compare effects across different endpoints possibly measured on different scales.

Blood concentrations of CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1 of the following groups were compared:

- Subjects with diabetes regardless of DR presence and status (DM) versus healthy controls (Cntrl).
- Subjects with diabetes without diabetic retinopathy (no DR) versus healthy controls (Cntrl).
- Subjects with diabetes with NPDR versus subjects with diabetes without diabetic retinopathy (no DR).
- Subjects with diabetes with PDR versus subjects with diabetes with NPDR.
- Subjects with diabetes with PDR versus subjects with diabetes without diabetic retinopathy (no DR).

Possible causes of heterogeneity were explored via subgroup analysis on the comparison DM versus Cntrl. Included studies were grouped according to the following characteristics, when available:

- DM type: 1, 2, or undefined / pooled (1 or 2).
- Study size (above median or below / at median number of total subjects).
- Region: according to the country where the study was conducted.
- Sample matrix (serum or plasma).

The same statistical analysis as described above was performed on the single subgroups. Type 1 and type 2 DM studies were kept separate in the analysis grouped by region, sample matrix, and study size. Papers investigating “type 1 or 2” DM (pooled data or unspecified type) were excluded from the same subgroup analyses.

Results

Selection Process and Characteristics of Identified Studies

Based on our search strategy, 10,495 unique records were retrieved from EMBASE, the Clarivate Integrity Biomarker module, or MEDLINE and screened for eligibility according to the criteria listed in the “Methods” section. Full texts or conference abstracts of 167 studies were analyzed more in-depth and 23 additional records were excluded for any of the following reasons: summary data were provided in a format different than mean and SD or SEM or 95% CI, values were below lower limit of quantification (indicating the use of high-sensitive assays may be needed for low abundant analytes), included disease subsets were incompatible with the comparison of interest or the order of magnitude of the provided data was questionable. The remaining 144 records were finally included in this review.

Blood levels of one or more markers of interest (CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1) were available for a total of 5604 healthy controls and 9627 subjects with DM, regardless of DR presence and status. Moreover, we were able to extract data for 2052 subjects with DM without ocular complications, 993 with NPDR and 951 with PDR, regardless of DME presence. We aimed to base our work on internationally recognized guidelines for DM and DR diagnosis: from the American Diabetes Association, the World Health Organization, or the ETDRS. Criteria used to diagnose DM and DR were, however, often not reported by the studies, which were included anyway in the analysis. The design was case-control and cross-sectional for the vast majority of the included studies and, when the study was longitudinal or prospective, the appropriate cross-sectional dataset was extracted, either at baseline or at follow-up, depending on study design. The characteristics of the eligible studies (reference, country, study design, DM / DR diagnostic criteria, sample matrix, analytical method, unit of measurement, and DM type) and the extracted data are summarized in a separate tabulated format for each protein in Supplementary Table S1.

Association of Circulating Markers With DM Presence, DR Presence, and DR Severity

We first confirmed that systemic levels of the chosen analytes were increased in subjects with DM compared to healthy individuals by performing meta-analysis using the random effect model. The systemic concen-

tration of all selected markers showed a significant increase in the diseased population when compared to healthy controls (Fig. 1A, black lines), both in type 1 and type 2 DM. Circulating levels of CRP, IL-6, sICAM1, and sVCAM1 were increased even in subjects with DM without ocular complications when compared to Cntrl (see Fig. 1A, blue lines).

We next addressed whether the same markers gradually increased with DR presence and in more severe disease stages. Although in the above comparison, - DM versus Cntrl - both type 1 and type 2 DM data contributed to the analysis, it must be noted that only very few type 1 data were available for the DR group comparisons. As shown in Figure 1B and 1C, all analytes tended to increase from no DR to NPDR to PDR. In particular, a significant increase in circulating levels of CRP, TNF α , and sVCAM1 was detected with presence of NPDR compared to no DR (see Fig. 1B, black lines). TNF α and sVCAM1 showed again significantly increased levels with increasing disease severity (PDR versus NPDR), together with IL-6 (see Fig. 1C). When comparing the two extreme groups (PDR versus no DR), we found a significant increase in the same four analytes (CRP, IL-6, TNF α , and sVCAM1; see Fig. 1B, red lines).

Overall, TNF α and sVCAM1 showed the most robust effect across all comparisons, with sVCAM1 having the highest and always significant extent of increase in the tested dataset (SMDs between 1.01 and 2.86). Both markers were also consistently increased to roughly the same extent (around 0.7 SMD for TNF α and >1 SMD for sVCAM1) with each increasing disease step: Cntrl < no DR < NPDR < PDR. It is interesting to note that IL-6 was observed to be significantly increased in the comparison PDR versus NPDR (see Fig. 1C), but not yet in the comparison of NPDR versus no DR (see Fig. 1B, black lines). The opposite was observed for CRP, which showed significance in the early stage NPDR versus No DR comparison (see Fig. 1B, black lines) but not in the later stage PDR versus NPDR one (see Fig. 1C). Both IL-6 and CRP were, in addition, significantly increased when comparing the extreme groups PDR versus no DR (see Fig. 1B, red lines). Although there was a trend toward an increase with disease severity for all of them (see Figs. 1B, 1C), the elevation has not reached significance for systemic levels of IL-1 β and sICAM1 neither for the comparison of NPDR versus no DR nor for PDR versus NPDR or PDR versus no DR. This might be due to the low number of available studies and included subjects for sICAM1, an explanation that may not apply to IL-1 β , for which few studies were available but including a reasonable amount of subjects (see “Discussion” section).

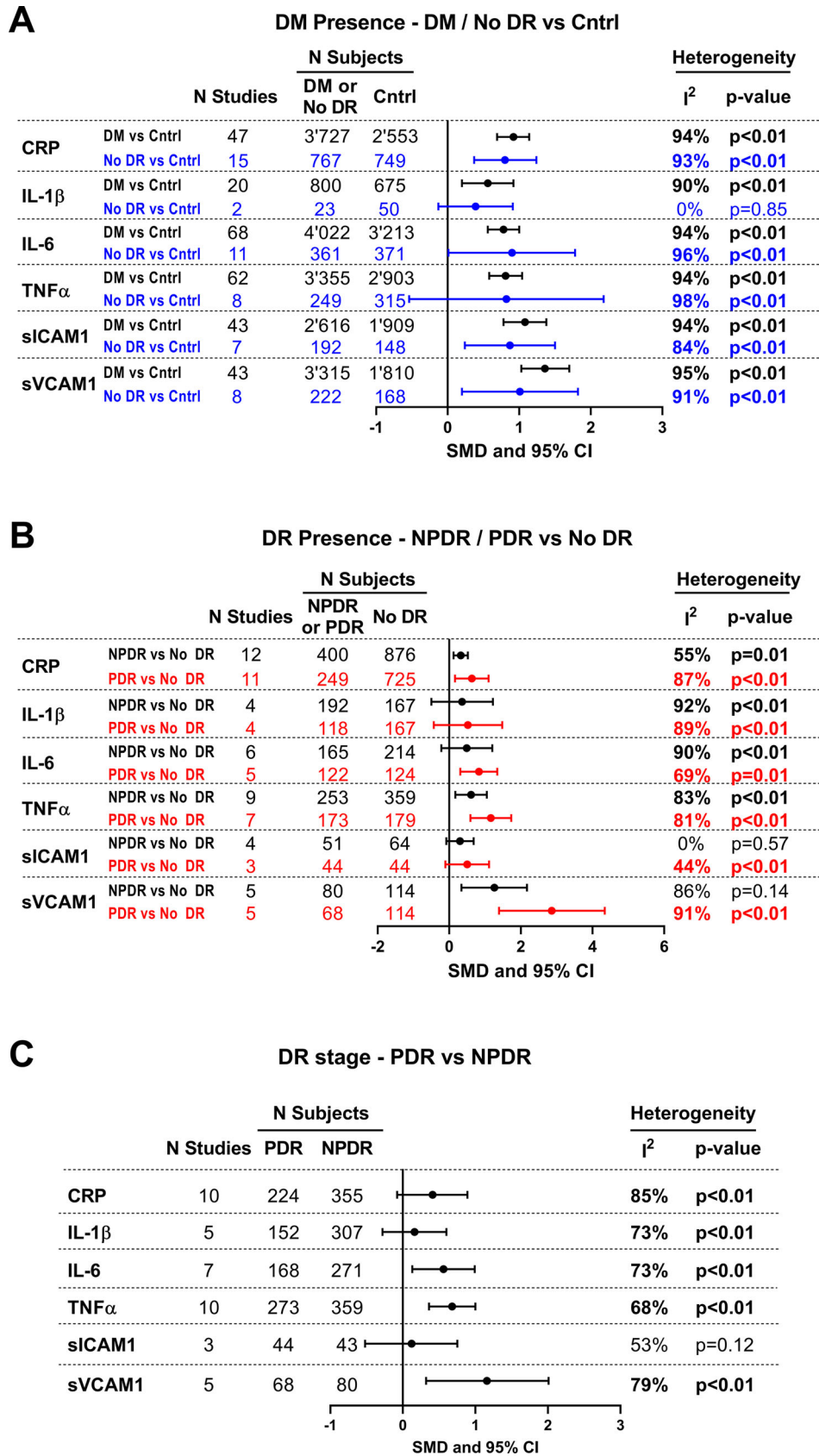


Figure 1. Statistical analysis summary results. Summary results of the statistical analysis (meta-analysis approach) are shown as standardized mean differences (SMDs) and 95% confidence interval (CI) for the following comparisons. (A) Subject with DM (pooled data regardless of DR status) versus healthy controls (Cntrl) in *black* and subjects with DM without DR (no DR) versus healthy controls (Cntrl) in *red*

←

blue. (B) Subjects with DM affected by NPDR versus subjects with DM without DR (no DR) in *black* and subjects with DM affected by PDR versus subjects with DM without DR (no DR) in *red.* (C) Subjects with DM affected by PDR versus subjects with DM affected by NPDR. When the CI does not cross the line of null effect (0), the result is significant ($p < 0.05$). The number (N) of studies and subjects included in each group comparisons are shown on the right side. The heterogeneity measure I^2 and corresponding p value are shown on the left; statistically significant I^2 measures are highlighted in *bold*.

The I^2 statistics indicated that heterogeneity was high for all proteins in the analysis of subjects with DM versus Cntrl, with values ranging from 90% to 98% (see Fig. 1A). The DR comparisons showed a variable but generally lower degree of heterogeneity (55% - 92%; see Figs. 1B, 1C), however, the number of included studies was considerably lower for the DR comparisons than for the DM versus Cntrl one. High heterogeneity suggests there may be subgroups in the dataset showing different effects. We therefore performed subgroup analysis in order to identify potential sources of heterogeneity.

Subgroup Analysis

The same statistical approach described above for the overall dataset was applied to subsets of studies categorized into the following subgroups, selected based on the available information: DM type, study size, region, and sample matrix. Given the relatively low number of studies available for the DR comparisons, we decided to perform subgroup analysis on the DM versus Cntrl comparison only. For the same reason, studies including “type 1 or 2” DM (pooled data or unspecified type) were excluded from the study size, region, and sample matrix subgroup analysis. Of note, most of the studies included subjects with type 2 DM, however, type 1 DM was still over-represented if considering that it only accounts for approximately 10% of the DM cases¹ (Fig. 2A). With regard to sample size, Figure 2B shows both the median number of subjects per study as well as the total number of subjects participating in all the studies: the number of total included subjects is lower for IL-1 β compared to the other markers, which may limit the interpretation of the subgroup analysis for this cytokine. The total number of subjects was generally higher for type 2 DM studies compared to type 1 DM, again with the exception of IL-1 β . The median number of subjects per study varied from a minimum of 57 (IL-1 β , type 2 DM) to a maximum of 105 (CRP, type 1 DM). Different geographic areas were well represented by the selected studies, as shown in Figure 2C, with the majority being conducted in Asia and Eastern Europe. Serum was found to be more frequently used as a sample matrix compared to plasma to measure all analytes (Fig. 2D).

The majority of the subgroup comparisons for CRP, IL-6, TNF α , sICAM1, and sVCAM1 (66.6 to 84%, depending on the analyte) still showed a significant increase in the circulating levels of the corresponding markers in the diabetic population compared to the healthy control subjects, further supporting the overall results (data not shown). Of note, this was also true for the two types of DM, type 1, and type 2, which generally showed the same result. Only 21% of the subgroups instead remained statistically significant for IL-1 β concentration (data not shown), possibly due to the lower amounts of available studies, 20 in total. The I^2 statistics was calculated and used to explore the extent of heterogeneity among the studies in each subgroup (Supplementary Table S2). The I^2 values remained high in most of the tested comparisons and significantly decreased below 75% - indicating moderate heterogeneity⁴² - only in 6.6 to 15.8% of the subgroups, depending on the analyte. Based on these results, none of the characteristics tested here could be identified as clearly driving heterogeneity among the selected studies. Thus, the source of the overall high level of heterogeneity of the data in this analysis remains unknown.

Relationship Between Ocular and Circulating Concentrations of Selected Markers

In order to explore the relationship between ocular and circulating levels of the analytes of interest, we reviewed the studies measuring matched VH levels in the eye and blood (plasma or serum) concentrations in the same population. No ocular levels of CRP were reported, whereas a limited number of studies measured circulating and ocular IL-1 β , IL-6, TNF α , sICAM1, or sVCAM1 in Cntrl and subjects with PDR who underwent ocular surgery.⁴³⁻⁴⁷ As summarized in Figure 3 and the Table, published studies agreed on considerably higher (>10 fold) levels of IL-6 in VH compared to plasma / serum, whereas the opposite was true for sICAM1 and sVCAM1. The picture is less clear for IL-1 β and TNF α , for which conflicting conclusions were drawn by different studies. Three studies^{43,45,46} also tested for correlation between systemic and ocular concentrations of the proteins of interest: no significant correlation could be found for any of the analytes. Additionally, the authors assessed the difference in

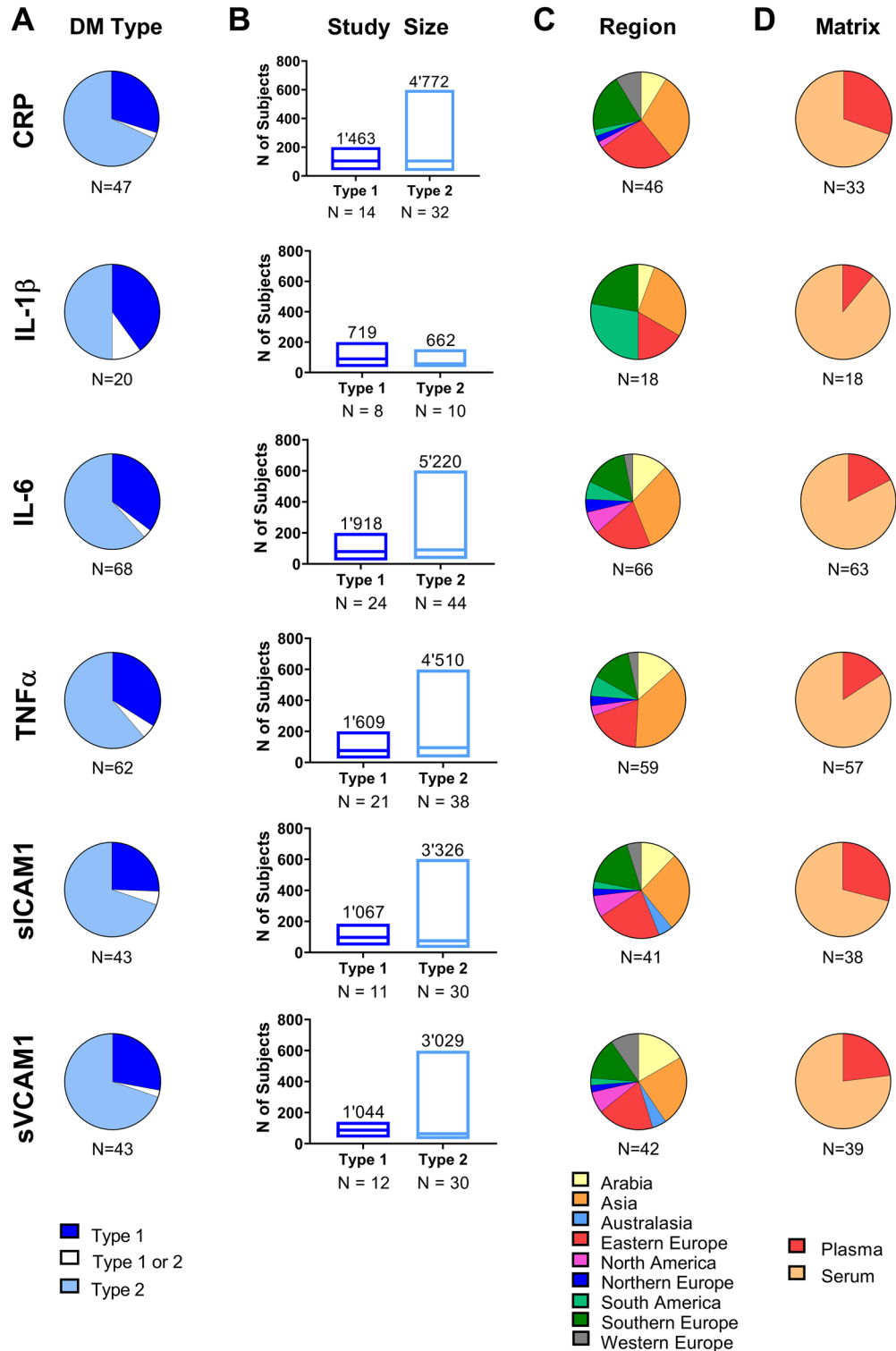


Figure 2. Distribution of the included studies into the considered subgroups. Distribution of the studies included in this review and statistical analysis (for the comparison DM versus Cntrl) into the considered subgroups. (A) DM type, (B) study size (median and range of number of participating subjects per study, divided by DM type), (C) region (according to the country where the study was conducted), and (D) sample matrix used for measurement of proteins of interest. Below each pie chart A, C, and D or box plot B the total number of considered studies is shown; note that this number is not always equal for the same analyte because some studies did not report the necessary characteristics to be categorized in all subgroups. The total number of subjects included in all the considered studies per analyte and DM type is shown above the boxes in B. Papers investigating “type 1 or 2” DM (pooled data or unspecified type) were excluded from the region, matrix and sample size subgroup analysis B, C, and D.

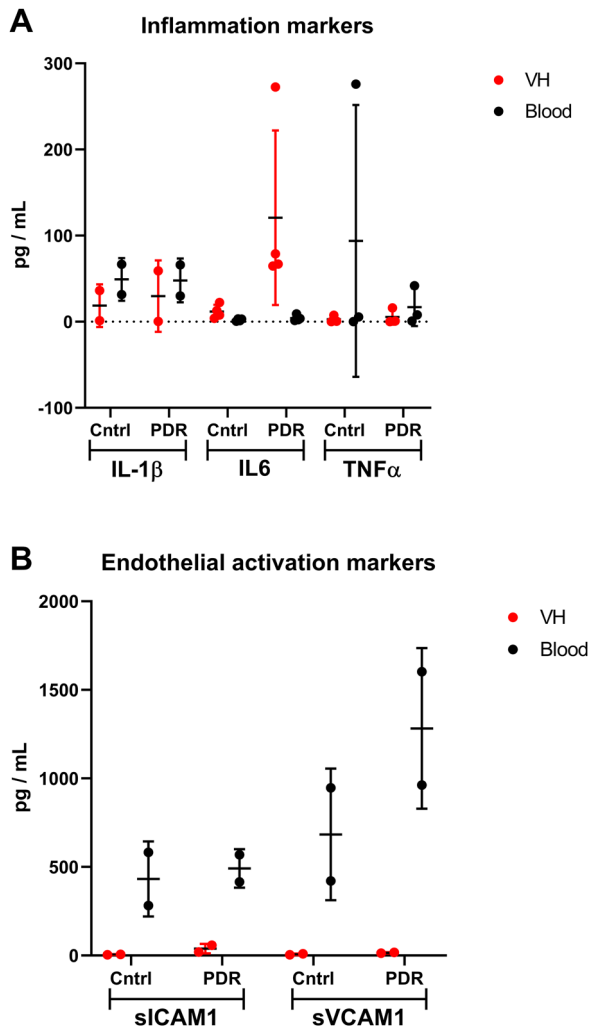


Figure 3. Ocular and circulating concentrations of IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1 in healthy control and subjects with PDR. Mean concentrations of vitreous humor (VH, red circles) and blood (plasma or serum, black circles) inflammatory (A) and endothelial activation (B) markers in matched Cntrl and PDR population (references in the Table). Mean and SDs of biomarker levels from each study are shown separately for Cntrl and PDR groups.

vitreous concentrations between subjects with PDR and control subjects: a significant ocular increase in IL-6, sICAM1, and sVCAM1 was detected by all studies in the PDR population; conflicting conclusions were again obtained for IL-1 β and TNF α levels in VH.

Discussion

Given the role of inflammation and endothelial activation in the pathogenesis of DM and its complications, many studies investigated the possibility of assessing circulating levels of proteins involved in these processes as biomarkers of disease. Here, we aimed to review the existing literature on selected systemic

inflammatory and endothelial activation markers in relation to DR presence and severity - defined as no DR, NPDR, and PDR - in a cross-sectional setting. We chose six markers based on their suggested involvement in DM / DR (CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1) and showed that all of them are significantly elevated in blood of subjects with diabetes compared to healthy controls, both in type 1 and type 2 DM, in line with previously published reviews and meta-analyses.^{25,27,28} Although statistical significance was not reached for each DR comparison in this population-based analysis, all the tested analytes showed a trend of increased blood levels with increasing retinopathy severity, partially confirming published data^{29-31,37} on a bigger dataset, mainly coming from patients with type 2 DM. As expected, larger SMDs were observed when comparing more diverse and extreme groups (DM versus Cntrl, no DR versus Cntrl, and PDR versus no DR) than when considering comparisons within a more homogeneous population (NPDR versus no DR and PDR versus NPDR). When looking at the significant changes with increasing DR severity, TNF α and sVCAM1 increased with each step of DR, higher CRP levels were associated with the early stage, from absence of DR to NPDR, whereas IL-6 levels rather were increased in the late stage disease, PDR.

Overall, our results fit well with the biology of the disease, where systemic low-grade inflammation and endothelial activation play an important role in development and progression of DR.^{10,13,48} The upregulation of circulating cytokines, on top of increasing age, disease duration, and a number of chronic factors in diabetes (such as HbA1c and blood pressure), contributes to the creation of a systemic pro-inflammatory environment. With onset of NPDR, vascular damage occurs in the retina including compromised retinal-blood barrier, which might increase adhesion of immune cells pre-exposed to the systemic inflammation. These events are followed by local cytokine production and immune cell infiltration / activation at the more advanced stages, all contributing to disease development and progression in the eye. The later vision-threatening PDR is additionally characterized by pronounced ischemia, leakage, and neovascularization as vascular phenotypes (Fig. 4).

Many of the analyzed markers or combinations of them have also been associated with other microvascular complications of diabetes, like nephropathy^{26,49-53} and neuropathy,^{51,53,54} suggesting a common mechanism between complications in different organs. Whether the changes in these biomarkers are independent or associated to each other and, possibly, regulated by common pathways remains to be clarified.

Table. Summary of Studies Assessing Concentrations of Selected Markers in Matched VH and Blood From Healthy Subjects (Cntrl) and Subjects With PDR

Outcome	Analyte	Adamic- Mroczek ^a 2008 (N = 15/19)	Koskela 2013 (N = 16/38)	Ma ^a 2011 (N = 31/76)	Shen ^a 2020 (N = 46/32)	Yuuki 2001 (N = 21/47)	Summary
Overall absolute levels in VH versus blood	IL-1 β		↓↓		=		?
	IL-6	↑↑	↑↑	↑↑		↑↑	↑↑
	TNF α	↑	↓↓			=	?
	sICAM1	↓↓	↓↓				↓↓
	sVCAM1	↓↓	↓↓				↓↓
Significant higher levels in VH of PDR versus Cntrl	IL-1 β		No		Yes		?
	IL-6	Yes	Yes	Yes		Yes	Yes
	TNF α	Yes	No			No	?
	sICAM1	Yes	Yes				Yes
	sVCAM1	Yes	Yes				Yes

↑↑ = Ratio VH to blood mean concentration is >10 in each individual study (VH levels are approximately 10 times higher than blood levels).

↓↓ = Ratio VH to blood mean concentration is <0.1 in each individual study (blood levels are approximately 10 times higher than VH levels).

^aStudy assessed correlation between VH and blood levels; no significant correlation was found for any of the analytes.

N = Number of Cntrl/PDR subjects.

For example, a decrease in IL-6 and CRP serum levels have been observed in patients with diabetes treated with a recombinant IL-1 receptor antagonist (anakinra), suggesting IL1 β may be upstream of these two proteins.⁵⁵

A crucial question to be addressed in future studies concerns the value of measuring multiple biomarkers: a combination of two or more markers of inflammation and endothelial activation may be more strongly associated to DR stage or show increased power in predicting disease progression than the single markers. This is valid also for the combination with other systemic factors that are known to be associated with DR progression, namely HbA1c, disease duration, blood pressure, and dyslipidemia.^{12,19,20} Here, analysis of the added value of biomarker combinations with regard to DR stage association could not be performed due to the lack of patient-level data and the low number of studies measuring more than one analyte of interest, as well as reporting HbA1c, disease duration, blood pressure, or lipid levels in the included DR patients. It is thus important that future prospective studies include measurements of all the above-mentioned parameters in order to draw conclusions about the individual markers or their combination with the highest power in diagnosing DR stages and / or predicting DR risk progression. The validity of this approach has been demonstrated in previous studies.^{56–58}

An open question in the field of DR is the relationship between the ocular and circulating levels of protein biomarkers. Few data were available in matched VH and blood samples, possibly due to difficulty in obtaining both samples from the same subject. The consistently higher IL-6 levels in vitreous compared to blood suggests that this cytokine might be locally produced by intraocular tissues or infiltrating immune cells. On the other hand, a spillover of blood from leaking or newly formed vessels may be the main source of sICAM1 and sVCAM1. IL-6, sICAM1, and sVCAM1 also showed significantly increased ocular levels in subjects with PDR versus Cntrl, in line with the results of our meta-analysis on systemic levels. Controversial conclusions were drawn by different studies for IL-1 β and TNF α , which were generally present at very low concentrations in blood and VH: the use of highly sensitive immunoassays might be needed in order to have a clear picture for these two cytokines. No correlation was found by any of the studies between ocular and circulating levels of IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1, however, the low sample size prevents a conclusive assessment of correlation. More studies including a higher number of subjects are therefore needed in order to truly understand the relationship between ocular and systemic concentrations of biomarkers in DR.

Our analysis presents limitations that need to be taken into consideration:

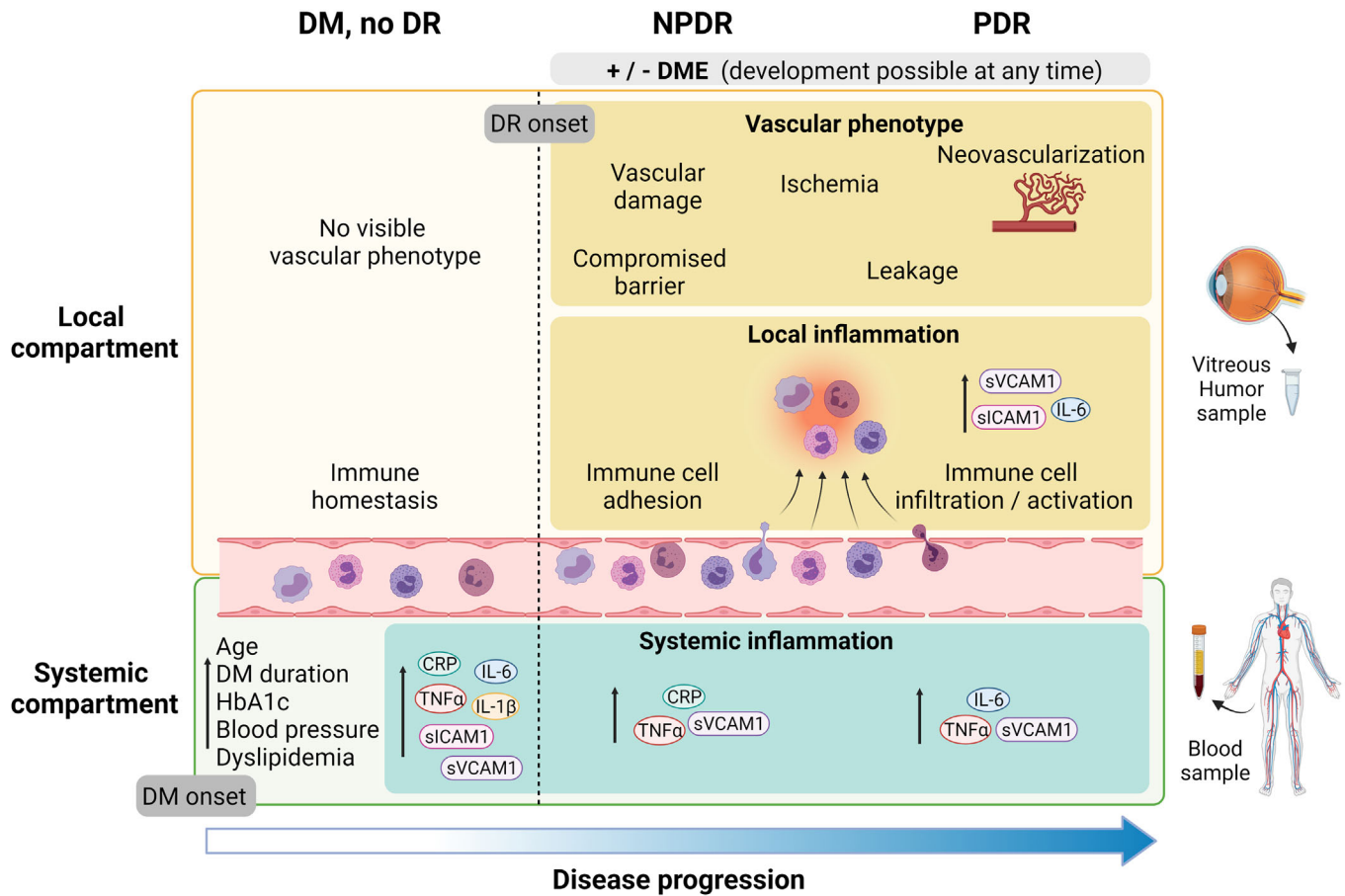


Figure 4. Systemic and local inflammation and endothelial activation during development and progression of DR as a consequence of systemic DM. Schematic representation of systemic and local inflammatory events and endothelial activation during development and progression of DR as a consequence of systemic DM. With increasing age and time from DM diagnosis a number of systemic factors significantly increase in subjects with diabetes, including HbA1c, blood pressure, and circulating markers of inflammation and endothelial activation (CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1). Before DR can be detected in fundus images of patients with diabetes (DM / no DR), the presence of a systemic pro-inflammatory environment is indicated by the significant upregulation of CRP, IL-1 β , IL-6, and TNF α as well as an endothelial cell activation is indicated by the significant upregulation of sICAM1 and sVCAM1. This is consistent with broader systemic inflammation and vascular impairment in DM. As vascular changes are not yet manifest and overall immune homeostasis is likely being maintained in the eye in this stage of the disease, a very low-grade local inflammation in the retina cannot be excluded. After onset of NPDR and manifestation of vascular fundus abnormalities, the retinal blood barrier becomes compromised and circulating immune cells, already pre-exposed to the systemic pro-inflammatory environment, might show increase adhesion to the vessel walls (leukostasis). At this disease stage, CRP, TNF α , and sVCAM1 are additionally increased in the systemic circulation compared to the DM / no DR stage. As disease progresses, the worsening of the vascular phenotype in the retina (pronounced ischemia, leakage and, eventually, neovascularization in the PDR stage) is accompanied by enhanced immune cell infiltration / activation and more pronounced local inflammation with ocular production of IL-6, sICAM1, and sVCAM1. Resident retinal cells as well as infiltrating immune cells could be sources of cytokine production. In order to better understand the interplay and contributions of local versus systemic biomarkers to DR progression, it is important to collect matched ocular and blood samples in future clinical studies, as shown on the right side of the figure. Note that only changes that showed statistical significance in our analysis are shown in the figure and that only data from papers with matched blood and vitreous sampling were included in this analysis, which generally led to a low number of data sets being included here. For NPDR, no data on ocular cytokine levels were available. In addition, matched data were available for vitreous, but not aqueous humor in the reviewed evidence. This figure was created with [BioRender.com](https://www.biorender.com).

- We did not perform a prospectively registered, systematic review and meta-analysis, but reviewed the literature for the available evidence on systemic inflammation and endothelial activation markers in DR. Some of the same statistical tools used for meta-analyses have nevertheless been used on this

dataset for data synthesis and interpretation of the results.

- As part of our literature strategy, we searched only the mentioned three databases up to the end of 2019 (EMBASE, Medline, and Clarivate Integrity Biomarker module).

- As part of the selection and analysis strategy, we used mean and SD of the systemic concentrations of the markers of interest as this was reported in the majority of articles, whereas we excluded the papers expressing the data as median and range only. We acknowledge that formulas for estimating mean and SD from median and range have been investigated in the literature.^{59,60} The discussion sections of these articles, however, show that there is no commonly accepted way to estimate mean and SD from median and range and thus we decided not to integrate any data expressed as median and range only.
- Type 1 DM was generally overrepresented in our dataset, accounting for more than 10% (up to 40% for IL-1 β) of the total subject number, as would be expected from epidemiological data.¹ This was relevant for the comparison DM versus Cntrl. In contrast, for the DR stage comparisons, the vast majority of the included records collected data from patients with type 2 DM.
- For some DR stage comparisons, the number of available studies was low (below 5) for sICAM1 and IL-1 β . When considering the number of subjects, sICAM1 was consistently investigated in the smallest population, however, this was not the case for IL-1 β , for which the included subject number was higher than for sVCAM1, one of the most robust biomarkers in our analysis. Thus, sample size may be a limitation for sICAM1 only.
- The high I² values indicated substantial heterogeneity between the selected studies, which was not clearly explained by any of the characteristics considered for the subgroup analysis (DM type, study size, region, and sample matrix), suggesting there may be other factors, that we cannot measure, contributing to heterogeneity. As noted in Section 10.10 of the Cochrane handbook,⁶¹ statistical heterogeneity may be inevitable due to methodological diversity. The studies considered here included different populations in terms of age, duration of disease, treatments, glycemic control, and other clinical characteristics, which could all be considered confounding factors. As diagnostic criteria for DM and DR as well as presence of DME were not always reported, differences cannot be ruled out in regard to this too. Another parameter that could explain the high I² values observed is the handling of the samples and methodology used to assess blood levels of inflammatory and endothelial activation markers: generally, immunoassays with fluorescent or colorimetric readout (ELISA) but from different vendors, occasionally flow cytometry and multi-

plex immunoassays. A meta-regression analysis to address the effect of these covariates on heterogeneity could not be performed as many studies did not report the necessary information.

- For the comparison of ocular and circulating concentrations of the selected proteins, we only considered studies selected for the analysis of the systemic biomarkers showing data from matched ocular samples, which generally led to a low number of datasets being included and no datasets available for NPDR. In addition, matched data were available for VH, but not AH in the reviewed evidence. Due to the very limited size of the dataset, these results should be interpreted with caution.
- Analysis of individual biomarkers and their combinations (e.g. with other systemic factors associated with DR [HbA1c, diabetes duration, blood pressure, and lipid levels]), could not be performed due to lack of patient-level data. Furthermore, the number of studies reporting levels of more than one systemic marker of interest for the included patients with DR was low.

In conclusion, our analysis supports the use of CRP, IL-1 β , IL-6, TNF α , sICAM1, and sVCAM1 systemic levels as diagnostic biomarkers of type 1 and 2 DM status and DR presence and severity, at least in type 2 DM. TNF α and sVCAM1 showed the most robust effect with significant increase at every DR step; other markers could potentially be used to monitor progression from no DR to NPDR (CRP) and from NPDR to PDR (IL-6). Further large and prospective studies are required to assess the specific use of these easily accessible biomarkers as described by the US Food and Drug Administration (FDA).^{62,63} Association to disease stage, as shown in this review, makes the analytes good diagnostic biomarker candidates that could aid in the diagnosis of early DR stages, when the vascular abnormalities are not yet visible in the fundus pictures. Only properly powered, prospective, longitudinal studies can, however, confirm or disprove their use as prognostic biomarkers (i.e. to identify patients at higher risk of progressing to more severe DR stages). Such biomarkers would be useful for patient selection and stratification in clinical trials. Similar studies will also inform on the value of the selected analytes as disease-monitoring biomarkers with the goal of tracking disease stage longitudinally in individual patients. In addition, inflammation and endothelial activation are relevant processes in DR development: the investigated biomarkers could be used to monitor the effect of novel therapies targeting these pathways, therefore as pharmacodynamics

biomarkers. Their use as predictive biomarkers should be evaluated too in dedicated studies, in order to enrich populations with higher chance of response to a certain therapy or even contribute to the appropriate treatment selection for individual patients in the future. It must be noted that even if the biomarkers are confirmed to be predictive at the population level, the strength of the single or combined biomarkers for prediction on the individual patient level will also need to be thoroughly investigated. Moreover, the collected evidence supports the view of inflammation and endothelial activation playing a role at the interface between the systemic diabetic disease and its local complications and the importance of collecting matched blood and ocular samples in order to investigate the relationship between systemic and local biomarkers.

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