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Reviewer #1:

1. The article is not particularly well written. There are many unclear or redundant sentences, unclear transitions and points. Several of the references are incorrectly summarized. Without line #s, there are too many problems to elucidate for the authors here.

Authors Response: Thank you for taking the time to read the review and offer your critique. All authors have participated in the revision in hope to remove redundant text and make the transitions more seamless. We have now added line numbers to help streamline the review process.

2. There is only a modest amount of relatively new data and the summary of prior data is relatively superficial and not always clear. It is difficult to get a clear take home message from many of the sections.

Authors Response: We have extensively revised the manuscript and updated the references in the revised text in addition to providing a more detailed description of the studies, so there is a clear take home message.
3. Many of the studies described are missing appropriate detail to permit fully understanding or appreciating the data implications.

Authors Response: Similar to the above response, we have provided a more detailed description of the studies to offer a better understanding of the mechanistic and clinical significance of the data being reviewed.

4. This is also true for the authors descriptions of their own studies

Authors Response: The focus of this series/invited review is coronary atherosclerosis in diabetics from the perspective of pathology with an emphasis on complications of calcification. This is a rather challenging topic considering peer review articles of diabetic coronary artery disease relative to lesion morphology from the view of the pathologist are limited. On the contrary, a disproportionate number of non-invasive coronary imaging studies of lesion morphology in diabetics, particularly involving modalities such as computed tomography are available. In this regard, we have focused on many of the pathologic studies in diabetics from our own laboratory although relevant papers from other prominent groups are also cited and discussed (see below).


5. Several descriptions of statistical analysis results are presented in a way that limits ready translation into clinical implications. There is also incorrect usage of statistical terms when describing results which adds further confusion to the data interpretations.

Authors Response: We have revised the descriptions of statistical analysis to be clarified and to avoid misinterpretation.

6. The article should replace the word diabetic with "diabetes" wherever possible

Authors Response: We have changed this terminology from “diabetic” to “diabetes”. In addition, type 1 diabetes and type 2 diabetes are abbreviated as T1D and T2D.
Reviewer #2:

1. The distinction between type 1 and type 2 diabetes is not clear in every part of the manuscript, and I would like to know more about the differences in phenotype and pathology as the phenotypes are different in some aspects as shown in the tables and figures

Authors Response: The vast majority of clinical data published use a general term “diabetes patients” without distinction as to type, i.e., type 1 or 2. However, the current manuscript has been revised with the differences between type 1 DM (T1D) and type 2 DM (T2D) clearly stated. We have added a paragraph showing the classification of diabetes in the introduction (see below).

(Page 3 to 4, Line 72 to 89)

“Diabetes is generally classified as either type 1 (T1D), an autoimmune disorder caused by the destruction of β-cells of the pancreas with consequent absolute deficiency of insulin secretion, to the more common type 2 (T2D) that results in a resistance to insulin with a relative insulin deficient, to insulin secretory defect. The clinical presentation of T1D and T2D in relation to cardiovascular disease however, is strikingly similar although distinctions are worth mentioning. In general, T1D is diagnosed at younger age and therefore, the time of exposure to diabetes-related CVD risk factors is longer. Moreover, the incidence of stroke is relatively low for T1D as compared to coronary heart disease. Tight glycemic control in T1D diabetes might also slow the process of coronary artery calcification (CAC), but not in T2D.

The diagnosis of diabetes is based on hyperglycemia and despite the
heterogeneity and markedly different genetic and mechanistic causes, both T1D and T2D exhibit a higher prevalence of cardiovascular complications. Therefore, intuitively hyperglycemia is considered responsible for the observed accelerated cardiovascular disease observed in patients with diabetes. The clinical diagnosis of diabetes falls under several definitions of fasting plasma glucose ≥126 mg/dL (7.0 mmol/L), 2-hour plasma glucose ≥200 mg/dL (11.1 mmol/L) after 75-g oral glucose load, hemoglobin A1c (HbA1c) ≥6.5% (48 mmol/mol), or random plasma glucose ≥200 mg/dL confirmed by repeat testing.⁶

2. Mechanistic information on influences of e.g. hyperglycemia on vascular cells is provided, but I think it would be clearer when effects of hyperglycemia on specific steps in plaque formation are described.

Authors Response: We have added discussion of the effect of hyperglycemia on atherosclerosis plaque formation.

(Page 4 to 5, Line 90 to 113)

“The primary complications of diabetes-related atherosclerosis are mediated by persistent hyperglycemia involving mechanistic processes driven by inflammation, oxidative stress, and epigenetic modulation.¹²,¹³ Although insulin resistance precedes the onset of prediabetes or diabetes mellitus, hyperglycemia develops in prediabetes and worsens with the development of diabetes. Clinical data suggest that hyperglycemia may exert long-term detrimental effects on the cardiovascular system, a phenomenon defined as “metabolic” or “glycemic memory” indicating a persistence of
hyperglycemic stress even after blood glucose normalization. The accumulation of advanced glycation end products (AGE) represent the lead candidate in this paradigm, which result in the non-enzymatic glycation of mitochondrial proteins, lipids, and nucleic acids. The result is the accumulation of highly reactive carbonyl groups, recognized as α-dicarbonyls or oxoaldehydes, products of which are 3-deoxyglucosone and methylglyoxal referred to as “carbonyl stress” resulting in the denaturing, browning, and cross-linking of target proteins.

AGEs not only inhibit DNA synthesis in target cells, but also contribute to vascular hyper-permeability, pathological angiogenesis, and thrombogenic reactions involving vascular endothelial growth factor (VEGF), and plasminogen activator inhibitor-1 (PAI-1) mediated through a NF-κB pathway and downstream interaction with the receptor for AGEs (RAGE). Therefore, AGE activity is central to an amplified “interminable feed-forward loop” in which AGE-mediated cellular perturbations are not easily reversed. Ultimately, AGE along with elevated free fatty acids (FFA) and activation of protein kinase C (PKC) and other mediators are thought to alter atherosclerotic lesions severity and complexity through low-grade inflammation accompanied by enhanced oxidized lipid uptake, direct endothelial/tissue injury, and induction of a pro-thrombotic state whereupon there are sustained deleterious effects even after the removal of hyperglycemic stimulus.  

3. The relation between insulin resistance and atherosclerosis/CAC mentioned in figure 5 but not specifically discussed in the text. While direct influences of impaired insulin-stimulated glucose uptake are unlikely, impaired insulin
signaling has been linked to endothelial dysfunction, macrophage activation and plaque formation in mouse studies. In man, the atherosclerosis risk associated with insulin resistance/pre-diabetes should be discussed as it is a type 2 diabetes-specific risk factor.

Authors Response: Figure 5 has now become Figure 6, which was revised to improve the mechanistic discussion in the text, as it applies to atherosclerosis progression in T2D with complications of CAC.

Figure 6. Plaque calcification in diabetes involves cardiovascular risk factors of atherosclerosis which also account for plaque progression, although in this setting,
hyperglycemia and insulin resistance appear to represent significant driving factors. The earliest form of calcification, microcalcification occurs in apoptotic vascular smooth muscle cells (VSMCs) and macrophages in conjunction with an increase in serum calcium-phosphorous (CaxP) product. There is strong evidence, particularly from murine data that OPG/RANKL/RANK activity regulates vascular calcification. RANKL controls calcification processes in vSMCs by inducing osteoblast-like activity, which is reminiscent of bone formation through endothelial-derived RANKL. Osteoprotegrin (OPG), also expressed by ECs and VSMC exhibits an affinity for RANKL and thus serves a mediator against calcification. Osteoblast-transformed vSMCs represent an addition source of OPG, which serves as a decoy for RANKL, thereby further blocking the downstream osteoclastogenic cascade. Therefore, existing regulators of vascular calcification include genetic factors in addition to an imbalance between the promotors and inhibitors of calcification, which are further dependent on hyperglycemia, insulin resistance and diabetic status.

Abbreviations: BMP=bone morphogenic protein; EPCs=endothelial progenitor cells; FGF=fibroblast growth factor; HDL-C=high density lipoprotein-Cholesterol; IL=interleukin; MCCs=myeloid calcifying cells; OPG=osteoprotegrin; ROC=reactive oxygen series; Runx2=Runt-related transcription factor-2; TNF=tumor necrosis factor; TGF=transforming growth factor; Wnt=wingless-type MMTV integration site family.

4. The authors mention that hyperglycemia influences calcification (p.16), but the mechanisms involved are not clear.
Authors Response: See response to question #3 above. Also, this issue is mentioned in text, where relevant.

5. The description of reno-cardiac axis in coronary artery disease is not clear. Disturbances in calcium handling in renal failure are described, but the connecting mechanisms should be discussed in more detail. For instance, FGF23 is a strong predictor of cardiovascular risk in renal failure, but I did not find this in the manuscript.

Authors Response: We initially did not expand on the role of chronic renal failure, as it’s an end-stage disease process, however 50% of patients with diabetes develop microalbuminuria and of these 1/3 will develop full brown renal failure. On page 18, final paragraph with continuation into pg19, we have expanded on this topic with the addition of pertinent preclinical and clinical studies.

(Page 18, Line 411 to 429)

“Approximately 50% of diabetic subjects develop microalbuminuria, which progresses towards established diabetic nephropathy in 1/3 of patients and elevated serum phosphate (sPi) is an independent risk factor for calcification.87 Phosphate homeostasis is maintained by the gut, bone, and kidney and is regulated by many hormones such as parathyroid hormone (PTH), and 1α,25-dihydroxyvitamin D3 (1α,25-(OH)2D3), and the more recently described FGF23 and its required cofactor Klotho.88, 89 Regarding the latter, FGF23 is a circulating phosphaturic hormone that is elevated in patients with chronic kidney disease and strongly associated with cardiovascular mortality.90, 91
Plasma FGF23 concentrations have also been independently linked to African Americans with type 2 diabetes mellitus and high CAC scores. The specific link between FGF23 and vascular calcification however, is unclear considering FGF23 or its co-receptor, klotho, does not appear to be present in human or mouse vascular SMCs or normal or calcified mouse aorta. Moreover, quantified coronary artery and thoracic aortic calcification by computed tomography in 1501 patients from the Chronic Renal Insufficiency Cohort (CRIC) study showed that baseline plasma FGF23 was not associated with the prevalence or severity of calcification even after multivariable adjustment. The absence of FGF23/klotho expression and failure to shows a direct association with vascular calcification possibly argues other explanation for the increase in cardiovascular events and mortality, as there is a described klotho-independent FGF23 effect on cardiac myocytes and left ventricular hypertrophy, which may underlie the pathogenesis of cardiovascular disease in chronic kidney disease.”

6. At present therapy for coronary artery disease in diabetes is suboptimal, and therefore I think the current clinical option for CAD prevention in diabetes and future developments should be discussed. For instance, oxidative stress in diabetes is well known but trials targeted at reducing oxidative stress have been negative so far.

Authors Response: In our view, the topic of prevention is outside the scope of the manuscript and therefore we did not address this in the text. Also, the manuscript is rather lengthy without this additional discussion.
7. Fig 5 contains too much text in its present form, this makes it rather crowded and hard to read.

Authors Response: The figure 6 (formerly Figure 5) has been revised, with markedly reduced text with focused addition of bone morphogenic mechanisms. Also, reference the response to your questions 3 and 4 above.
Reviewer #3:

I found this review to be thorough and informative, but at times disorganized. The pathologies of diabetes and calcification are complicated, which makes it difficult to discuss them in a clear, concise manner. The authors do an excellent job discussing clinical data and the mechanisms of calcification. However, the beginning of the manuscript was difficult to follow. It is sometimes unclear whether the authors were comparing type-1 to type-2 diabetes, or coronary calcification to carotid calcification, or medial and intimal calcification.

If the paper instead focused on the pathology of calcification and its association to diabetes, it would be easier to follow. The sections on healed plaque rupture, and arterial remodeling, seem to be tangential to the story. Otherwise, the manuscript focuses well on the link and potential mechanisms underlying the pathologies of vascular calcification and diabetes.

Authors Response: Thank you for your kind comments. We agreed with your comments, and have modified the beginning of the review to focus on the different types of diabetes and the differed in clinical presentation. In addition, we tried to avoid confusion in terms of which type of diabetes, type 1 vs. type 2, coronary vs. carotid, and intimal vs. medial calcification. We believe the understanding and the role of healed plaque rupture in plaque progression are important in order to explain the diffuse nature of the disease in diabetes. Arterial remodeling section has been shortened however, we believe it is connected to the excessive inflammation seen in both type 1 and 2.
1. Type 1 diabetes and type 2 diabetes have distinct pathologies, which makes it difficult to group the two conditions. The authors make comparisons between type-1 and type-2 diabetics, but offer little explanation for the differences between the pathology of the two conditions and how they may contribute to the differences in calcification observed between type-1 and type-2 diabetics.

Authors Response: We reported that calcification area was histologically greater in only T2D, but not in T1D as compared to non-diabetes (Burke AP, Arterioscler Thromb Vasc Biol. 2004;24:1266-1271), which has been added in the text. This difference may be more to do with our study population of sudden coronary death includes cases never known to have clinical coronary heart disease prior to sudden death. We also observed a shift in the number of lesions without calcification decreasing with increase in HbA1C and a significant reciprocal increase in sheet type of calcification with escalating HbA1C levels, which is consistent with clinical data (Carson AP, et al. Diabetes Care. 2015;38:66-71) (shown in Figure 4).

(Page 6, Line 151 to 153)

“Calcified matrix area was significantly greater for only T2D (12.1±11.2%), but not T1D (7.8±9.1%) as compared to non-diabetes (11.4±13.5%, T1D, P=0.9; T2D, P=0.05).”

2. The authors also discuss gender-specific effects on calcification, but offer little explanation for the underlying mechanism.

Authors Response: We have expanded the discussion in the text (see below). We believe we only mentioned gender differences in thrombosis, but not in calcification. The
literature showed diabetes was strongly associated with coronary calcification in women as compared to men (3.6 times vs. 2.1 times higher prevalence compared to non-diabetes) (Dabelea D, et al., Diabetes 2003;52:2833-2839). For T2D, the differences between men and women the differences are less clear, although age differences between men and women continue until the seventh decade. (Otsuka F, et al., ATVB 2014;34:724-36)

(Page 5 to 6, Line 127 to 139)

“An overall sex-independent risk of thrombosis irrespective of cause was also noted for non-diabetic subjects while the etiology of plaque ruptures was significantly higher in males (p<0.05) relative to plaque erosion, the latter was more frequent in females (p<0.05). Similar sex-based trends for diabetes subjects were also noted for ruptures and erosions as well, although differences did not achieve statistical significance likely because of the limited number of cases. The lower incidence of plaque rupture in diabetes corroborates a previous autopsy study by Davies,21 which accounted for thrombi in 84% of men and 59% in women without diabetes as compared to only 34% of thrombi from rupture in diabetes patients of either sex. It has been postulated that the differences are mostly likely related to hormonal difference, especially androgenicity (low sex hormone-binding globulin and free androgen index); however, other studies showed only a weak relationship of between androgen levels and CAC.22, 23 In addition, high estrogen levels have been implicated as the underlying reason for the differences between men and women for progression of atherosclerosis.24”
3. The authors found that total calcification area was increased in diabetics, but percent calcification area was not different between non-diabetics and patients with DM (Figure 4). This suggests that lesions are bigger in DM, and that the extent of calcification is proportional to lesion size. Is this true? Is that the case only for carotid lesions? What about coronary or aortic lesions?

Authors Response: We analyzed total calcification area and percent calcification area using samples from CEA; however sample size was varied. Therefore, the result of percent calcification area was not shown appropriately. We deleted the table from the figure 5 (formerly Figure 4) and only showed total calcification area in the text. In coronary lesions we have mentioned that coronary calcification area correlated with plaque burden, but not with% stenosis.

(Page 14, Line 327 to 331)

“The extent of carotid calcification was investigated in our registry of 68 CEA specimens, which included radiographic examination from 14 patients with T2D and 54 controls without diabetes. Total area of calcification by radiography was significantly greater in patients with T2D as compared to controls (T2D = 165±313 mm² vs. controls = 66±53 mm², P=0.03) (Figure 5).”

4. The authors should provide a stronger justification for focusing on coronary and carotid calcification while overlooking aortic calcification. Do the findings in diabetic vs. non-diabetic patients not apply to aortic calcification?
Authors Response: We were asked to focus on coronary and carotid calcification in this review and not on the aortic. Although aortic lesions also show calcification but correlation with stenosis to our knowledge has not been reported. CARDIA study showed a good correlation between coronary and aortic calcification with risk score, suggesting that coronary calcification further improves risk prediction.

5. Type-2 diabetes and diabetes-mellitus are used interchangeably throughout the manuscript, it would be better to use one term consistently throughout.
Authors Response: We agree with the reviews comment and have used “type 2 diabetes (T2D)” rather than diabetes-mellitus to avoid confusing if information was available.

6. The sections describing mechanisms of vascular calcification are dated, and should be updated with reference citations to more recent literature. For example, Towler, Chen and Demer have all made recent progress elucidating the role(s) of oxidative stress, hyperglycemia, and type 2 diabetes in eliciting cell transdifferentiation and vascular calcification. The last paragraph on page 14 discusses only mechanisms of high phosphate induced calcification, not other potential initiators of the disorder.
Page 16 paragraph 1 - the authors cite a study which showed decreased inflammatory marker expression in diabetic lesions - this is a surprising result, and should be discussed in more detail especially since the review authors have
drawn a correlation between increased inflammatory cell presence in the vascular calcified lesions of diabetics

Authors Response: We have partially rewritten the discussion on the mechanisms of diabetic calcification and redrawn the figure 5 now figure 6 in the revised manuscript. We have added the references from Towler, Chen and Demer where they are applicable to calcification in diabetes (see below). Although the reviewer wished us to elaborate on decrease of inflammatory marker expression in diabetes, we choose to delete this discussion as it is somewhat controversial and not consistent with pathology studies.

(Page 15, Line 344 to 346)

“Vascular calcification has been broadly divided into three types by Demer and Tintut: inflammatory, metabolic and genetic, with the latter being mostly medial.”

(Page 17, Line 407 to 410)

“Chen et al. observed that exposing bovine vSMCs to high levels of glucose resulted in higher Runx2 and osteocalcin expression, as well as stimulated alkaline phosphatase activity and mineralization capacity compared to cells exposed to normal glucose levels.”

(Page 20, Line 460 to 471)

“In culture studies, it has been shown that Msx2 enhances osteogenic differentiation of aortic myofibroblasts in response to BMP production. Reactive oxidative species and
inflammatory cytokines upregulate endothelial cells production of BMP. Msx2 expression enhances the elaboration of Wnts increasing the expression of ALP and MVs production; ALP deplete the inhibitors of calcification, such as inorganic pyrophosphate thus enhancing calcification of MVs. The authors have shown in LDLR-/- mice fed a high fat diet containing high cholesterol developed obesity, hyperlipidemia, hyperinsulinemic diabetes and aortic atheroma with calcification. The atherosclerotic calcifying aorta showed high expression of Msx 1 ans Msx2 (Drosophila muscle segment homeobox gene (msh) also known as Msx), and osteopontin expressing macrophages. The same authors generated SM22-Cre;Msx1(fl/fl;Msx2fl/fi); LDLR-/- mice and showed dramatic reduction of 31% in aortic calcification.