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Histopathological assessment of calcification and inflammation of calcific aortic valves from patients with and without diabetes mellitus

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Abstract

Background—Calcific aortic valve disease (CAVD) is the most common valvular heart disease and likely evolves from inflammatory pre-conditions in the valve. Type II diabetes mellitus (DMII) has been associated with pathogenesis of CAVD, however, the mechanism initiating CAVD in DMII is not well understood and the human valve pathology in DMII has not been described. We therefore performed quantitative histological analyses of aortic valves of CAVD patients with and without DMII.

Methods—CAVD human aortic valves (n=45) obtained after surgical valve replacement were examined macroscopically with gross measurements of calcified areas. Inflammation and calcification were assessed by immunohistochemistry and immunofluorescence staining.

Results—Calcification was increased in diabetic patients according to gross measurements ($p<0.01$) and alizarin red staining ($p=0.05$). Early calcification markers, including Runx2 ($p=0.02$) and alkaline phosphatase (ALP, $p=0.03$) were significantly elevated in diabetic patients. Furthermore, in diabetic patients we found significantly increased expression of annexin II ($p=0.04$) and annexin V ($p=0.04$), both of which are thought to play a role in microcalcification formation via apoptosis or extracellular vesicle release. Macrophage numbers were comparable in both groups ($p=0.41$), while the expression of the pro-inflammatory protein S100A9 ($p<0.01$) was significantly decreased in diabetic individuals. Evaluation of lymphocytes revealed similar CD8 ($p=0.45$) and CD4 ($p=0.92$) T cell counts in diabetic and non-diabetic aortic valves.

Conclusion—Aortic valves from diabetic patients show more calcification, while inflammation is similar in both patient populations. Considering the generally accepted theory of an inflammation-dependent mechanism of calcification, these data suggest that in patients with

CAVD requiring valve replacement, diabetic patients could be molecularly in a more advanced disease stage with a higher grade of mineralization than non-diabetic patients.

Keywords

calcific aortic valve disease (CAVD); type II diabetes mellitus; calcification; inflammation

1. Introduction

Calcific aortic valve disease (CAVD) is the most common valvular heart disease and is a significant cause of morbidity and mortality (Otto, 2006; Bertazzo *et al.*, 2013). It is viewed as a progressive disease that is initiated by alterations in valvular cell biology that progresses to leaflet thickening, neovascularization and calcium deposition to calcific aortic stenosis (Aikawa, Nahrendorf, Sosnovik, *et al.*, 2007; Otto, 2008; Rajamannan, 2009). Surgical or interventional valve replacement is the only effective treatment of symptomatic aortic valve stenosis, as pharmacological therapies have not proven to be sufficient (Cowell *et al.*, 2005; Rossebo *et al.*, 2008; Chan *et al.*, 2010; Green *et al.*, 2014; Martínez-Martínez, 2014).

Calcification evolving from inflammatory conditions predominantly results from active osteogenesis within aortic valve leaflets (Mohler *et al.*, 2001). Previous observations suggest that this is mediated through osteoblast-like differentiation of valvular interstitial cells (Yetkin and Waltenberger, 2009). Three stages of CAVD have been proposed (Aikawa and Otto, 2012): During *the inflammatory initiation phase*, pro-osteogenic factors, which are released by activated macrophages, induce the transformation of valvular interstitial myofibroblasts into osteoblast-like cells. Apart from macrophages, immune cells, including CD4 and CD8 T cells, and the pro-inflammatory protein S100A9, which is predominantly expressed by activated macrophages, indicate these inflammatory processes. In *the propagation phase*, an early stage of calcification, the pro-inflammatory conditions promote the formation of microcalcification consisting of hydroxyapatite accumulation on a bone-like matrix of collagen and bone-matrix proteins (Mohler *et al.*, 1997; Mohler *et al.*, 2001), leading to calcium deposition and leaflet stiffening. Regulation of valvular osteogenesis occurs via activation of specific transcription factors including *Runx2* (Rajamannan *et al.*, 2011), which is necessary for osteoblast and osteocyte differentiation and maturation (Wirrig and Yutzey, 2013). *Runx2* and its downstream target *alkaline phosphatase (ALP)*, which also serves as an early marker of osteoblast differentiation, have been increased in high fat diet rabbit and mouse models of CAVD (Rajamannan *et al.*, 2002; Aikawa, Nahrendorf, Figueiredo, *et al.*, 2007; Miller *et al.*, 2009). Since *annexins* are implicated in various processes, including microcalcification, apoptosis and extracellular vesicle release (Shanahan *et al.*, 2011; New *et al.*, 2013), their expression in aortic valve leaflets was investigated. *Annexins* are calcium-regulated membrane-binding proteins (Gerke *et al.*, 2005), described to play a role in matrix mineralization in hypertrophic bone and cartilage - possibly via modulating Ca^{2+} entry into extracellular matrix vesicles during hydroxyapatite formation. In contrast to *irreversible late-stage calcification*, earlier stages might be targeted by medical therapies aiming at the attenuation of osteogenic and pro-inflammatory changes whereas the advanced late stage is characterized by reduced inflammation and excessive calcification progressing to the point of no return (Aikawa and Otto, 2012).

Inflammation plays a central role in the pathogenesis of adult glucose disorders, notably type II diabetes mellitus (DMII) (Barzilay *et al.*, 2001; Sjöholm and Nystrom, 2006). In addition to the observation that chronic inflammation and activation of the immune system are involved in the pathogenesis of DMII (Esser *et al.*, 2014), numerous studies have identified diabetes as an important risk factor for CAVD (Gleissner *et al.*, 2007; Wilhelmsen *et al.*, 2008; Emerging Risk Factors *et al.*, 2010; Yeh *et al.*, 2010; Gleissner, 2015). However, relatively little is known about the effect of diabetes on progression of CAVD. In fact, the disease progression in DMII patients may be accelerated and involve different pro-calcific pathways. Therefore, the current study was designed to evaluate the inflammation and calcification processes in the aortic valves in CAVD patients with or without diabetes.

It has been previously concluded that DMII contributes to CAVD pathogenesis by promoting early mineralization of the aortic valve (Le Quang *et al.*, 2014). However, this conclusion was derived from observations in a mouse model and inflammation was not assessed. Recently published data point towards significant differences regarding inflammatory and calcification mechanisms in mouse and human (Thubrikar *et al.*, 1986; Ingersoll *et al.*, 2010; Miller *et al.*, 2011; Cheek *et al.*, 2012; Seok *et al.*, 2013).

2. Methods

2.1. Tissue Collection

Human aortic valves were obtained with patient consent (Institutional Review Board protocol 2010P002567/BWH) from CAVD patients aged 51–88 years, undergoing aortic valve replacement surgery (17 diabetic patients, 28 non-diabetic patients) at Brigham and Women's Hospital, Boston, USA (Table 1). Patients with renal failure (GFR < 30 ml/min/1.73m²) were excluded. Patient medical history including past surgical history, laboratory results and pharmacological therapies were collected.

2.2. Measurements of gross calcification

Standardized overview pictures of the aortic valves were obtained by adhering to the same recording conditions: valve fragment on a white background, exposure time smaller than hundredth of a second (standard adjustment of front camera of Samsung Galaxy S4 mini), same angle (90°) and same distance (approx. 30 cm/12 inch) to leaflet. The percentage of positive area of calcified nodules was calculated with the software ImagePro Plus (6.0 Media Cybernetics, Inc., Rockville, MD, USA). All aortic valves had portions of the attachment of the aortic root and the tip of the valve, which enabled us to calculate uncalcified distance measurements for conclusions about disease progression.

2.3. Histology and immunohistochemistry

Tissue samples were frozen in OCT compound and 6-µm sections were prepared using a Cryostat CM3050 S (Leica Microsystems, Buffalo Grove, IL, USA). All samples were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA) for general morphology. Tissue sections were fixed in 37% buffered formaldehyde (American MasterTech Scientific, Lodi, CA, USA). For the staining process, Bluing Reagent (VWR International, Radnor, PA, USA), buffered with Tris-buffered saline, was used to ensure a

constant pH during staining. Tissue sections were dehydrated in 70%, 95% and 100% ethanol (Fisher Scientific, Waltham, MA, USA) and cleared with xylene (Fisher Scientific, Waltham, MA, USA). Slides were mounted with a permanent mounting medium (Vector Laboratories, Burlingame, CA, USA) and coverslipped.

Alizarin red staining (American MasterTech Scientific, Lodi, CA, USA) was performed for all samples for detection of calcium deposits. Tissue sections were fixed and dehydrated with -20°C cold acetone (Fisher Scientific, Waltham, MA, USA) and cleared with an acetone/xylene solution (50% acetone and 50% SafeClear II Xylene substitute, both purchased from Fisher Scientific, Waltham, MA, USA). Slides were mounted with a permanent mounting medium (Vector Laboratories, Burlingame, CA, USA) and coverslipped.

For immunohistochemistry, tissue sections were fixed in -20°C acetone (Fisher Scientific, Waltham, MA, USA) and blocked with 0.3% hydrogenperoxidase (Fisher Scientific, Waltham, MA, USA) and Protein Block Serum-Free (DAKO, Carpinteria, CA, USA). For washing and dilution steps phosphate-buffered saline (PBS, Boston BioProducts, Ashland, MA) was used.

All primary antibodies were diluted in 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA), which was prepared with PBS (see Table 2). All primary antibodies were incubated for 90 minutes at room temperature. Secondary antibodies were a ready-to-use biotinylated goat anti-mouse and anti-rabbit link (Dako, LSAB Kit) and biotinylated rabbit anti-goat IgG (Vector Laboratories Burlingame, CA, USA) used at 1:100 dilution (diluted in 5% normal horse serum; see Table 3). All secondary antibodies were incubated for 45 minutes at room temperature. The conventional streptavidin peroxidase method (Dako, LSAB Kit) was performed for each antibody and the reaction was visualized with a 3-amino-9-ethylcarbazol substrate (AEC Substrate Chromogen, Dako). Tissues were counterstained with Gill's No. 3 Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA), mounted with glycerol gelatin (Sigma-Aldrich, St. Louis, MO, USA) and coverslipped. For negative control samples, primary antibodies were replaced by PBS.

To confirm coexpression annexin V with CD68, double immunofluorescence staining was performed. Tissue sections were fixed in acetone (-20°C) and blocked with 5% normal serum of the species of the secondary antibody diluted in PBS with 1% albumin from bovine serum.

Goat anti-annexin V was first primary antibody (1:100; R-20, Santa Cruz, Dallas, TX, USA) and coupled with rabbit Alexa Red 594 (1:500; Life Technologies, Grand Island, NY, USA) as second primary antibody. Mouse second primary antibody against CD68 (Dako, Clone KP1, Carpinteria, CA, USA) was coupled with goat Alexa Green 488 (1:300; Life Technologies, Grand Island, NY, USA). Primary antibodies were incubated overnight (14 hours), secondary antibodies were incubated for 40 minutes. Nuclei were counterstained with DAPI (4',6-Diamidin-2-phenylindol; Nuc Blue Fixed Cell Stain from Life Technologies, Grand Island, NY, USA). Slides were mounted with fluorescent mounting medium (Dako, Carpinteria, CA, USA) and coverslipped.

2.4. Quantitative analysis

For measurements of gross valve pictures, the area of calcification nodules as well as the distance from nodule to tip of the valve were measured using the software ImagePro Plus (6.0 Media Cybernetics, Rockville, MD, USA). To measure the calcified areas after alizarin red staining, whole tissue images were taken with an Omnyx™ VL4 slide scanner (GE Healthcare, Pittsburgh, PA, USA). The percentage of positive staining was calculated using the software ImagePro Plus (6.0 Media Cybernetics, Rockville, MD, USA).

For quantification of immunohistochemistry staining, five high power field images per tissue were captured at 400× magnification (Eclipse 50i, Nikon Instruments, Melville, NY, USA) with a cooled CCD camera (DS-Fi1c, Nikon Instruments, Melville, NY, USA). Positive stained cells in the fibrosa layer of the valve and in the area around calcified nodules were quantified. Adjacent positive staining in the spongiosa layer was also included while the ventricularis layer was always excluded.

The RGB images were transformed into binary images using color deconvolution software as described previously (Ruifrok and Johnston, 2001; Taylor and Levenson, 2006). These images were then analyzed using quantification features of the imaging software NIS-Elements AR (Advanced Research) 3.1 (Nikon Instruments, Melville, NY, USA). For each antibody, a threshold was determined in order to quantify positive stained area.

2.5. Statistic Analysis

Differences between non-diabetic and diabetic patients were evaluated by a two-tailed unpaired Mann Whitney test. $P < 0.05$ was considered significant. Unless otherwise noted, all values are expressed as means \pm standard error of the mean (mean \pm SEM). Statistical analyzes were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Patient cohort

Our study included 17 diabetic and 28 non-diabetic CAVD patients. As CAVD is an age-dependent disease, similarly aged groups are necessary to minimize the influence of age on the assessed parameters. Diabetic patients showed characteristic metabolic features, such as higher BMI, glucose and HbA1c values. Clinically, non-diabetic and diabetic patients had similar AS severity, as expressed by measurements of aortic mean pressure gradient, ejection fraction and blood pressure. Aortic valve area was slightly larger in diabetic compared to non-diabetic patients at the time of valve replacement (Table 1).

3.2 Aortic valve leaflet macrocalcification is increased in diabetic patients

Gross overview images of the aortic valve leaflets removed from CAVD patients were used to identify macroscopic valve calcification. The percentage of positive area of calcified nodules within valve tissue was significantly greater in diabetic compared to non-diabetic individuals (Fig. 1A). Consistently, the distance between the calcification nodules and the tip of the leaflet was significantly smaller in diabetic patients suggesting a more advanced stage

of calcification in diabetic patients (Fig. 1B). However, potential contraction of calcified leaflets may have influenced the measurements of the distance to the tip of the leaflet.

Histological staining for calcium deposition using alizarin red in resected aortic valve leaflets of diabetic patients was stronger than in non-diabetic patients (Fig. 1C3). Those samples with macroscopic visible calcification showed an even greater difference between diabetic and non-diabetic patients ($p=0.05$; Fig. 1C4).

3.3. Early calcification markers are increased in diabetic patients

Based on differences in macrocalcification, molecular markers for early calcification were investigated. Expression of *Runx2* ($p=0.02$; Fig. 2A) and *ALP* ($p=0.03$; Fig. 2B) were significantly higher in diabetic patients suggesting that many cells may undergo osteogenic differentiation and that calcification is still active in these individuals. In diabetic patients we found higher expression of *annexin II* ($p=0.04$; Fig. 2C). However, *annexin V* expression was lower in the diabetic group ($p=0.04$; Fig. 2D). Since Annexin V may be released from macrophages (New *et al.*, 2013), we performed co-labeling of Annexin V and CD68, a macrophage marker. Immunofluorescence staining confirmed colocalization of annexin V with the CD68-positive macrophages (Fig. 3).

3.4. Inflammation and immune response are not increased in diabetic patients

Insulin resistance, as a central feature of the metabolic syndrome and DMII, is thought to be associated with the pathogenesis of CAVD (Utsunomiya *et al.*, 2014). Therefore, it was expected that diabetic patients more frequently show signs of inflammation. In early aortic valve lesions, T lymphocytes, such as CD4 and CD8 T cells, (Olsson *et al.*, 1994; Wallby *et al.*, 2002) and macrophages (Otto *et al.*, 1994) are the predominant inflammatory cell types.

There was no difference in the number of *CD4* and *CD8* T cells between the two patient groups (Fig. 4A, 4B). Macrophages (*CD68* cells) were similarly infrequent in both groups (Fig. 4C). Previous studies have shown that the pro-inflammatory calcium-binding S100A proteins associate with inflammation and calcification, as found in serum analysis and histological assessments (Yan and Bowman, 2014). *S100A9* is preponderantly expressed by macrophages (Croce, 2010) and described to mediate macrophage recruitment into inflamed tissue. (Croce *et al.*, 2009) We found a significantly lower expression of S100A9 in diabetic patients compared to non-diabetic patients ($p=0.002$; Fig. 4D).

Control stainings were performed for *Runx2*, *ALP*, *CD68* and *S100A9* on healthy aortic valves ($n = 3$) obtained from autopsies and expectedly showed no relevant stainings (Fig. 5).

4. Discussion

It has been previously concluded that DMII contributes to CAVD pathogenesis by promoting early mineralization of the aortic valve (Le Quang *et al.*, 2014). However, this conclusion was derived from observations in a mouse model and inflammation was not assessed.

Recently published data point towards significant differences in inflammatory and calcification mechanisms between mouse and human (Thubrikar *et al.*, 1986; Ingersoll *et al.*, 2010; Miller *et al.*, 2011; Cheek *et al.*, 2012; Seok *et al.*, 2013). Therefore, the present study

specifically examined the role of DMII to provide insights into the pathology of human CAVD. Here we show that diabetic CAVD is accompanied by a greater degree of calcification, whereas inflammation is similar in diabetic and non-diabetic individuals. This data suggests that, at the time of valve replacement, diabetic patients may be in a more advanced disease stage than non-diabetic patients. Whereas clinical severity of AS appeared to be comparable in both groups or even less severe in the diabetic individuals (Table 3), diabetic patients show less or equal inflammatory signs and more early calcification. Likewise, glucose levels seem to play a role in the severity of calcification as diabetic patients with higher HbA_{1c} levels (> 6.5%) show a trend towards more macrocalcification as detected by alizarin red staining (data not shown). In addition, the calcification process may also differ in diabetic individuals with progression *via* different mechanisms unassociated with inflammation.

The presence and upregulation of Runx2 in diabetic patients found in the present study strongly supports the concept that CAVD is an actively regulated process (Wirrig and Yutzy, 2013). It indicates that at the time of aortic valve surgery more valve interstitial cells (VIC) are undergoing osteogenic differentiation, a process promoted by Runx2 (Yang *et al.*, 2009); (Ladich *et al.*, 2011). This interpretation related to early calcification is supported by our data that the direct downstream target of Runx2, ALP, was likewise elevated in diabetic patients. Notably, ALP promotes mineral deposition into the extracellular matrix by providing inorganic phosphate through hydrolysis of pyrophosphate (Orimo, 2010).

Annexins II, V and VI are abundant in bone matrix vesicles (Kirsch *et al.*, 1997; Wu *et al.*, 1997; Kirsch *et al.*, 2000; Thouverey *et al.*, 2009) and play a role in microcalcification via facilitating Ca²⁺ entry into the vesicles and the formation of hydroxyapatite (Genge *et al.*, 1990). Most reports on the role of annexins in humans were focused on their role in bone tissue. We found that annexin II expression, primarily detected in close proximity to calcific lesions, was increased in diabetic CAVD patients, suggesting participation of annexins in microcalcification, with similarities to skeletal bone development.

By contrast, we found decreased annexin V expression in diabetic patients. Immunofluorescence double labeling revealed co-localization of annexin V and CD68 suggesting that annexin V may be released from macrophages via extracellular vesicles or apoptotic bodies. However, since the number of macrophages was low, the levels of annexin V were also decreased in diabetic patients. Several annexins have been reported to interact with S100 proteins (Rety *et al.*, 1999; Lewit-Bentley *et al.*, 2000; Rety *et al.*, 2000) and the formation of phosphatidylserine - annexin V - S100A9 - hydroxyapatite complexes may be involved in the secretion of calcifying matrix vesicles from macrophages as described by our group (New *et al.*, 2013). The significant decrease of S100A9 in diabetic patients may be a consequence of reduced macrophage or rather granulocyte numbers and annexin V expression. It could also be envisaged that diabetic factors such as hyperlipidemia and high oxidative stress, which are present in the diseased valve, may conjoin with inflammatory processes within the tissue and together enhance calcification. Likewise, it is plausible that calcification in diabetic patients may undergo different pathways independent from inflammation (e.g. apoptosis). Due to multifaceted roles of annexins, further investigation is necessary to pinpoint their exact functions in aortic stenosis and DMII.

Calcification of the aortic valve is also triggered by inflammation induced by radiation, infectious disease and metabolic syndrome (Aikawa, Nahrendorf, Sosnovik, *et al.*, 2007). Metabolic syndrome has been shown to be independently associated with presence (Katz *et al.*, 2006) and progress of CAVD (Katz *et al.*, 2009) and aortic stenosis (Briand *et al.*, 2006). Contrary to expectations, inflammation was diminished in diabetic patients. T lymphocytes, which have been suggested to have a mediating role in the pathogenesis of aortic stenosis, (Wu *et al.*, 2007) and particularly S100A9, which was found in different inflammatory disorders, (Bouma *et al.*, 2004; Burke *et al.*, 2004; Johansson *et al.*, 2008; Lood *et al.*, 2011) were less expressed in valves explanted from diabetic individuals. These results are in contrast with the findings of Natorska *et al.* who reported elevated signs of inflammation in diabetic patients by analyzing the expression of C-reactive protein (CRP) in aortic stenosis, concluding that enhanced inflammation might contribute to faster disease progression (Natorska *et al.*, 2012). However, in our observational study we were restricted to surgically removed valve leaflets from patients with end-stage disease. This might have contributed to lower detection of inflammation, which limits the force of conclusions for earlier disease stages.

At present, plasma levels of S100A8/A9 (MRP-8/14) serve as indicators of the risk for cardiovascular disease and can be used as biomarkers for acute cardiovascular events, such as atherosclerotic plaque rupture and thrombosis (Healy *et al.*, 2006; Morrow *et al.*, 2008; Wang, 2014). Serum analysis in addition to histological measurements of inflammatory biomarkers in CAVD individuals could potentially differentiate between general inflammation in the body and locally confined inflammation within the valve tissue. Potentially, S100A9 may also be used in the staging of aortic stenosis severity, high levels arguing for early disease stages, low levels for advancement. As S100A9 is involved in the regulation of vascular inflammation, early targeting of S100A9 may furthermore provide a novel therapeutic approach to diverse forms of appearances of vascular injuries.

As the calcification process during CAVD is currently seen as an inflammation-dependent process divided into three phases – initiation, propagation, and late stage – (Aikawa and Otto, 2012), our findings may suggest that surgically explanted aortic valves with severe aortic stenosis of diabetic patients are more mineralized with higher expression of osteogenic markers than the tissue of non-diabetic patients in a similar end-stage phase. In our study, the criteria for grading aortic stenosis severity, aortic valve area and mean pressure gradient, differ significantly between both patient groups. Aortic valve area is higher and mean pressure gradient lower in diabetic patients, which classifies them clinically as less advanced in disease, whereas on the contrary molecular markers such as Runx2 and ALP have shown that CAVD progresses faster in diabetic patients. Consequently, the inevitable question is if at the time of aortic valve replacement diabetic patients are indeed clinically in less severe stages but molecularly considerably further in disease progression, notably calcification. Future studies involving comparison proteomics and transcriptomics analyses with larger sample sizes are warranted.

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Abbreviations

AVA	aortic valve area
BMI	body mass index
BP	blood pressure
CAVD	calcific aortic valve disease
DMII	type II diabetes mellitus
LVEF	left ventricular ejection fraction
MPG	mean pressure gradient
VIC	valve interstitial cell

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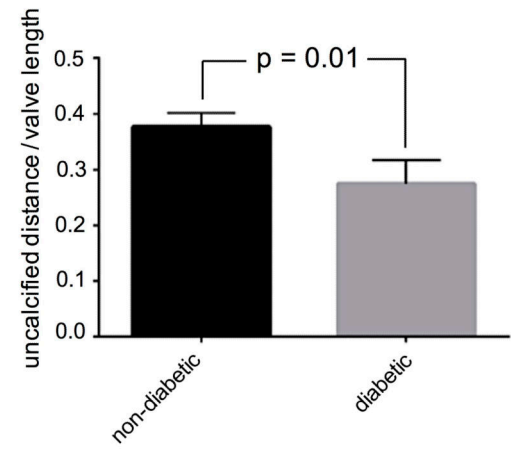
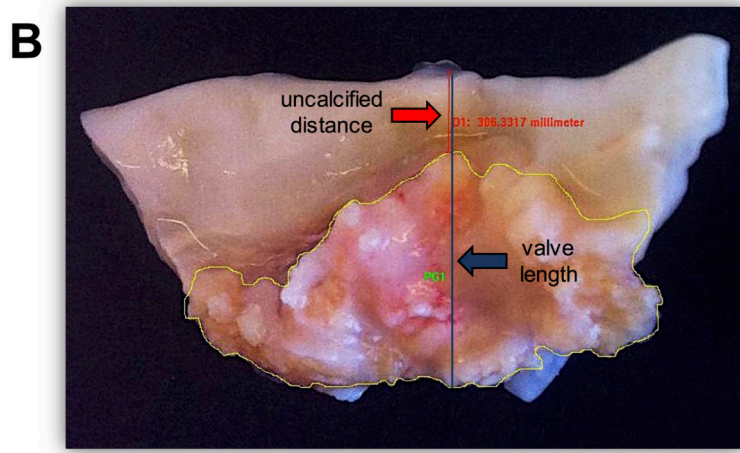
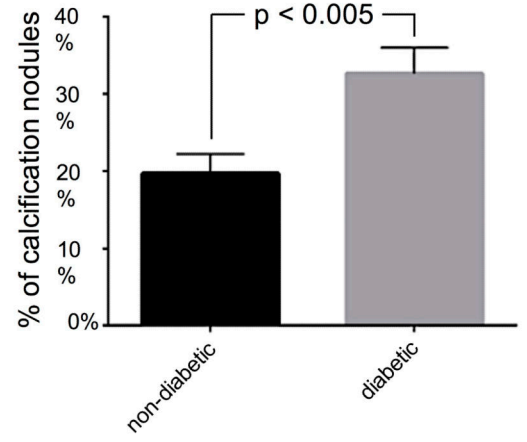
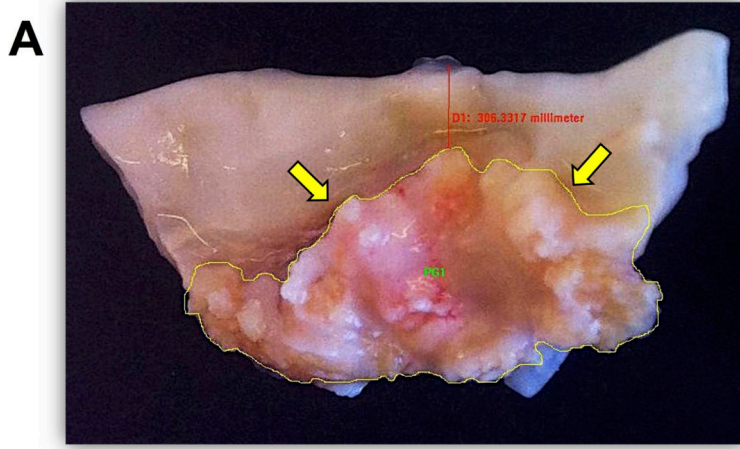
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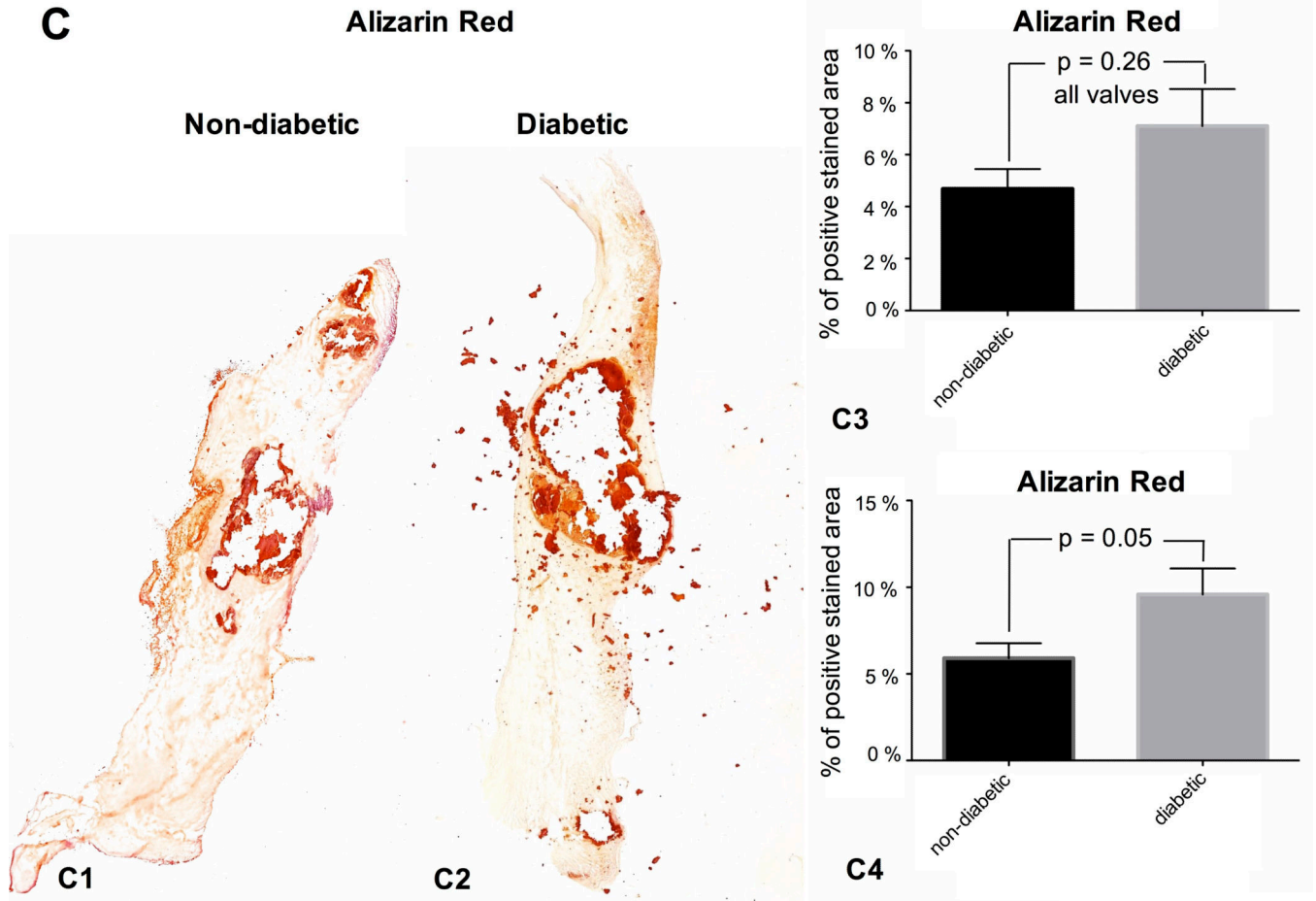
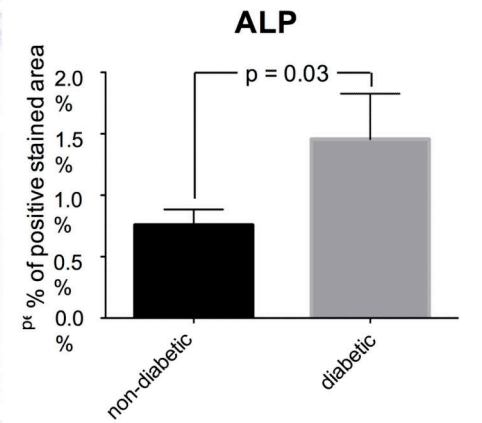
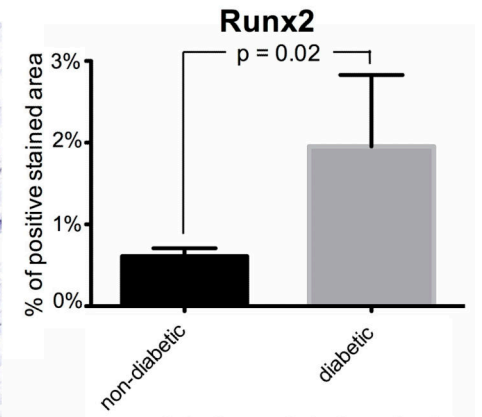
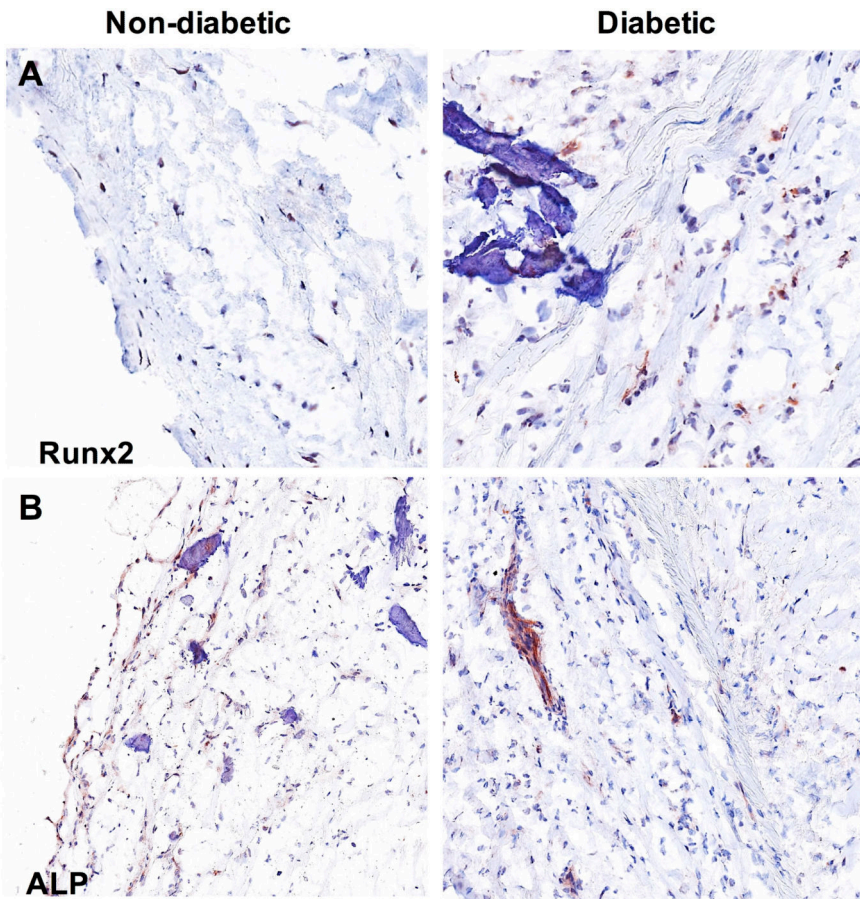


Fig. 1. MACROCALCIFICATION

Yellow arrows – border of calcification area; red arrow – distance from calcification nodule to tip of the valve; blue arrow – total valve length.

The area of macroscopic calcification nodules is larger in diabetic than in non-diabetic valve tissue (*n* = 64) (1A). The ratio of the distance from calcification nodule to the tip of the valve relative to the total length of the valve is shorter in diabetic compared to non-diabetic valve tissue (*n* = 69) (1B). Alizarin red staining reveals larger calcification nodules in diabetic than non-diabetic valve tissue. Whereas the comparison of all valve tissues (1C3; *n* = 45 (17 – 28)) did not reveal a major distinction between the two patient groups, there was a significant difference when comparing valve tissue with macroscopically detectable calcification (1C4; *n* = 34 (12 – 22)). Representative valve tissue sections of a non-diabetic (1C1) and a diabetic patient (1C2) (×40 magnification).



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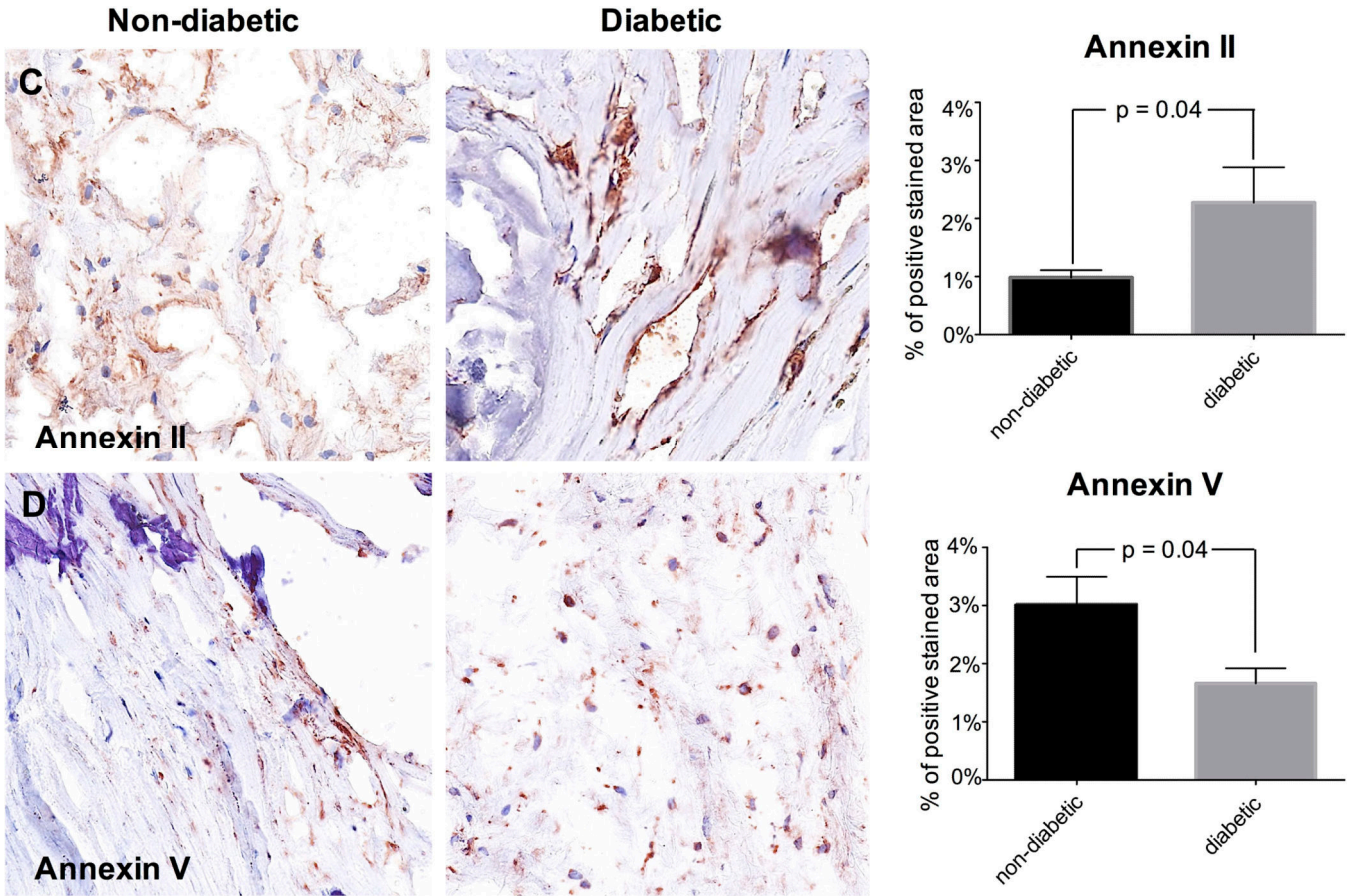


Fig. 2. MICROCALCIFICATION

Runx2 is more expressed in diabetic than in non-diabetic patients. Representative valve tissue sections of a non-diabetic and a diabetic patient ($\times 400$ magnification) (2A; $n = 45$ (17 – 28)). ALP is more expressed in diabetic than in non-diabetic patients. Representative valve tissue sections of a non-diabetic and a diabetic patient ($\times 200$ magnification) (2B; $n = 45$ (17 – 28)). Annexin II is more expressed in diabetic than in non-diabetic tissue sections that are strongly calcified ($n = 34$ (12 – 22)). Representative valve tissue sections of a non-diabetic and a diabetic patient ($\times 40$ magnification) (2C). Annexin V is more expressed in non-diabetic than in diabetic tissue sections that are strongly calcified ($n = 34$ (12 – 22)). Representative valve tissue sections of a non-diabetic and a diabetic patient ($\times 400$ magnification) (2D).

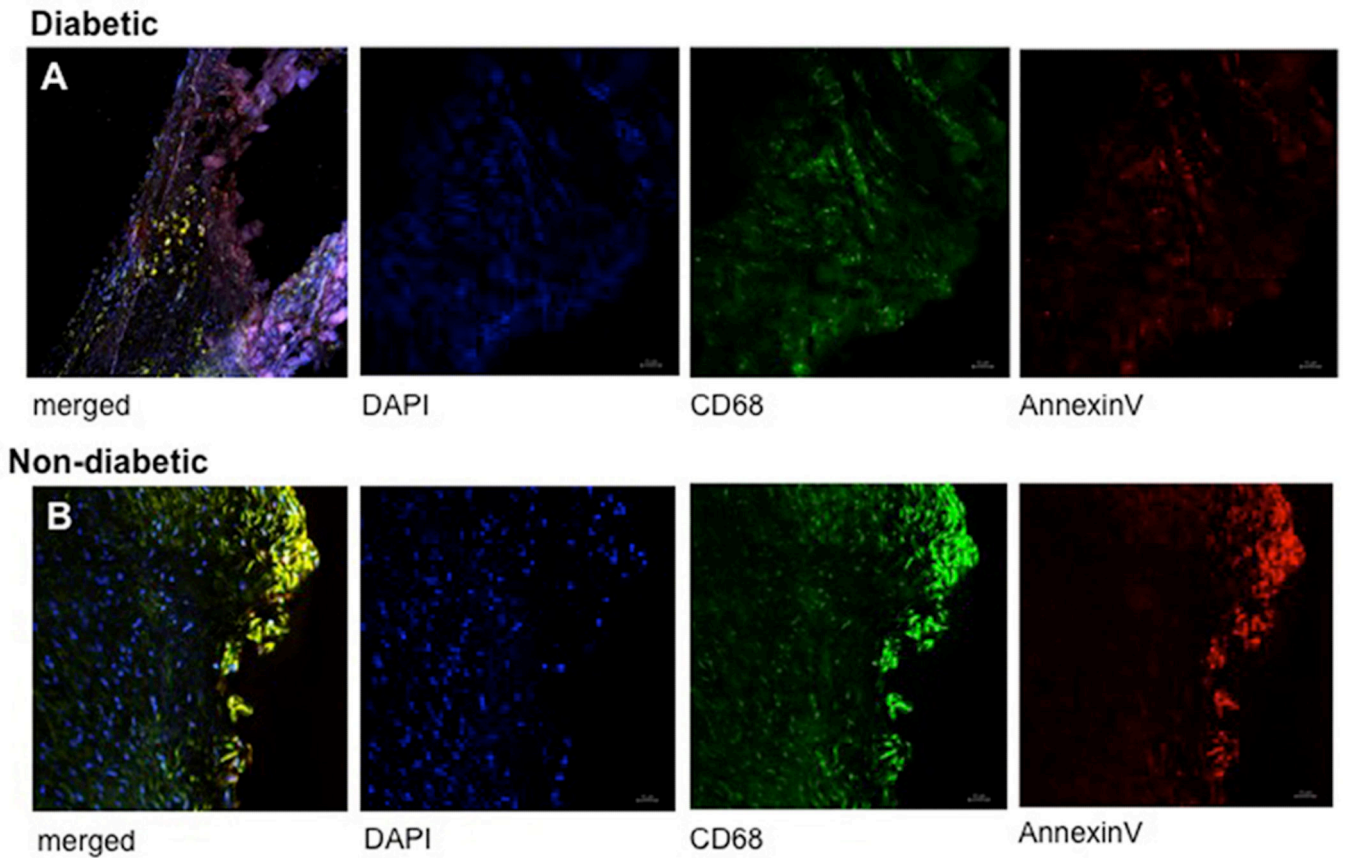
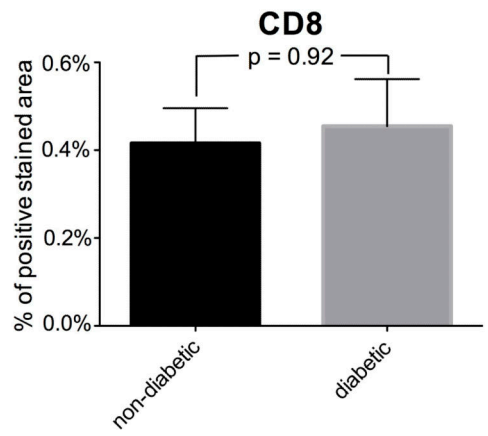
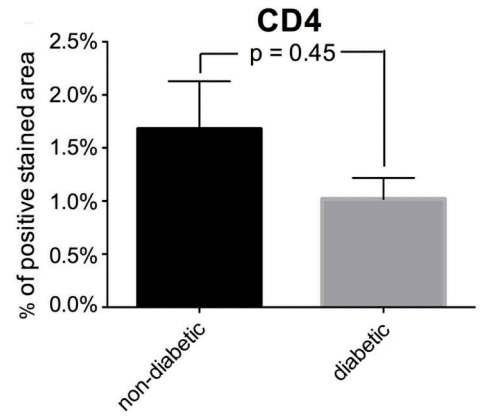
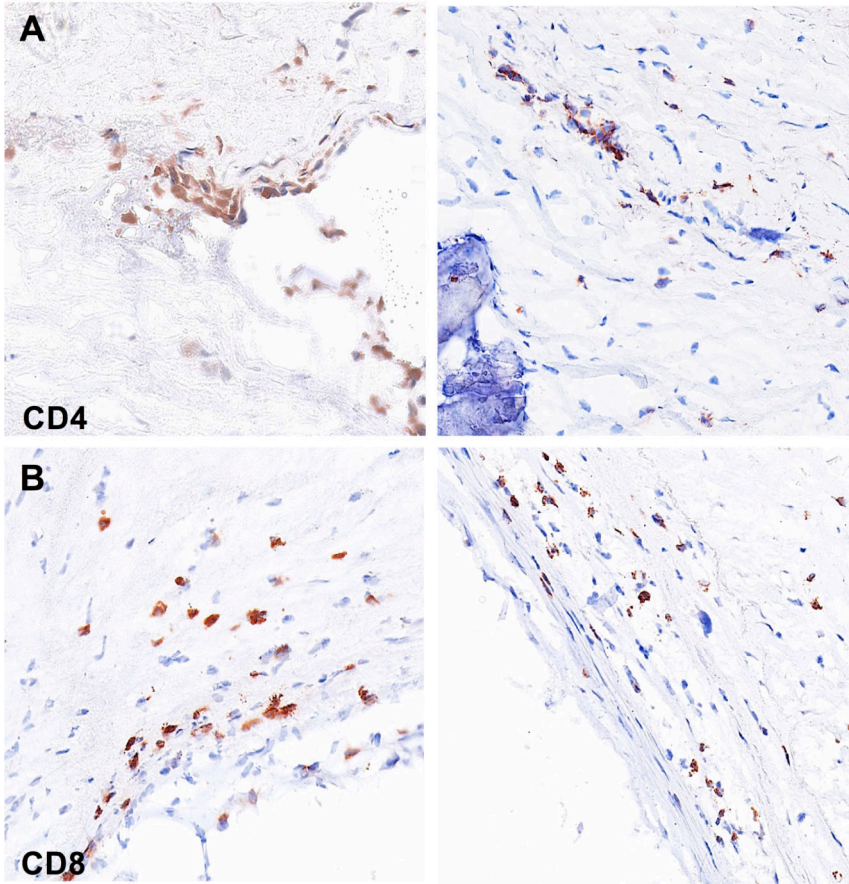


Fig. 3. COLOCALIZATION ANNEXIN V AND CD68

Immunofluorescence double labeling confirms colocalization of annexin V with CD68-positive macrophages, suggesting that annexin V may be released from macrophages via extracellular vesicles or apoptotic bodies. Representative valve tissue sections of a non-diabetic (3A) and a diabetic patient (3B) ($\times 400$ magnification).

Non-diabetic

Diabetic



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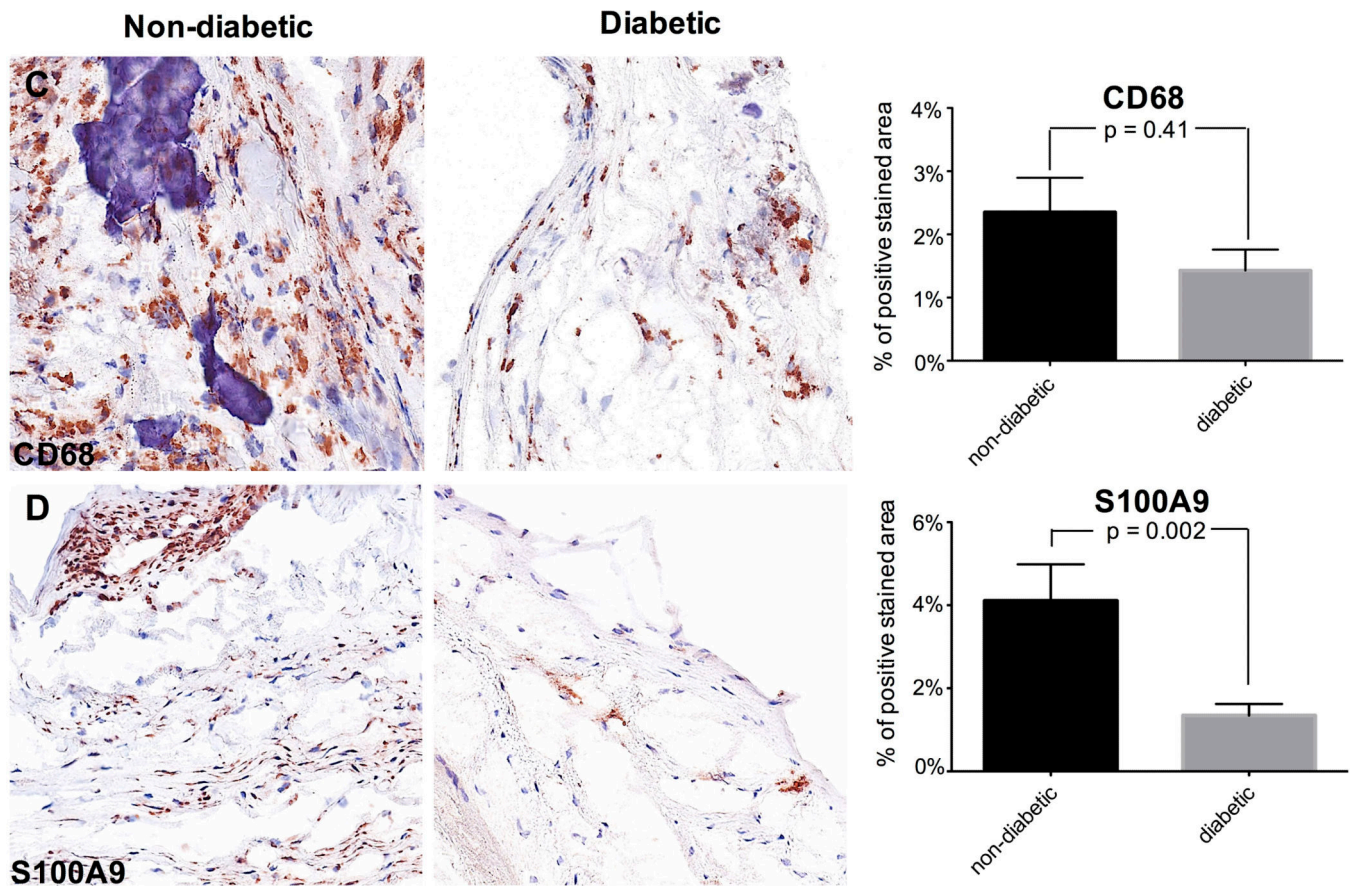


Fig. 4. INFLAMMATION

Representative valve tissue sections of a non-diabetic and a diabetic patient ($\times 400$ magnification) (4A-D). No difference could be detected in the presence of $CD4^+$ and $CD8^+$ T cells (4A, 4B) nor CD68-positive macrophages (4C) between the two patient groups ($n = 45$ (17 – 28)). By contrast, S100A9 is significantly lower expressed in diabetic patients compared to non-diabetic patients (4D; $n = 45$ (17 – 28)).

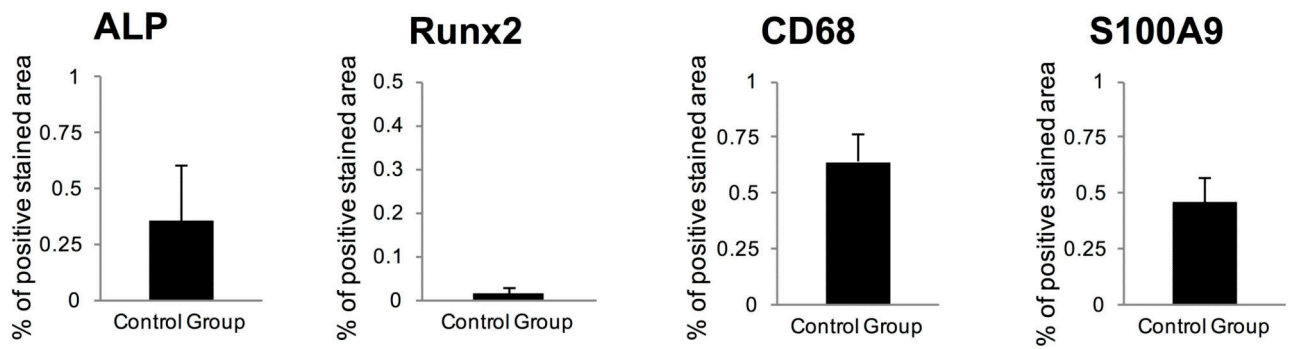
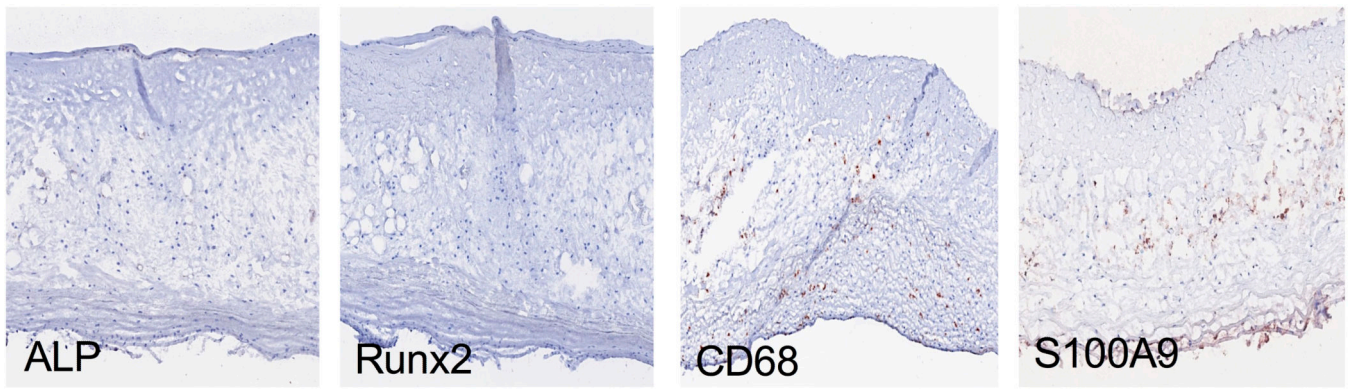


Fig. 5. CONTROL CAVD STAINING

Table 1

Clinical Baseline Characteristics. Continuous variables are recorded as mean \pm SD. Categorical variables are recorded as number and percentage

	All patients (n = 45)	Non-diabetic pa- tients (n = 28)	Diabetic patients (n = 17)	p value
Age (years)	68 \pm 11	67 \pm 11	71 \pm 10	NS
Gender (male)	27(60.0%)	15 (55.6%)	12 (70.6%)	NS
BMI (kg/m ²)	28.6 \pm 5.8 ^c	26.9 \pm 4.6 ^c	31.4 \pm 6.7 ^c	0.020 [*]
Smoker ^a , n (%)	18 (46.5%)	13 (46.4%)	6 (46.7%)	NS
Bicuspid valves, n (%)	14 (31.11%)	11 (39.29%)	3 (17.65%)	0.002 [*]
AVA ^a (cm ²)	0.72 \pm 0.18 ^c	0.68 \pm 0.03 ^c	0.80 \pm 0.04 ^c	0.032 [*]
LVEF ^a (%)	61 \pm 9	61 \pm 10	60 \pm 9	NS
MPG ^a (mmHg)	48 \pm 14 ^c	51 \pm 14 ^c	43 \pm 11 ^c	0.037 [*]
Diastolic BP ^a (mmHg)	76 \pm 12	76 \pm 13	75 \pm 9	NS
Systolic BP ^a (mmHg)	135 \pm 17	136 \pm 16	133 \pm 19	NS
Total cholesterol (mg/dl) ^a	174 \pm 62	159 \pm 41	194 \pm 84	NS
HbA _{1c} (%) ^a	6.2 \pm 1.3	5.5 \pm 0.4	7.1 \pm 1.5 ^c	< 0.0001 [*]
Glucose (mg/dl) ^a	110 \pm 40	96 \pm 13	132 \pm 56 ^c	0.007 [*]
Hemoglobin (g/dl) ^a	13.9 \pm 1.6	14.2 \pm 1.3	13.4 \pm 2.0	NS
GFR (ml/min/1.73m ²)	71.0 \pm 14.2 ^c	73.7 \pm 15.0 ^c	66.6 \pm 12.2 ^c	NS
Antihypertensive me- dication ^a	40 (93.0%)	26 (92.9%)	14 (93.3%)	NS
Statin n (in %)	35 (77.8%)	23 (82.2%)	12 (70.6%)	NS
Insulin n (in %)	-	-	5 (29.4%)	-

Data are shown as n (in %) or mean \pm SD.

* statistically significant ($p < 0.05$).

^a data not for all patients available.

^b calculated with CKD-EPI formula.

^c deviant values.

Table 2

Primary Antibodies for IHC Staining

Antibody	Source	Clonality	Dilution	Developing time	Company	Catalog number
CD 4	mouse	monoclonal	1:25	15 min.	Dako, Clone 4B12	M7310
CD8	mouse	monoclonal	1:50	10 min.	Dako, Clone C8/144B	M7103
CD68	mouse	monoclonal	1:700	15 – 20 min.	Dako, Clone KP1	M0714
annexin II	mouse	monoclonal	1:200	6 – 12 min.	Invitrogen, Clone ZO14	03-4400
annexin V	goat	polyclonal	1:75	10 – 12 min.	Santa Cruz, R-20	Sc-1929
S100A9	rabbit	polyclonal	1:35	20 min.	Proteintech	14226-1-AP
Runx2	mouse	monoclonal	1:25	9 min.	Abcam	Ab115899
ALP	rabbit	monoclonal	1:100	12 min.	Abcam	Ab108337

Table 3

Secondary Antibodies for IHC Staining

Antibody	Source	Directed against	Dilution	Company	Catalog number
biotinylated link	goat	anti-mouse, anti-rabbit	-	Dako, LSAB Kit	K0675
biotinylated IgG	rabbit	anti-goat	1:100	Vector Laboratories	BA-5000