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Plasma Levels of Advanced Glycation End Products are Associated with Haemolysis-related Organ Complications in Sickle Cell Patients

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Summary

Oxidative stress plays an important role in the pathophysiology of sickle cell disease (SCD). Plasma levels of advanced glycation end products (AGEs) are increased under oxidative conditions and are associated with disease severity in diabetes and inflammatory diseases. We questioned whether AGEs are increased in sickle cell patients and whether they are associated with SCD related complications. Plasma levels of the AGEs pentosidine, N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) were measured using single-column high performance liquid chromatography with fluorescence detection (pentosidine) and ultra performance liquid chromatography-tandem mass spectrometry (CML and CEL). Plasma levels of pentosidine and CML were increased in HbSS/HbSβ^0-thalassaemia (n=60) and HbSC/HbSβ^+-thalassaemia (n=42) patients during steady state as compared to healthy HbAA controls (n=30) without increments during painful crisis. CEL levels were comparable between all groups. Pentosidine and CML levels correlated significantly to haemolytic rate during the clinically asymptomatic state while pentosidine was significantly related to the number of haemolysis-related organ complications. The increased plasma AGE levels in sickle cell patients and their association with haemolysis and haemolysis-related complications suggest AGEs might be implicated in the pathophysiology of the haemolytic phenotype of SCD. Measurement of AGEs might be useful in predicting organ complications in SCD.

Keyword: sickle cell disease, advanced glycation end products, oxidative stress, haemolysis, organ damage

Introduction

Sickle cell disease (SCD) is characterized by unstable auto-oxidative sickle haemoglobin (HbS) (Hebbel *et al*, 1988), chronic intravascular haemolysis (Reiter *et al*, 2002), recurrent ischemia reperfusion injury (Nath *et al*, 2005) and low grade inflammation (Akohoue *et al*, 2007), all contributing to an increased generation of reactive oxygen species (ROS) potentially contributing to the characteristic widespread organ damage (van Beers *et al*, 2008).

ROS inflict direct oxidative damage to tissue by reacting with cellular proteins (interfering with their functions), membrane structures, DNA and extracellular matrix (Botto *et al*, 2002;Jain & Shohet, 1984;Rice-Evans *et al*, 1986). Oxidative stress also results in increased production and accumulation of advanced glycation end products (AGEs), which are not only well-established markers of oxidative stress (Koyama *et al*, 2007;Genuth *et al*, 2005;Gerrits *et al*, 2008), but are themselves oxidatively active and have been demonstrated to play a role in the pathophysiology of organ complications (nephropathy, retinopathy, neuropathy and ischemic heart disease) in diabetes and auto-immune inflammatory diseases (Ahmed, 2005;Lieuw *et al*, 2004;Miyata *et al*, 1998;Beisswenger *et al*, 1995).

AGEs are generated by non-enzymatic glycation and oxidation of proteins, lipids and nuclear acids in the Maillard reaction (Ahmed, 2005). AGEs are associated with several pathological mechanisms which could contribute to organ damage and disease severity. Cross-linking of AGEs with intra- and extracellular proteins affects their conformational structure resulting in distortion of normal tissue architecture (Goldin *et al*, 2006). This is especially important in the cardiovascular system where thickening of the basement membrane and reduced elasticity in blood vessels cause reduced filtration rate across the vessel lumen and diminished arteriolar vasodilatatory capacity (Goldin *et al*, 2006;Greenwald, 2007;Aronson, 2003). Another mechanism, through which AGEs inhibit vasodilatation, is by inducing resistance to nitric oxide and suppressing nitric oxide synthesis (Soro-Paavonen *et*

al, 2010). Furthermore, the interaction of AGEs with their receptor RAGE, which is expressed on different cells including endothelial cells and macrophages, enhances the production and expression of pro-inflammatory cytokines, adhesion molecules and oxidants (Ahmed, 2005;Brett *et al*, 1993;Pertynska-Marczewska *et al*, 2004;Liu *et al*, 2009). In diabetes, AGEs have been shown to increase vascular permeability (Hirose *et al*, 2010) which is a major and early characteristic of diabetic vasculopathy (Viberti, 1983). AGEs are also known to induce apoptosis in different cell types and thereby play a significant role in the pathology of various diseases and organ complications (Yamagishi *et al*, 2002;Lim *et al*, 2008;Sekido *et al*, 2004).

A recent study showed elevated plasma levels of AGEs in paediatric sickle cell patients during the clinically asymptomatic state without further increments during painful crisis (Somjee *et al*, 2005). However, patients with macrovascular (cerebrovascular accidents or abnormal transcranial Doppler study) or microvascular (evidence of retinopathy or nephropathy) SCD related organ complications were excluded from this study. As oxidative stress is associated with both acute and chronic organ complications in sickle cell patients (Klings & Farber, 2001;Morris *et al*, 2008;Fibach & Rachmilewitz, 2008), we studied whether AGEs are elevated in adult sickle cell patients and whether they are associated with SCD related organ complications.

Patients and methods

Study population

Adult sickle cell patients (≥18 years), including sickle cell anaemia (HbSS) and compound heterozygous states sickle β^0-thalassaemia (HbSβ^0-thal), sickle β^+-thalassaemia (HbSβ^+-thal) and sickle haemoglobin C disease (HbSC) visiting the out-patient clinic during the clinically asymptomatic state (defined as being free of SCD related acute events, such as painful crisis,

priapism, acute chest syndrome, strokes, sequestration crises and infections during at least 4 months prior to study participation) and patients admitted for a painful crisis (defined as musculo-skeletal pain not otherwise explained and recognized as such by the patient) at the Academic Medical Centre and Slotervaart Hospital, Amsterdam, The Netherlands were eligible for the study. Exclusion criteria were: diabetes mellitus, renal failure, auto-immune inflammatory diseases, active infection and pregnancy. Healthy race matched volunteers with normal haemoglobin (HbAA) served as controls. Haemoglobin (Hb) types were confirmed in all participants by high performance liquid chromatography (HPLC). All participants received verbal and written explanation of the study and subsequently gave written informed consent. The protocol was reviewed and approved by the local medical ethical committee and conducted in agreement with the Helsinki declaration of 2000.

Blood sample Collection

Blood from patients was obtained upon their visit to the outpatient clinic or their presentation at the emergency department. Standard blood counts were performed in EDTA-anticoagulated blood (Cell-Dyn 4000, Abbott, Illinois, USA). Lactate dehydrogenase (LDH) and bilirubin levels were measured in heparinized plasma with spectrophotometry (P800 Modular, Roche, Basel, Switzerland). Blood samples were centrifuged within 30 minutes of withdrawal at 3000 rpm (4 ^{o}C) for 15 minutes and serum and plasma samples were stored at -80^{o}C until further analysis.

Laboratory analysis

The AGEs (pentosidine, N$^{\varepsilon}$-(carboxymethyl)lysine (CML) and N$^{\varepsilon}$-(carboxyethyl)lysine (CEL)) were measured in EDTA anti-coagulated plasma. Pentosidine was measured with single-column HPLC with fluorescence detection and CML and CEL were determined by

ultra performance liquid chromatography-tandem mass spectrometry as described previously (Scheijen *et al*, 2009;Teerlink *et al*, 2004). Inter-assay coefficients of variation were 4.9% for pentosidine, 3.7% for CML and 5.1% for CEL. The intra-assay coefficients of variation were below 4% for all these AGEs. Soluble vascular cell adhesion molecule-1 (sVCAM-1) levels were determined in serum using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). Plasma levels of von Willebrand Factor antigen (vWF:Ag) were determined in citrate plasma using a homemade ELISA.

Data analysis

For data analysis, patients were classified according to genotype into two groups: patients with the relatively severe genotypes HbSS and HbSß^0-thal grouped in one group (HbSS/HbSβ^0-thal) and patients with the relatively milder HbSC and HbSß$^+$-thal genotypes collected in the other group (HbSC/HbSβ$^+$-thal) (Powars *et al*, 2002). SCD related organ complications were grouped as haemolysis related (pulmonary hypertension, leg ulcers, priapism, ischemic stroke, cholecystolithiasis and microalbuminuria) or viscosity-vaso-occlusion related (acute painful crisis, acute chest syndrome, retinopathy and avascular necrosis of bone) complications (Kato *et al*, 2007).

Statistical analysis

As data were not normally distributed, statistical tests for nonparametric data were used. For multiple group comparisons of continuous variables the Kruskal–Wallis test was employed. The Mann–Whitney U-test was used for comparison between two groups. The Wilcoxon Signed Rank Test was used for a paired analysis of patients who were included both during asymptomatic state and painful crisis. For correlation studies, the Spearman Rank correlation coefficient (r_s) was determined. To analyze the association of plasma AGEs with haemolysis

and fetal Hb (HbF), a multivariate stepwise regression model with forward entry and removal was constructed with one of the AGEs as the dependent variable and group (healthy control, patient during steady state or painful crisis), age, Hb, reticulocyte counts, LDH, total bilirubin and HbF as the independent variables. P <0.05 was considered statistically significant (SPSS 16.0, Chicago, IL, USA).

Results

Sixty HbSS/HbSβ^0-thal patients (54 HbSS, 6 HbSβ^0-thal) and 42 HbSC/HbSβ^+-thal (32 HbSC, 10 HbSβ^+-thal) were included during the asymptomatic state and 27 HbSS/HbSβ^0-thal patients (25 HbSS, 2 HbSβ^0-thal) and 8 HbSC/HbSβ^+-thal patients (6 HbSC, 2 HbSβ^+-thal) were included during acute painful crisis. Thirty healthy race matched HbAA volunteers served as controls. Table 1 shows clinical and biochemical characteristics of the participants.

Plasma levels of pentosidine were significantly higher in asymptomatic state HbSS/HbSβ^0-thal ($P < 0.0001$) and HbSC/HbSβ^+-thal patients ($P = 0.002$) than in healthy controls but did not increase further during painful crisis (Figure 1A). CML levels were significantly higher in asymptomatic state HbSS/HbSβ^0-thal patients as compared to controls ($P = 0.007$; Figure 1B) but were comparable between controls and asymptomatic state HbSC/HbSβ^+-thal patients. No further increases in CML levels were observed during painful crisis. Also in a paired analysis of 25 patients included both during asymptomatic state and painful crisis, there were no increases in plasma levels of pentosidine and CML during painful crisis (data not shown). CEL levels were comparable between controls and patients during both asymptomatic state and painful crisis (Figure 1C).

Pentosidine and CML levels were higher in sickle cell patients with 1, 2 and 3 or 4 haemolysis-related organ complications than in patients without these complications (Figures

2A and 2C). Also when dividing the patients into 2 groups of patients with at least one haemolysis-related complication versus patients without these complications, pentosidine and CML levels were higher in the former group (Figures 2B and 2D). As haemolysis is more severe in HbSS/HbSβ^0-thal patients and there were more patients in the relatively milder HbSC/HbSβ^+-thal patients who were not tested for all listed organ complications, we also analyzed the differences in pentosidine and CML levels in the HbSS/HbSβ^0-thal group alone, where the differences between patients with and those without haemolysis-related complications were more pronounced (Figures 2B and 2D). AGEs were not associated with viscosity-vaso-occlusion related organ complications (data not shown).

In a sub-analysis of the 60 HbSS/HbSβ^0 patients, pentosidine and CML concentrations were significantly related to haemolytic rate (Table 2). Plasma concentrations of pentosidine and CML were also inversely related to HbF% (Table 2). In a multivariate stepwise regression analysis, adjusting for age, group, HbF and the markers of haemolytic anaemia, only LDH was significantly related to both pentosidine (0.55 x LDH; $P < 0.0001$) and CML (0.35 x LDH; $P < 0.0001$).

Both pentosidine and CML levels were significantly related to sVCAM-1 levels in HbSS/HbSβ^0-thal patients during asymptomatic state. During painful crisis the correlation between AGEs and sVCAM-1 was stronger than during the asymptomatic state (Table 3). Pentosidine levels in asymptomatic state patients also correlated with plasma levels of vWF:Ag (Table 3).

The measured AGEs did not correlate with age or BMI in sickle cell patients (data not shown). In controls, only pentosidine levels were related to age (Sr = 0.49, $P = 0.006$). Nineteen patients during asymptomatic state (16 HbSS/HbSβ^0-thal and 3 HbSC/HbSβ^+-thal) and 8 patients during painful crisis (5 HbSS/HbSβ^0-thal and 3 HbSC/HbSβ^+-thal) were on hydroxyurea therapy. AGE levels were not different between patients with and those without

hydroxyurea therapy (data not shown). As AGEs are cleared by the kidneys, we analysed correlations between AGEs and plasma creatinine as a biomarker of renal function. However, AGEs were not related to creatinine levels in both controls and sickle cell patients (data not shown).

Discussion

In the current study, plasma levels of the AGEs pentosidine and CML, which are established biomarkers of oxidative stress, were higher in clinically asymptomatic sickle cell patients as compared to healthy controls, without further increments during painful crisis. These findings confirm the observations by Somjee et al, who found increased levels of AGEs in children with SCD without further increases during painful crisis (Somjee *et al*, 2005). In our study, we measured three different AGEs with specific analytical techniques and pentosidine and CML, but not CEL, were significantly related to the haemolytic rate and accordingly significantly associated with endothelial activation as reflected by sVCAM-1 and vWF;Ag levels and with the presence of haemolysis related organ damage. These data suggest that AGEs may play an important role in the pathophysiology of chronic haemolysis associated organ complications but not of viscosity-vaso-occlusion associated complications in SCD (Kato *et al*, 2005;Landburg *et al*, 2010). However, further studies are needed to elucidate the mechanistic contributions of AGEs to haemolysis related organ complications in SCD.

Due to continuous intravascular haemolysis, sickle cell patients have highly increased plasma levels of cell-free haem. The hydrophobic haem rapidly intercalates into the plasma membrane of (endothelial) cells where it releases its iron (Jeney *et al*, 2002), which potentiates endothelial cell activation and damage by catalyzing non-enzymatic generation of ROS and AGEs (Jeney *et al*, 2002;Galaris & Pantopoulos, 2008). Indeed, several studies have

shown an association between haemolytic rate and endothelial activation in SCD (Schnog *et al*, 2003;Kato *et al*, 2006), which could, at least in part, be explained by the haemolysis driven production of AGEs. In line with this observation, serum levels of sVCAM-1 correlated with plasma levels of pentosidine and CML. Also a possible ameliorating effect of HbF on oxidative stress, through both lower percentage of the auto-oxidative HbS and reduced haemolysis, is suggested by the inverse correlations of pentosidine and CML with HbF (Hebbel *et al*, 1988). Total glutathione and its reduced form (GSH), a major intracellular antioxidant, have been reported to be decreased in sickle cell patients (Tatum & Chow, 1996;Wetterstroem *et al*, 1984). Somjee et al (2005) reported a strong inverse correlation between plasma AGEs and GSH levels in sickle erythrocytes. This might suggest that increased production of ROS and AGEs, mainly due to the auto-oxidative HbS, causes excessive consumption of the intracellular antioxidants rendering the erythrocytes more susceptible to oxidative damage. On the other hand, reduced GSH may be a rate-limiting factor in the increased formation of AGEs since GSH is an essential cofactor for glyoxalase 1, the enzyme that efficiently detoxifies the major AGE precursor methylglyoxal to S-d-lactoyl-gluthathione (Brouwers *et al*, 2010).

While AGEs in the study patients were increased during the clinically asymptomatic state, no further increments were detected during painful crises, suggesting that factors contributing to local vaso-occlusive painful crisis with potential ischemia-reperfusion injury have no significant effect on plasma levels of AGEs. This observation confirms findings by earlier studies which also did not detect further increases in AGEs (Somjee *et al*, 2005) and isoprostanes (another marker of oxidative stress) (Klings *et al*, 2001) during painful crisis. This is confirmed by the fact that pentosidine and CML correlated only with haemolysis-related organ complications and not with viscosity-vaso-occlusion related complications. AGEs may therefore potentially be useful as biomarkers of haemolysis related complications

in the long term follow-up of sickle cell patients. AGEs are produced within hours, are cleared by the kidneys and most plasma AGEs are irreversibly bound to serum albumin, which has a half-life of approximately 20 days (Jones *et al*, 1983). Therefore, AGE half-life is not likely to have affected our results during painful crisis.

The correlations between AGEs and markers of endothelial activation (sVCAM-1 and vWF:Ag) suggest that AGEs might be implicated in endothelial damage, both via accumulation in vascular wall and activation of RAGE (Ahmed, 2005). Interestingly, during painful crisis, without further increments in AGEs, the correlations of pentosidine and CML with sVCAM-1 were stronger than in asymptomatic state. While the exact mechanism for this enhancing effect of painful crisis on the relationship between AGEs and endothelial activation needs to be studied further, one possible explanation is an up-regulating effect of increased inflammation during painful crisis on endothelial expression of RAGE (van Beijnum *et al*, 2008;Fiuza *et al*, 2003). Potential increased endothelial expression of RAGE during painful crisis could amplify effects of AGEs on endothelial activation.

In contrast to pentosidine and CML, plasma levels of CEL were not different between controls and sickle cell patients. This might be explained by the fact that unlike pentosidine and CML, production of CEL, via modifications of proteins by methylglyoxal, is highly dependent on hyperglycemia (Ahmed *et al*, 1997), which is rare in SCD.

Since AGEs were strongly related to LDH on multivariate regression analysis, one might question whether AGEs are just a biomarker of haemolytic rate in SCD. However, while AGEs seem primarily to be downstream products of the excessive intravascular haemolysis in SCD, given the strong evidence about their role in pathophysiology of (organ complications in) other disease states, AGEs most likely have additional role in the pathophysiology and disease severity of SCD.

In conclusion, plasma levels of pentosidine and CML are increased and associated with haemolysis and haemolysis-related organ complications in sickle cell patients, suggesting that AGEs might be implicated in vascular damage and chronic organ complications in SCD. Measurement of these AGEs might be useful in assessing disease severity and predicting organ complications in SCD.

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Authorship Contributions

E.N. designed and performed research, analysed data and wrote the manuscript; D.P.B., H.M.O. and K.F critically reviewed and edited the manuscript, C.G.S. analysed data and edited the manuscript, J.J.B.S. and B.J.B. designed research, participated in data analyses and in writing the manuscript.

Conflict of Interest Disclosure

All authors declare no conflicts of interest.

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Table 1. Baseline characteristics.

	Controls	Asymptomatic State		Painful Crisis	
		HbSC/HbSβ$^+$	HbSS/HbSβ^0	HbSC/HbSβ$^+$	HbSS/HbSβ^0
	(n = 30)	(n = 42)	(n = 60)	(n = 8)	(n = 27)
Age, y	39 (19 – 59)	30 (18 – 57)*	25 (18 – 57)*	25 (20 – 48)	25 (19 – 48)
Gender (male/female)	19/11	27/15	35/25	8/0	14/13
BMI (Kg/m^2)	25.4 (23.4 – 27.2)	23.4 (21.4 – 28.2)	21.3 (19.4 – 24.0)*¥	21.3 (18.8 – 28.4)	22.7 (19.6 – 26.0)
Hb (mmol/L)	7.9 (7.5 – 8.6)	6.9 (6.6 – 7.3)*	5.5 (4.9 – 6.1)*¥	6.9 (6.6 – 7.1)*	5.2 (4.4 – 5.8)*¥
Leukocytes (10^{12}/L)	5.3 (4.4 – 6.8)	6.4 (5.0 – 8.4)*	9.8 (7.9 – 10.9)*¥	8.4 (6.4 – 10.2)*	11.9 (10.6 – 14.0)*¥†
Platelets (10^{12}/L)	244 (205 – 296)	209 (161 – 336)	371 (284 – 481)*¥	183 (127 – 236)	334 (282 – 394)*¥
LDH (U/L)	177 (153 – 202)	230 (183 – 260)*	383 (304 – 504)*¥	223 (198 – 398)*	460 (425 – 641)*¥†
sVCAM-1 (ng/mL)	413 (368 – 474)	777 (630 – 924)*	987 (782 – 1254)*¥	741 (592 – 1109)	946 (811 – 1434)

Hb haemoglobin; LDH lactate dehydrogenase. Numbers are medians with 25th and 75th percentiles, age expressed as medians with range.
* Significantly different as compared to healthy controls ($P < 0.05$).
¥ Significantly different as compared to HbSC/HbSβ$^+$ within one state ($P < 0.05$).
† Significantly different as compared to asymptomatic state ($P < 0.05$).

Table 2. Correlation analyses of plasma levels of pentosidine and CML with markers of haemolysis and HbF in 60 HbSS/HbSβ^0-thalassaemia patients in asymptomatic state.

	Pentosidine (nmol/L)		CML (nmol/L)	
	Spearman's rho	*P*-value	Spearman's rho	*P*-value
Haemoglobin (mmol/L)	-0.29	0.024	-0.33	0.009
Haematocrit (L/L)	-0.43	0.032	-0.43	0.030
Reticulocyte %	0.34	0.018	0.28	0.054
LDH (U/L)	0.26	0.048	0.40	0.002
HbF (%)	-0.33	0.012	-0.35	0.006

Spearman Rank correlation coefficient (r_s) was used. CML N^{ε}-(carboxymethyl)lysine; LDH lactate dehydrogenase; HbF faetal haemoglobin

Table 3. Correlations between markers of endothelial activations (sVCAM-1 and vWF:Ag) and pentosidine and CML in 60 HbSS/HbSβ^0-thalassaemia patients in asymptomatic state.

	sVCAM-1 (ng/mL)				vWF:Ag (%)			
	Asymptomatic State		Painful Crisis		Asymptomatic State		Painful Crisis	
	Spearman's rho	*P*-value	Spearman's rho	*P*-value	Spearman's rho	*P*-value	Spearman's rho	*P*-value
Pentosidine (nmol/L)	0.27	0.035	0.45	0.020	0.28	0.032	0.36	0.074
CML (nmol/L)	0.28	0.031	0.56	0.002	Not related		Not related	

sVCAM-1 soluble vascular adhesion molecule-1, vWF:Ag von Willebrand antigen.
Spearman Rank correlation coefficient (r_s) was used. CML N^{ε}-(carboxymethyl)lysine.

Legends to the figures

Figure 1

Plasma levels of AGEs in healthy controls (CTRL) and HbSC/HbSβ$^+$ (SC) and HbSS/HbSβ^0 (SS) patients during asymptomatic state (AS) and painful crisis (CR). (A) While pentosidine levels are higher in both HbSC/HbSβ$^+$ (SC-AS; n = 42; $P = 0.002$) and HbSS/HbSβ^0 (SS-AS; n = 60; $P < 0.0001$) patients during asymptomatic state than in healthy controls (n = 30), they do not increase further during painful crisis (SC-CR, n = 8 and SS-CR, n = 27 respectively). Within the patients during steady state, HbSS/HbSβ^0 patients have higher pentosidine levels than HbSS/HbSβ$^+$ patients ($P < 0.0001$) (B) Levels of N$^\varepsilon$-(carboxymethyl)lysine (CML) are comparable between healthy controls and asymptomatic state HbSC/HbSβ$^+$ patients but significantly higher in asymptomatic state HbSS/HbSβ^0 patients ($P = 0.007$). Also CML is higher in steady state HbSS/HbSβ^0 patients than steady state HbSS/HbSβ$^+$ patients ($P = 0.008$). (C) Plasma levels of N$^\varepsilon$-(carboxyethyl)lysine (CEL) are comparable between healthy controls and sickle cell patients both during asymptomatic state and painful crisis. Note the interruptions in the scale on Y axes.

Figure 2.

Plasma levels of pentosidine and N$^\varepsilon$-(carboxymethyl)lysine (CML) in association with haemolysis related complications. (A) Pentosidine levels are higher in sickle cell patients with 1, 2 and 3 or 4 haemolysis-related organ complications compared to patients without these complications ($P = 0.004$, Kruskal-Wallis test). (B) When divided in two groups of patients without and those with at least one haemolysis-related organ complication, plasma levels of pentosidine were significantly higher in the latter group. This applied both when the differences were analyzed across all sickle cell patients in asymptomatic state ($P = 0.006$) and

when the differences were analyzed for HbSS/HbSβ^0 patients alone (P = 0.004). (C) CML levels are higher in sickle cell patients with 1, 2 and 3 or 4 haemolysis-related organ complications compared to patients without these complications (P = 0.044, Kruskal-Wallis test). (D) Also when divided in two groups of patients without and those with at least one haemolysis-related organ complication, plasma levels of CML were higher in the latter group, though the differences were not statistically significant. Leg ulcers, pulmonary hypertension, ischemic strokes, priapism, cholecystolithiasis and microalbuminuria were counted as haemolysis-related complications of SCD. Note the interruptions in the scale on Y axes.

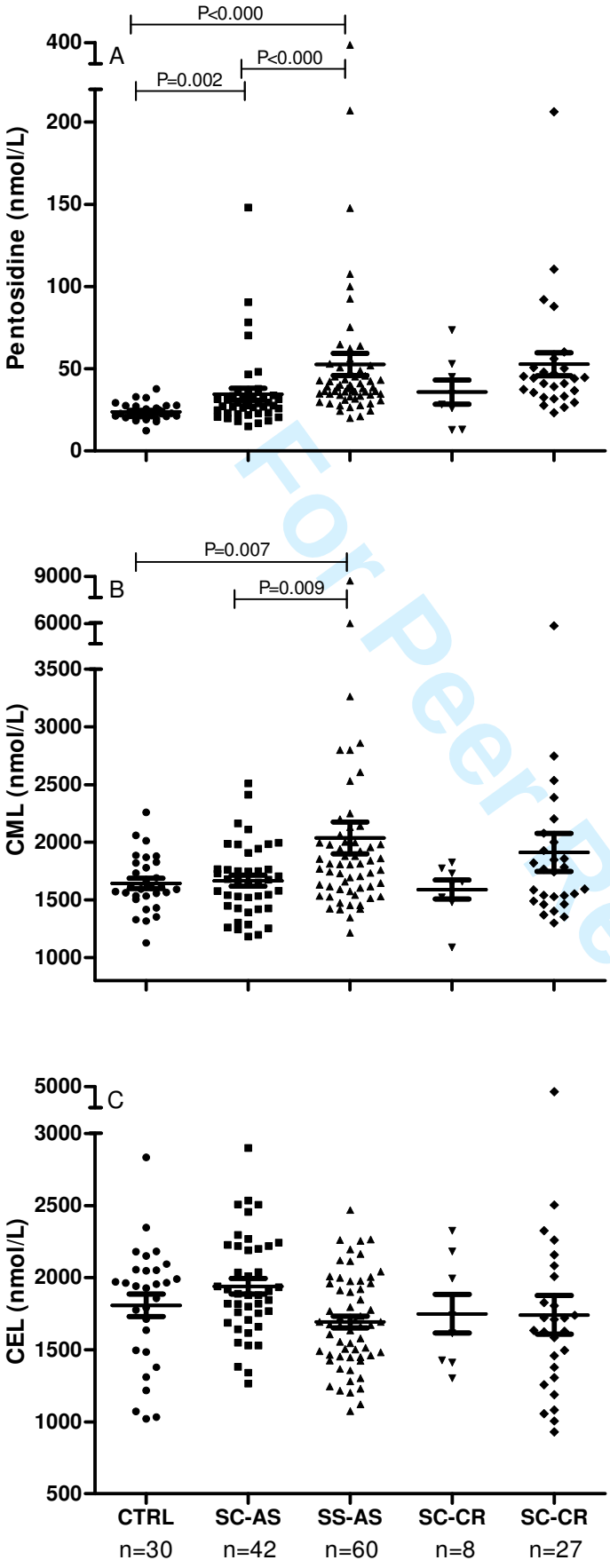
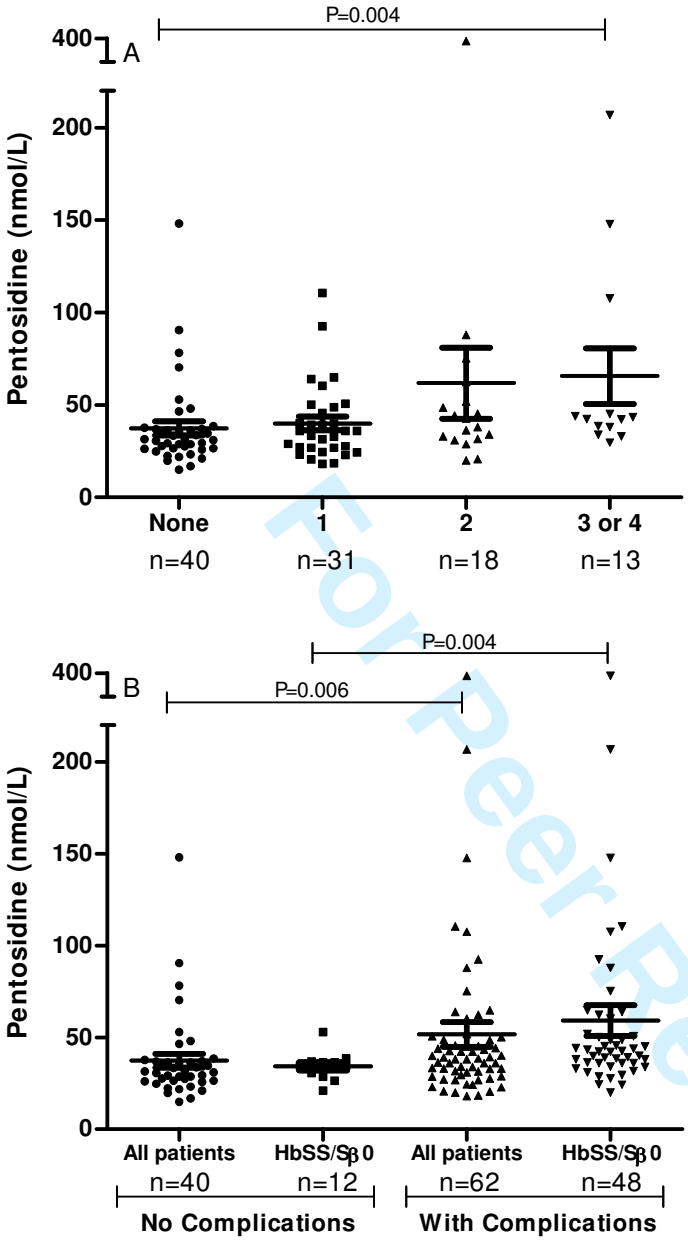


Figure 1.



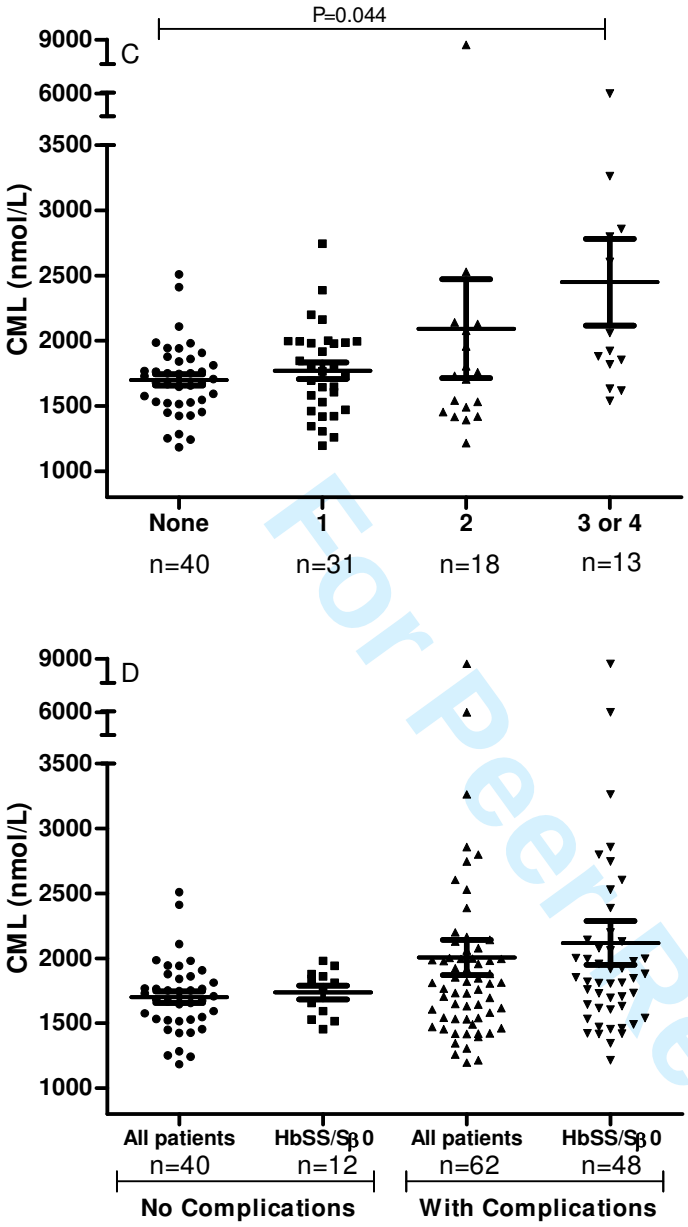


Figure 2.