Vasculo-toxic and pro-inflammatory action of unbound haemoglobin, haem and iron in transfusion-dependent patients with haemolytic anemias

Supplementary Text and Figures

Material and Methods

Haptoglobin and Haemopexin measurement

Serum haptoglobin (Hp) of β-thal samples were measured at the Blood Analysis Center of Heidelberg University. Serum haemopexin (Hx) was measured using the human Haemopexin ELISA kit Ab108859 (Abcam). The assays were performed according to manufacturer's instructions and monitor the total serum levels of Hp and Hx (bound and unbound). The total Hb/haem binding capacity of Hp and Hx was measured as the sum of Haptoglobin μmol/l and Haemopexin μmol/l.

Serum Hb/haem measurement

Total Hb/haem concentrations in patient sera were determined colorimetrically using the QuantiChrom Haem assay kit DIHM-250 from BioAssay Systems according to manufacturer's instructions. This assay accounts for total serum haemoglobin and haem measurement, bound and not bound to scavengers and proteins. 'Free' Hb and haem measurement defined as non-haptoglobin bound haemoglobin (NHBHB) and non-haemopexin bound haem (NHBH) were obtained using the formula: [Total serum Hb/haem μmol/l – (Haptoglobin μmol/l + Haemopexin μmol/l)].

Serum Iron Measurement

Serum iron parameters (serum iron, UIBC, Tf saturation, serum ferritin) of β-thal samples were measured at the Blood Analysis Center of Heidelberg University. Serum iron parameters (serum iron, UIBC, Tf saturation) of SPH and SCD samples were measured using the SFBC (serum iron) and UIBC (unsaturated iron binding capacity) kits (Biolabo) following manufacturer's instructions. Transferrin saturation was calculated using the formula SFBC/(SFBC+UIBC) x100.

Serum NTBI measurements

Non-transferrin bound iron (NTBI) measurement was conducted using the ultrafiltration method. 90µl of serum were incubated with 10 µl of 800 mM nitrilotriacetic acid (NTA) containing 20µM Fe (pH 7.0) at 23°C for 30 minutes. The mixture was then ultra-filtered at 10000 g at 4ºC for 60 minutes using 10 kDa molecular weight cut-off Amicon Ultra 0.5 ml filtration units (Millipore). Clear ultrafiltrate was recovered containing NTA-mobilized iron and iron content was determined by the bathophenanthroline disulphonic acid (BDA) method. After 30 minute-incubation with a 1:1 mixture of 60mM BDA and 120mM thioglycolic acid, absorbance at 537 nm and iron concentration was quantitated by interpolation from a standard curve . Standards were prepared using iron for atomic absorption spectrometry (#16596, Sigma-Aldrich) in 80mM NTA (0-20µM). The 800mM NTA solution used was previously treated with 20µM iron to normalize the background iron that contaminates reagents. Thus the zero standard contains the added background iron and gives a positive signal . When unsaturated Tf is present in sera, this additional background iron can be donated to vacant Tf sites resulting in a loss of the background signal and yielding a negative NTBI value. Values are expressed as µM NTBI.

Erythropoietin and Hepcidin measurement

Serum Erythropoietin (EPO) was measured using the human EPO Elisa Kit DEP00 (R&D). Serum hepcidin was measured using the Bioactive hepcidin 25 ELISA kit (DRG Diagnostics, Marburg, Germany). The hepcidin concentration was extrapolated against a standard curve by using a logistic model of the Curve Expert software. All assays were performed according to manufacturer's instructions. Hepcidin/iron and hepcidin/ferritin ratios are calculated for each patients and donors as following: (hepcidin/ serum iron or serum ferritin) x 100.

Serum Soluble Adhesion Molecule and Nitrotyrosine measurements

Soluble adhesion molecules sVCAM-1, sICAM-1, sE-selectin, sP-selectin were measured using the ELISA kit DVC00, DCIM00, DSLE00, BBE6 (R&D Systems), respectively. Serum nitrotyrosilated proteins were measured using the OxiSelect Nitrotyrosine ELISA Kit (STA-305, Cell Biolabs).

Measurements of markers of Oxidative Stress

Serum thiobarbituric acid reactive substances (TBARS) levels were measured using the QuantiChrom TBARS Assay Kit (BioAssay Systems) according to the manufacturer's instructions. Results were

expressed in μM malondialehyde (MDA). Serum advanced oxidized protein products (AOPP) were quantified using the Human AOPP ELISA Kit (MBS028634, Biozol). Results were expressed in μmol/l AOPP.

Cytokine measurements

Interleukin 6 and 1β (IL6; IL1β), tumor necrosis factor α (TNFα), monocyte chemoattractant protein 1 (MCP-1) and vascular endothelial growth factor (VEGF) were determined in human serum samples applying Multiplex bead-array based technology. Measurements were performed on a BioPlex200 System using the Bio-Plex Pro Cytokine Reagent Kit and Bio-Plex Pro Human Cytokine sets (Bio-Rad) according to manufacturer's instructions. Cytokine levels are expressed as picograms per ml serum.

Serum LDLs and Oxidized LDLs

Serum low-density lipoprotein (LDL) measurements were performed using an Olympus AU400 analyzer at the Claude Bernard Institute (Paris, France). Serum oxidized LDL (oxLDL) levels were measured using the CSB-E07931h ELISA kit (Cusabio) according to according to manufacturer's instructions.

Patient Data

The current human study is a retrospective analysis of serum samples from cohorts of transfusion-dependent patients with β-thal major (n=59) and intermedia (n=7), hereditary spherocytosis (SPH, n=14) and sickle cell disease (SCD 1 – simple transfusions, n=16[65]; SCD 2 – exchange transfusions, n=19[66]) and compared to samples from healthy subjects (n=17 and 34). The β-thal cohort has been analyzed separately from the other cohorts, which required two groups of healthy control subjects. Patients' cohorts were compared to healthy subjects without underlying haemolysis or other disease conditions. Summary of the demographic and biochemical data at the time of the study is provided for each cohort in Tables 1, 2 and 3 and detailed information of each patient is included in Supplementary Table 1, 2, 3 and 4.

Ethics approval statement: All patient data were anonymized and confidentiality was maintained during the study process according to the Helsinki Declaration. All subjects provided written informed consent for the use of their stored samples for the present research. Research was approved by the Ethical Committee of the New York Blood Center (New York, USA) and the Al Shifa Hospital (Gaza, Palestine).

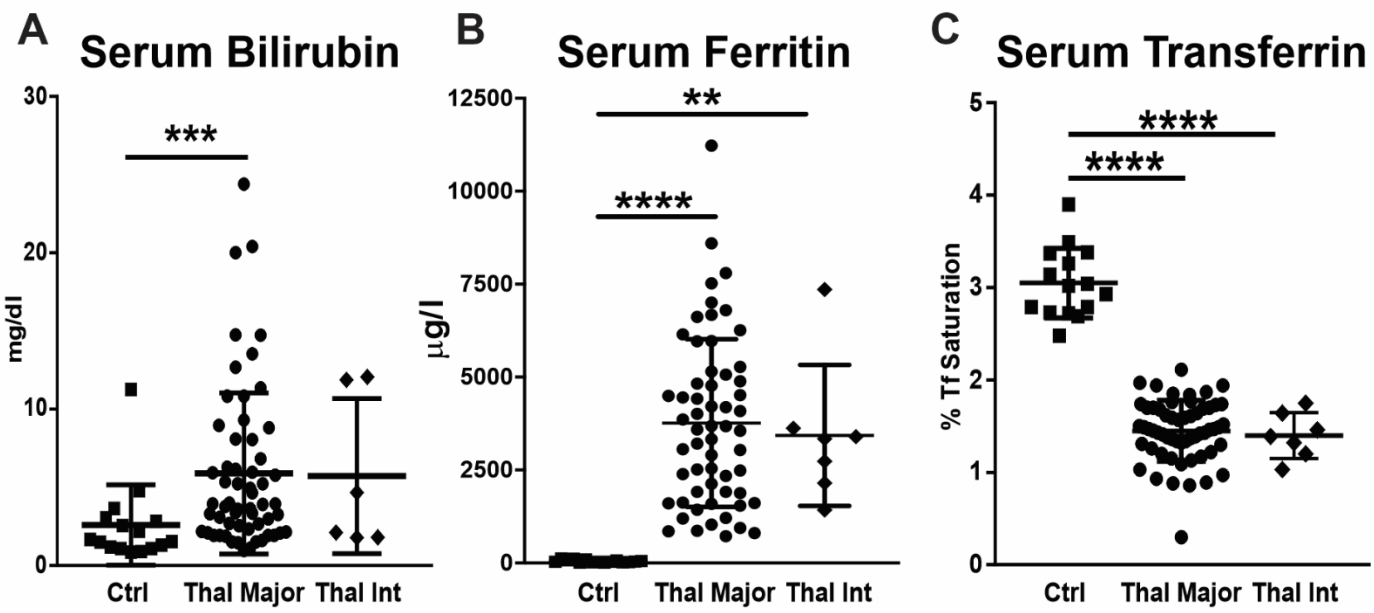
Patient consent statement: All subjects provided written informed consent for the use of their stored samples for the present research.

Statistical Analysis

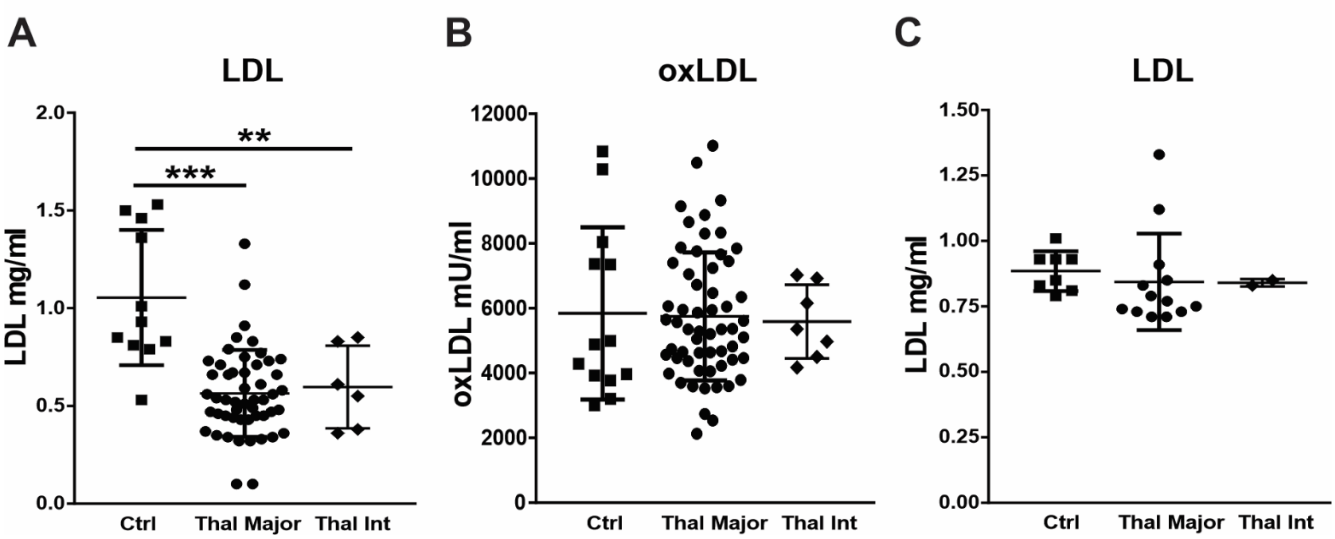
Comparisons were performed with 1-way ANOVA followed by Bonferroni post-test, using GraphPad Prism v6. Where indicated, comparisons were performed between healthy subjects (Ctrl) and a patient cohort using 2-sided Welch *t*-tests. A P value <0.05 was considered significant. In the figures results are expressed as mean ± standard deviation (SD). Correlation analyses were performed with Spearman's r for associations between different variables.

Parallel analyses were run for each cohort. Descriptive statistics (mean, sd, median, IQR, min, max) were generated for each of the parameters and the data were tested for normality using the Shapiro-Wilk test. In order to determine the relationship between biomarkers, correlation matrices with Spearman correlations were generated. Parameters were evaluated for multicollinearity by inspecting the VIF (variance inflation factors). In addition, we examined missing data patterns and percentages for each of the biomarker variables.

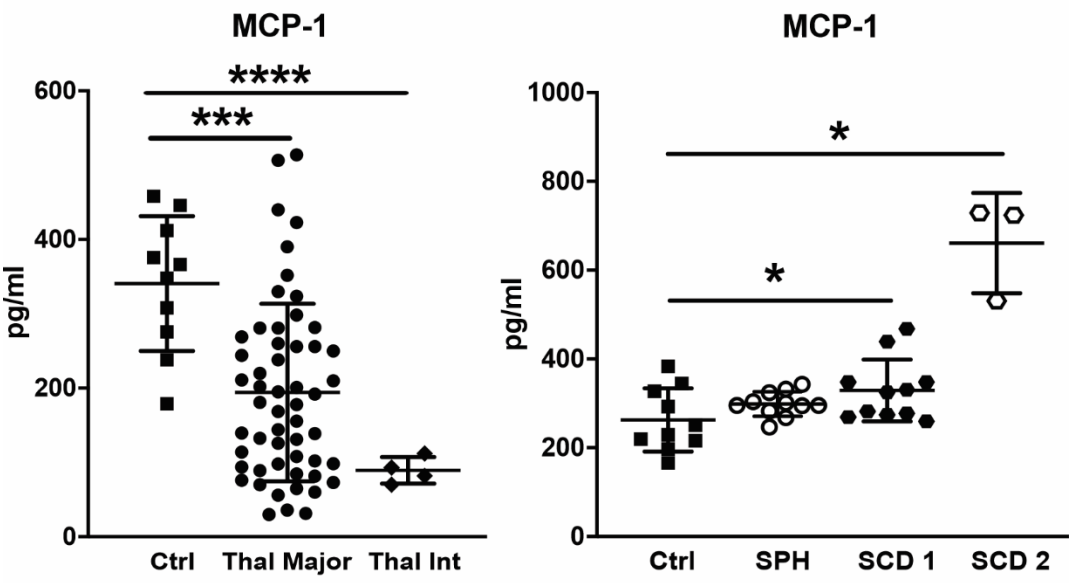
For the β-thalassemia major cohort, the biomarkers had 0% to 15% missing data but two-thirds of the biomarkers had less than 10% missing data. For the SCD/SPH cohort biomarker missing data had a range from 0% to 33% with half of the biomarkers having less than 10% missing data. For the SCD/SPH cohort the VEGF and MCP1 biomarkers were not included in the analysis due to substantial missing data (47%). We imputed missing values using multivariate imputation by chained equations (100 imputation datasets, 50 iterations). We ran multiple regression linear models to assess the relation between each of the iron parameters with each of the three domains consisting of sets of biomarkers: adhesion molecules, oxidative stress markers and cytokines. These models were run as multivariable linear regression models and pooled beta estimates with 95%CI and p-values were generated. In addition to creating estimates from the imputed datasets we also conducted a parallel Complete Case Analysis and examined statistical results across the two methods. All statistical analyses and the correlation plots (Figure 8) were conducted using R version 3.6.1 (2019-7-05) Copyright (C) 2019 The R Foundation for Statistical Computing (packages: stats, olsrr, corrplot, mice).



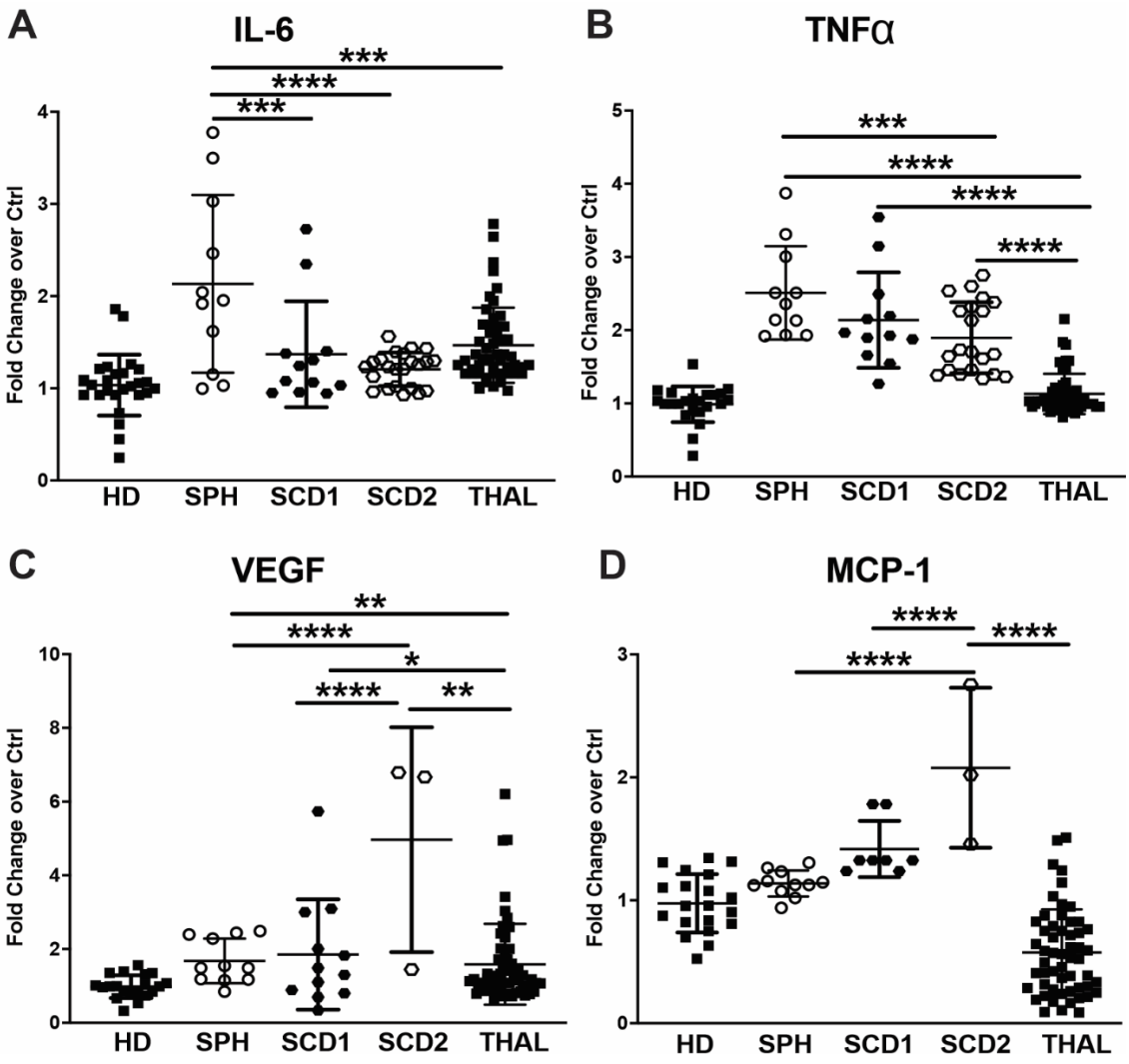
Supplementary Figure 1. Serum ferritin and transferrin analyzed in β-thalassemia patients. Measurement of (A) serum bilirubin, (B) ferritin and (C) transferrin in sera of healthy subjects (Ctrl), as well as patients with β-thal int and major. Values represent mean ± standard deviation (SD). **P<0,01; ***P<0,001; ****P<0,0001.



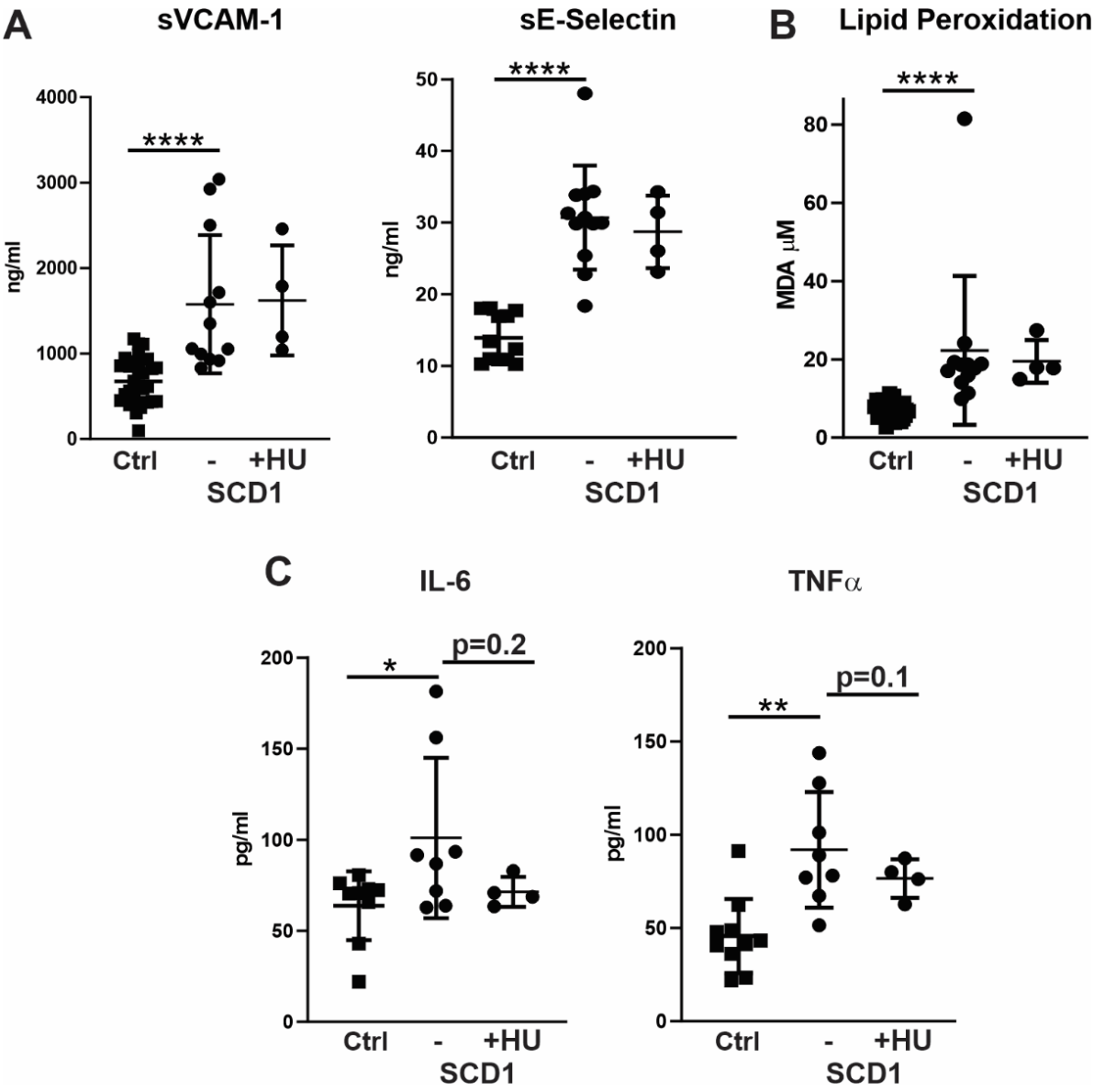
Supplementary Figure 2. Serum LDL and oxLDL in β-thalassemia patients. (A) Measurement of low-density lipoproteins (LDL) and (B) oxidized low-density lipoproteins (oxLDL) in sera of healthy subjects (Ctrl) and patients with β-thal int and major; (C) LDL levels in a subgroup of healthy subjects (Ctrl) and patients with β-thal int and major with average 0,8 mg/ml LDL levels. oxLDL levels for these patients are shown in Figure 5 of the main text. Values represent mean ± standard deviation (SD). **P<0,01; ***P<0,001.



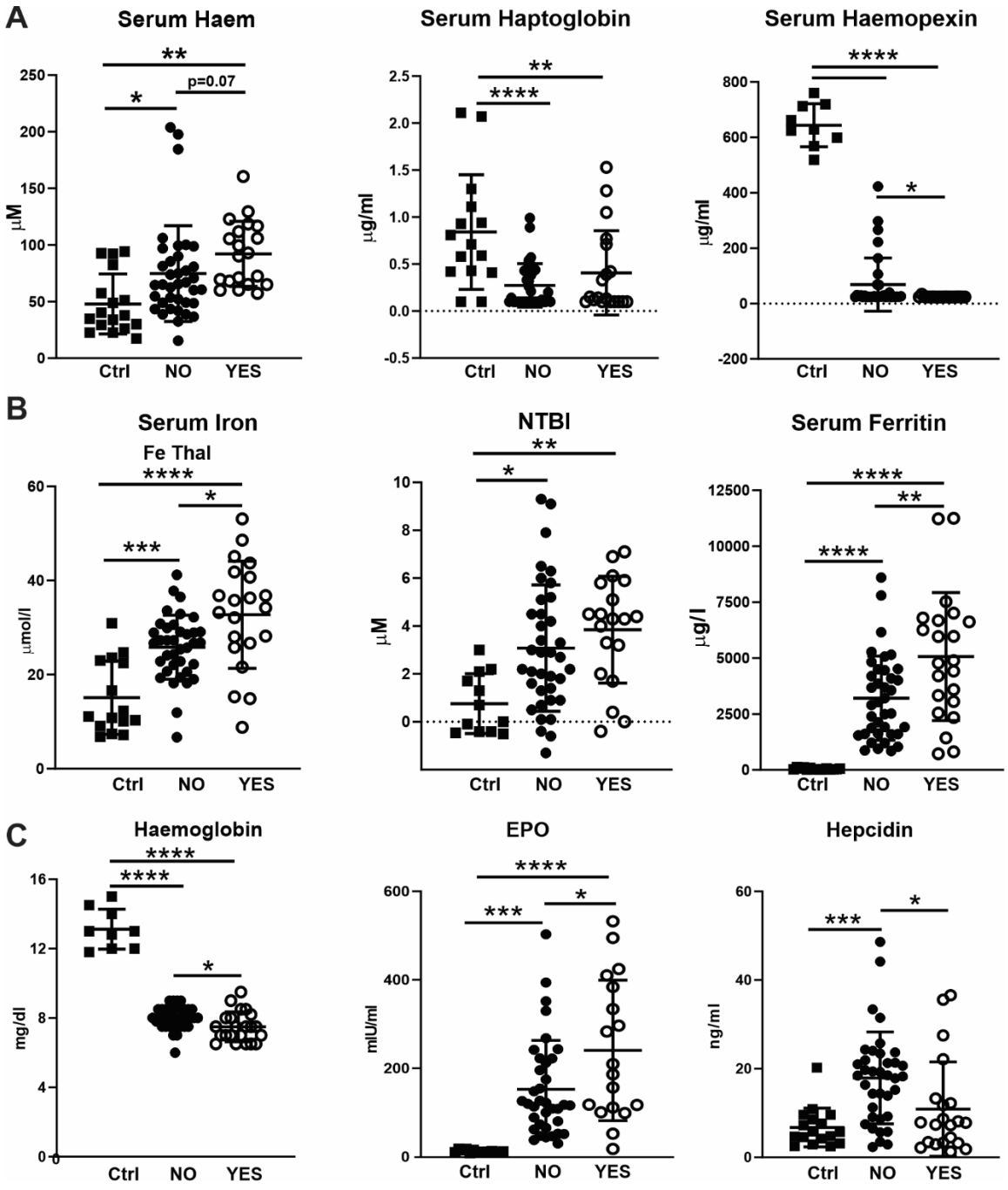
Supplementary Figure 3. MCP-1 levels in patients with haemolytic diseases. Measurement of MCP-1 in sera of healthy subjects (Ctrl), patients with β-thal int and major, as well as patients with sickle cell disease (SCD 1; SCD 2) and spherocytosis (SPH). Values represent mean ± standard deviation (SD). *P<0,05; ***P<0,001; ****P<0,0001.



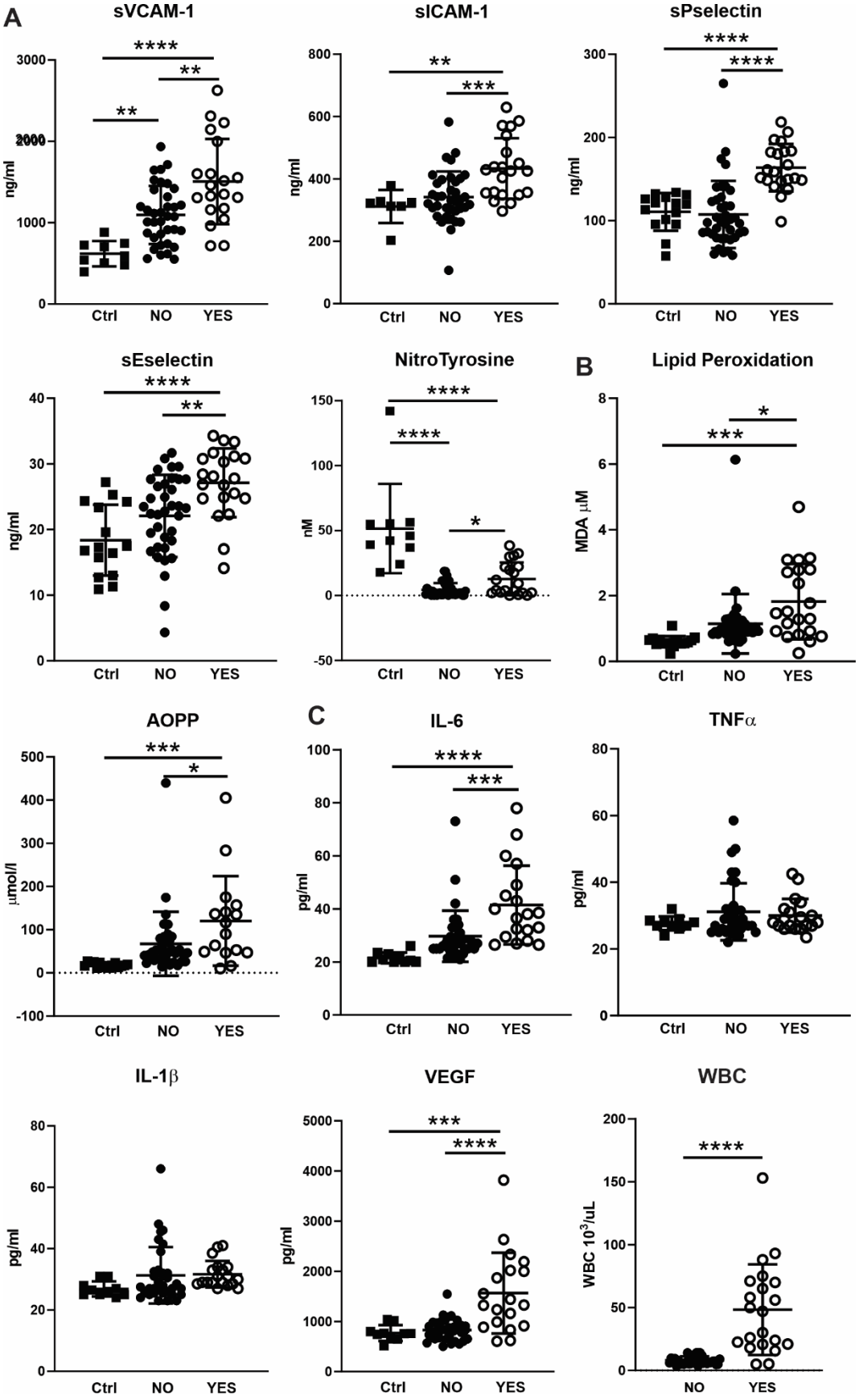
Supplementary Figure 4. Cytokines in patients with haemolytic diseases. (A) IL-6, (B) TNFα, (C) VEGF and (D) MCP-1 in sera of healthy subjects (Ctrl) and patients with spherocytosis (SPH), sickle cell disease (SCD1; SCD2) and β-thal major expressed as fold change over Ctrl values. Values represent mean ± standard deviation (SD). Significant comparisons between SPH, SCD1, SCD2 and β-thal are shown. ****P<0,0001. While IL-6, TNFα and VEGF are increased in all disease groups compared to healthy subjects, MCP-1 levels are increased in SPH and SCD patients but decreased in β-thal patients compared to healthy subjects. SPH patients show higher IL-6 and TNFα levels compared to SCD and β-thal patients. SCD patients receiving exchange transfusions (SCD2) show higher VEGF and MCP-1 levels compared to SPH and β-thal patients. β-thal patients show lower TNFα, VEGF and MCP-1 levels compared to SPH and SCD patients. Overall β-thal patients show more moderate inflammation compared to SCD and SPH patients.



Supplementary Figure 5. Effect of hydroyurea treatment in sickle cell disease. Measurement of (A) sVCAM-1 and sE-selectin, (B) lipid peroxidation (MDA), (C) IL-6 and TNFα in sera of healthy subjects (Ctrl) and patients with sickle cell disease receiving simple transfusion (SCD1) treated (+ HU) or not (- HU) with hydroxyurea. Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ****P<0,0001.



Supplementary Figure 6. Effect of splenectomy on haem and iron parameters and anemia in transfusion-dependent β-thalassemia major patients. Measurement of (A) cell-free Hb/haem, haptoglobin and haemopexin, (B) serum iron, NTBI and ferritin, (C) Hb, EPO and hepcidin in sera and blood samples of healthy subjects (Ctrl) and patients with β-thal major non-splenectomized (NO; n=38) and splenectomized (YES; n=21). Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ***P<0,001; ****P<0,0001.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ng/ml
Ctrl
NO
YES
sICAM-1
ng/ml
sPselectin
ng/ml
sEselectin
ng/ml
NitroTyrosine
nM
B
Lipid Peroxidation
MDA µM
AOPP
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C
IL-6
pg/ml
TNFα
pg/ml
IL-1β
pg/ml
VEGF
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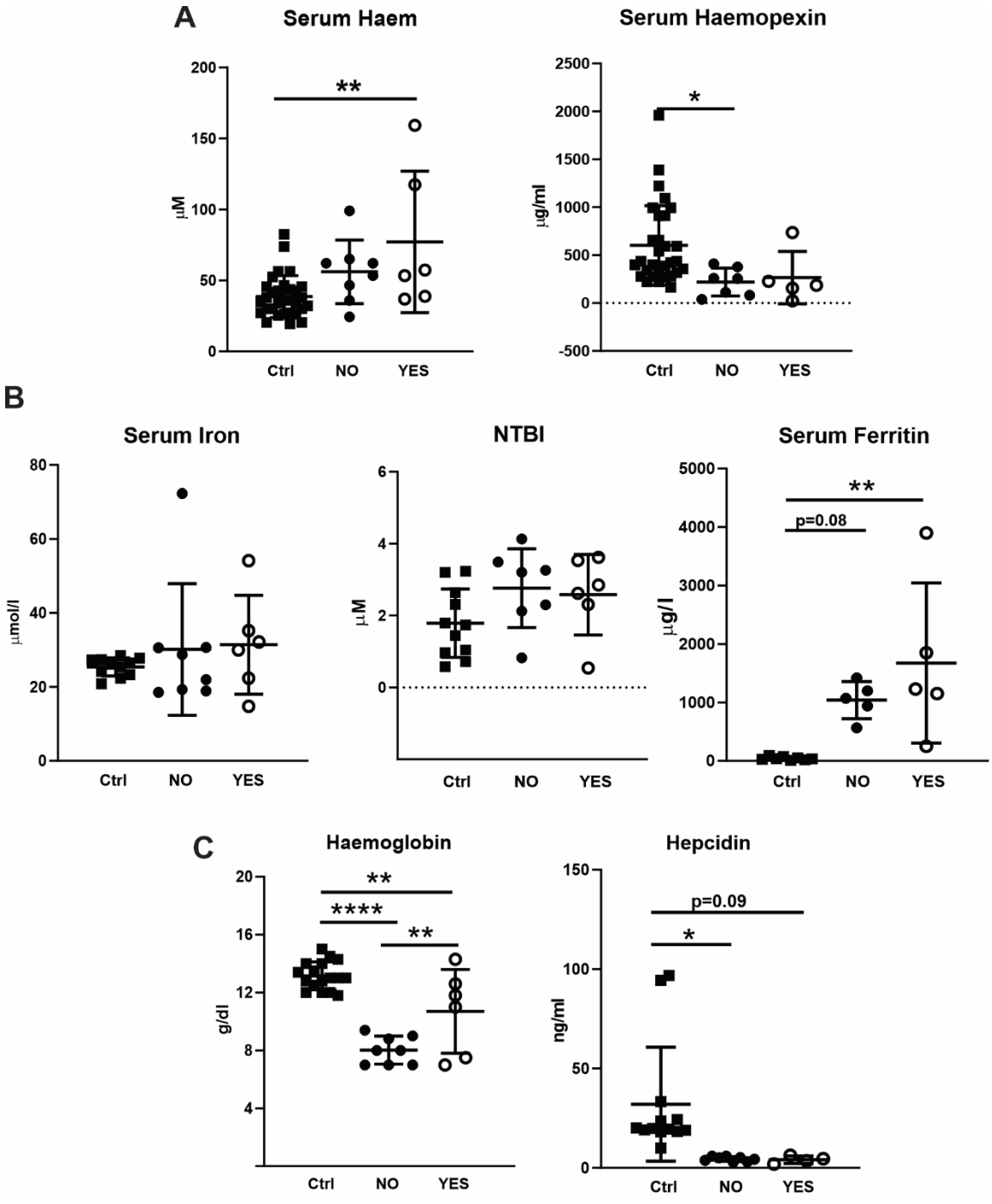
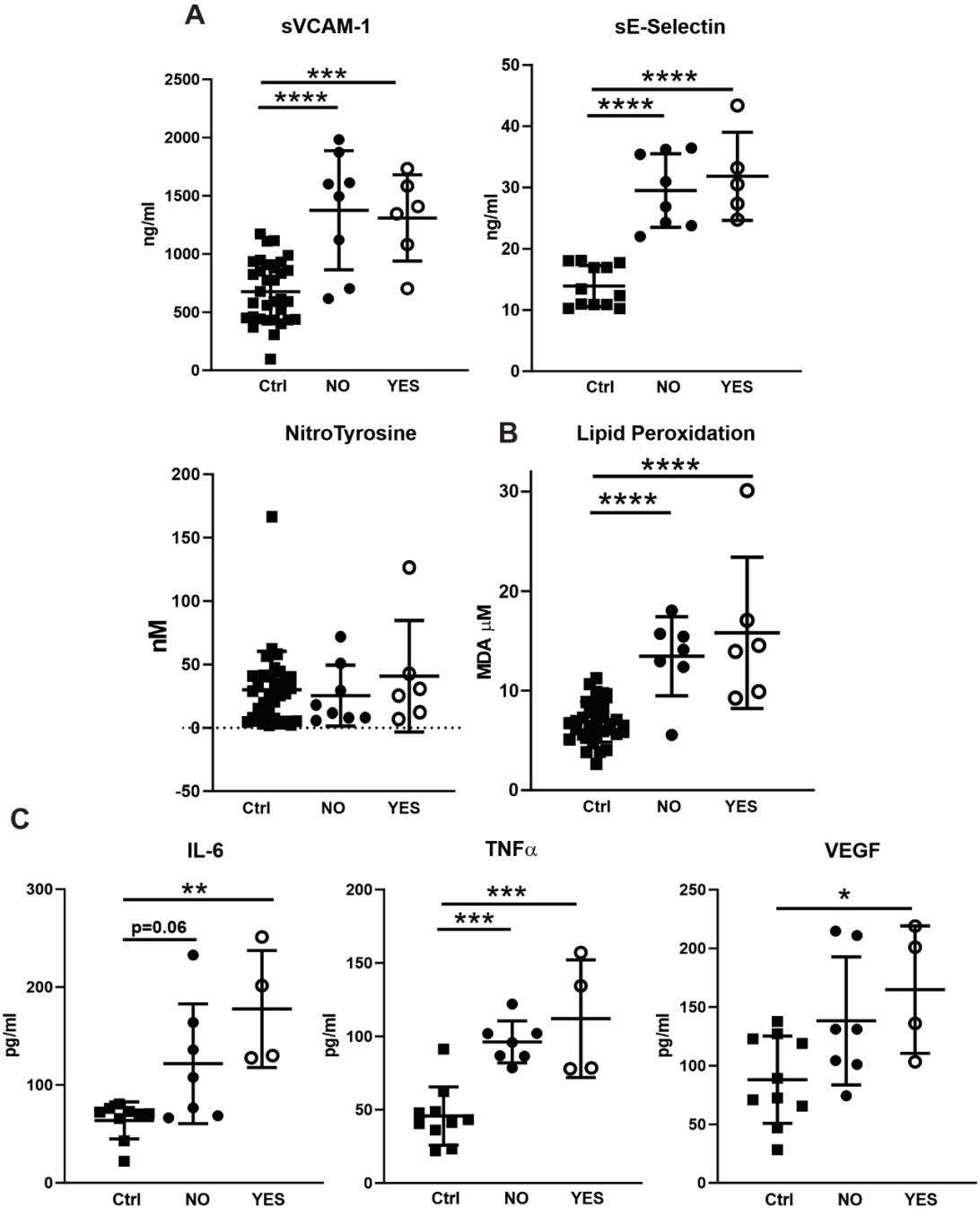
WBC
WBC 10^3/uL

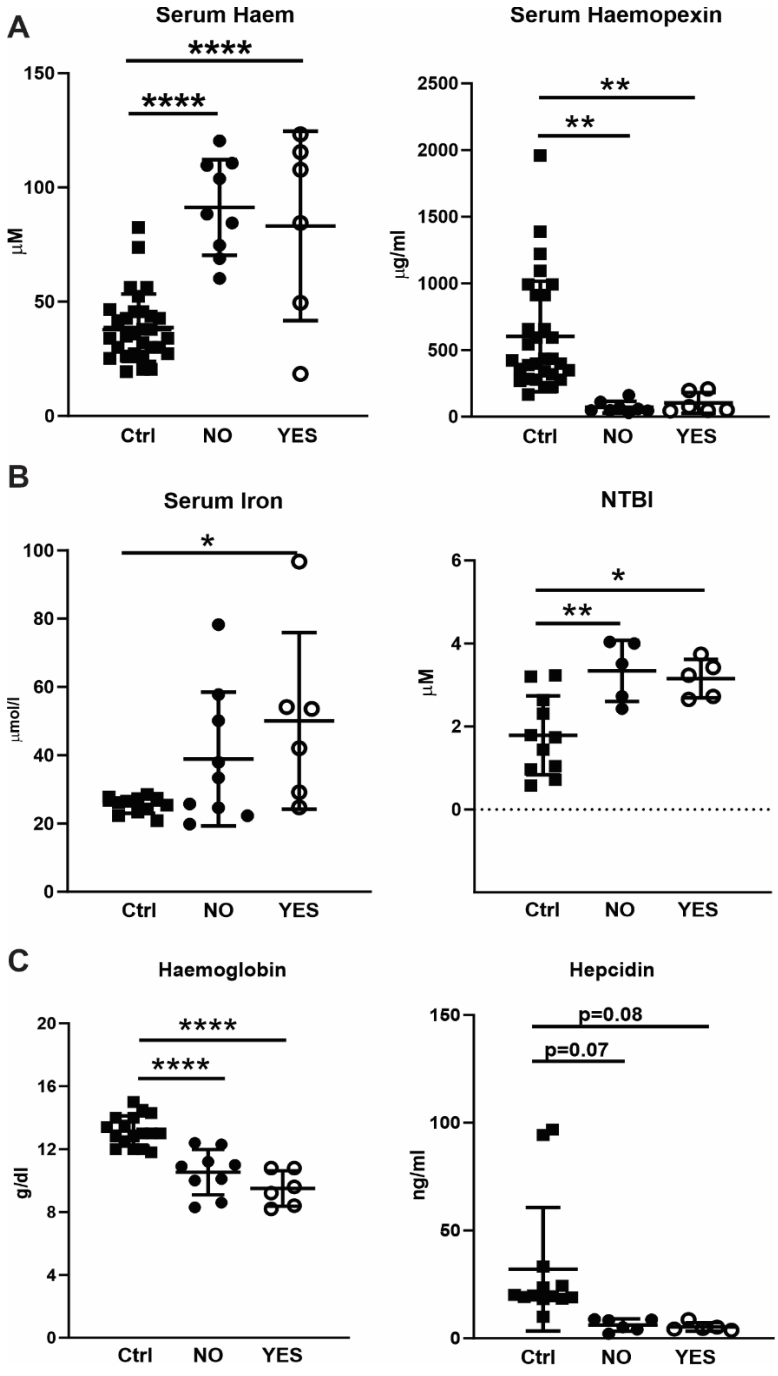
Supplementary Figure 7. Effect of splenectomy on markers of vascular dysfunction, oxidative stress and inflammation in transfusion-dependent β-thalassemia major patients. Measurement of (A) sVCAM-1, sICAM-1, sP-selectin, sE-selectin and nitrotyrosine, (B) lipid peroxidation (MDA) and advanced oxidized protein products (AOPP), (C) IL-6, TNFα, IL1β, VEGF and WBC in sera and blood samples of healthy subjects (Ctrl) and patients with β-thal major non-splenectomized (NO; n=38) and splenectomized (YES; n=21). Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ***P<0,001; ****P<0,0001.



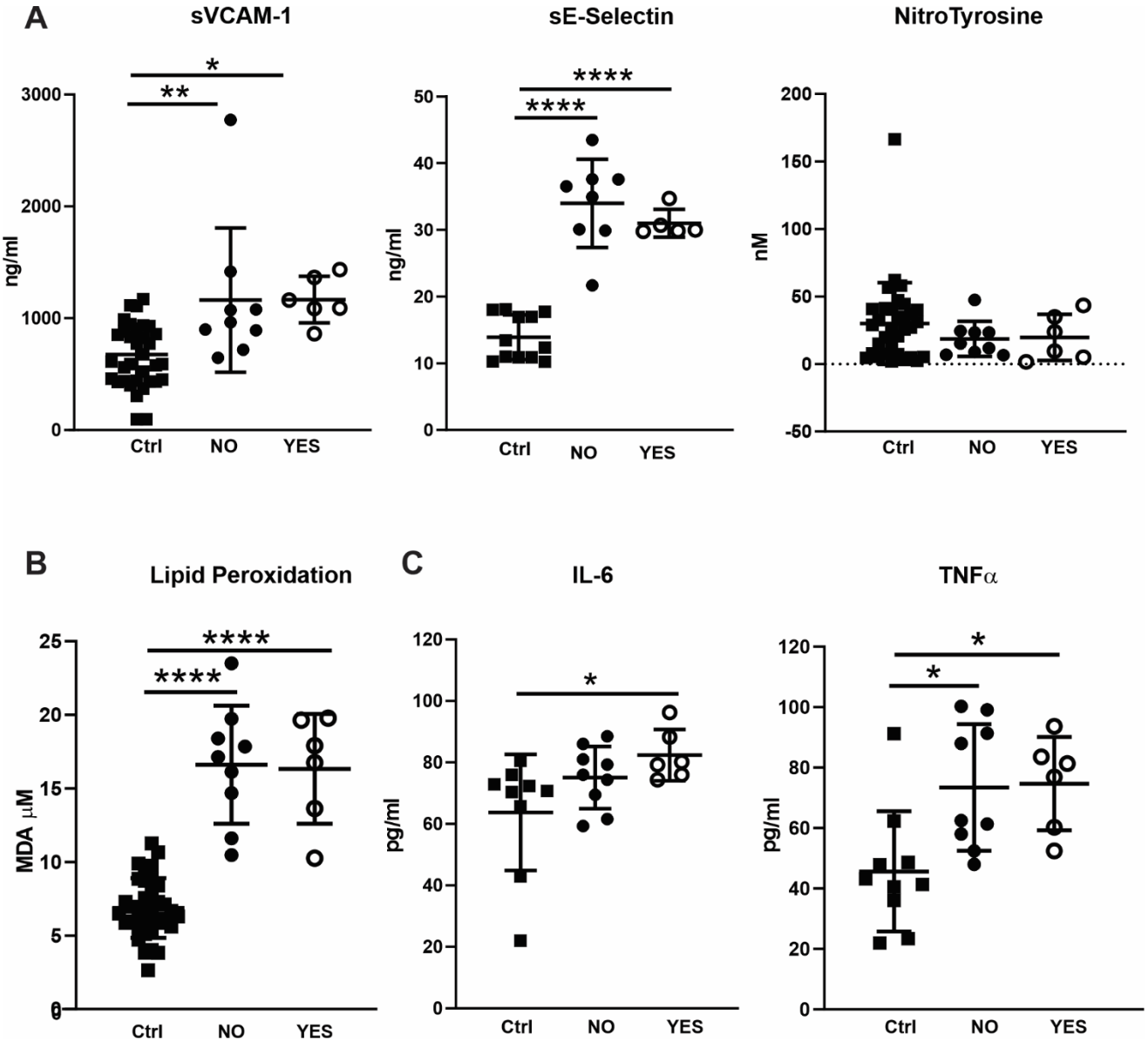
Supplementary Figure 8. Effect of splenectomy on haem and iron parameters and anemia in transfusion-dependent spherocytosis patients. Measurement of (A) cell-free Hb/haem and haemopexin, (B) serum iron, NTBI and ferritin, (C) Hb and hepcidin in sera and blood samples of healthy subjects (Ctrl) and patients with spherocytosis (SPH) non-splenectomized (NO; n=8) and splenectomized (YES; n=6). Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ****P<0,0001.



Supplementary Figure 9. Effect of splenectomy on markers of vascular dysfunction, oxidative stress and inflammation anemia in transfusion-dependent spherocytosis patients. Measurement of (A) sVCAM-1, sE-selectin and nitrotyrosine, (B) lipid peroxidation (MDA) and (C) IL-6, TNFα and VEGF in sera of healthy subjects (Ctrl) and patients with spherocytosis (SPH) non-splenectomized (NO; n=8) and splenectomized (YES; n=6). Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ***P<0,001; ****P<0,0001.



Supplementary Figure 10. Effect of splenectomy on haem and iron parameters and anemia in sickle cell disease patients receiving exchange transfusions. Measurement of (A) cell-free Hb/haem and haemopexin, (B) serum iron and NTBI, (C) Hb and hepcidin in sera and blood samples of healthy subjects (Ctrl) and patients with sickle cell disease receiving exchange transfusion (SCD2) non-splenectomized (NO; n=9) and splenectomized (YES; n=6). Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ****P<0,0001.



Supplementary Figure 11. Effect of splenectomy on markers of vascular dysfunction, oxidative stress and inflammation in sickle cell disease patients receiving exchange transfusions. Measurement of (A) sVCAM-1, sE-selectin and nitrotyrosine, (B) lipid peroxidation (MDA), (C) IL-6 and TNFα in sera of healthy subjects (Ctrl) and patients with sickle cell disease receiving exchange transfusion (SCD2) non-splenectomized (NO; n=9) and splenectomized (YES; n=6). Values represent mean ± standard deviation (SD). *P<0,05; **P<0,01; ****P<0,0001.