SUPPLEMENTARY APPENDIX

Germline *GATA2* variant disrupting endothelial eNOS function and angiogenesis can be restored by c-Jun/AP-1 upregulation

Giulio Purgatorio,[1] Elisa Piselli,[1] Giuseppe Guglielmini,[1] Emanuela Falcinelli,[1] Loredana Bury,[1] Valeria Di Battista,[2] Fabrizia Pellanera,[2] Francesca Milano,[2] Caterina Matteucci,[2] Cristina Mecucci[2#] and Paolo Gresele[1#]

[1]Section of Internal and Cardiovascular Medicine, Department of Medicine and Surgery, University of Perugia and [2]Hematology and Bone Marrow Transplantation Unit, Department of Medicine and Surgery, University of Perugia, Perugia, Italy.

[#]PG and CM contributed equally as co-senior authors.

 doi:10.3324/haematol.2021.278450

Received: February 1, 2021.

Accepted: June 16, 2021.

Pre-published: July 8, 2021.

Correspondence: *PAOLO GRESELE* - paolo.gresele@unipg.it

SUPPLEMENTARY METHODS

Denaturing high-performance liquid chromatography and sanger sequencing

DNA was extracted from unsorted PB and/or BM cells of patients using the salting out method, quantified with a Qubit 1.0 fluorimeter using the Quant-i-T dsDNA HS Assay Kit (Invitrogen) and samples quality was assessed using Tapestation visualization (Agilent 2100 Bioanalyzer). Mutational analysis was performed by Denaturing High Performance Liquid Chromatography (DHPLC, Wave® MD system; ADS BIOTEC Inc. Omaha, NE) and/or Sanger sequencing (ABI 3500 Genetic Analyzer, Applied Biosystem) in all family members. The full coding sequence of the *GATA2* gene was screened; the appropriate forward and reverse primers are available upon request.

Blood outgrowth endothelial cells isolation

Blood outgrowth endothelial cells (BOEC) were isolated from peripheral blood of GATA2 deficiency patients and from age- and sex-matched healthy controls and cultured in EGM-2 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), as previously described[22]. Blood samples were diluted 1:1 with PBS and peripheral blood mononuclear cells (PBMCs) isolated by density-gradient centrifugation over Lymphoprep (StemCell Technologies), as described[23]. PBMCs were washed twice with PBS and seeded in six-well culture plates (Corning, New York, USA) coated with 50 µg/mL rat tail collagen type 1 (BD Biosciences, Franklin Lakes, New Jersey) and medium was changed every second day from the moment in which colonies started to appear (usually from day 6 post-seeding); colonies were used until passage 8. After differentiation BOECs were characterized by flow-cytometry (CytoFlex, Beckman Coulter), incubating 10^5 cells with fluorochrome-conjugated antibodies directed against CD45, CD14, CD31, CD146 and CD309 (Beckman Coulter). VWF expression was evaluated by immunofluorescence, after fixing BOEC with 4% paraformaldehyde for 20 minutes on coverslips and then permeabilized with 0.1% Triton X-100 for 10 minutes. After blocking with PBS containing 1% BSA cells were incubated with a rabbit anti-VWF polyclonal antibody (Dako, Denmark A/S, 1:250) as primary antibody and an anti-rabbit Alexa 488-conjugated as secondary antibody (Abcam,1:1000) for 1h. F-actin was stained with rhodamine phalloidin (ThermoFisher, Massachussets, USA). Nuclei were stained using the prolong Diamond Antifade Mountant with DAPI.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChiP) assay was performed using the SimpleChiP® enzymatic Chromatin IP Kit #9002 (Cell Signaling, Danver, Massachussets, USA), according to manufacturer's instructions[24]. Cells were fixed with formaldehyde to cross-link histone and non-histone proteins to DNA. Chromatin was then digested with Micrococcal Nuclease into 150-900 bp DNA/protein fragments. Histone and non-histone proteins were precipitated using histone H3(D2B12) XP®

rabbit mAb (ChIP Formulated) as positive control, normal rabbit IgG as negative control, anti-GATA2 antibody-ChiP grade (Abcam) and c-Jun Rabbit mAb (Cell Signaling). Cross-links were then reversed by adding 6 µl 5M NaCl and 2 µl proteinase K and purified DNA was used as template for quantitative PCR, using primers to *eNOS* promoter regions where AP-1 (For: CTCAGCCCTAGTCTCTCTGC; Rev:GGTTCTTGGGGATAGAGGCC) and GATA2 (For:GGTGCCACATCA CAGAAGGA; Rev: CACAATGGGACAGGAACAAGC) transcription factors bind DNA, as previously described[15].

Protein expression: western blotting

BOECs were lysed in protein extraction buffer (Tris 40mM, NaCl 0.3M, EDTA 1mM, Na_3VO_4, NaF, pH 7.4, additioned with NP-40 and protease inhibitors) for 30 minutes in ice, centrifuged for 10 minutes at 10,000xg and protein concentration in the supernatant was assessed by the Bradford dye-binding method, as described[26]. Protein lysates were separated by 7.5% SDS-PAGE electrophoresis and then electrotransfered onto a nitrocellulose membrane (Bio-Rad, California, USA). GATA2 expression was assessed using a rabbit monoclonal (Abcam,1:2000) as primary antibody and an anti-rabbit HRP-conjugated as secondary antibody (1:5000,Bethyl). e-NOS expression was assessed with a rabbit anti NOSIII polyclonal as primary (1:1000, Millipore) and an anti-rabbit HRP-conjugated as secondary antibody (1:5000, Bethyl, Montgomery, USA). C-Jun/AP-1 expression was assessed using a rabbit monoclonal (Cell Signaling, 1:1000) as primary antibody and an anti-rabbit HRP-conjugated as secondary antibody (1:5000,Bethyl). RUNX1 expression was evaluated using a rabbit monoclonal (Abcam, 1:1000). Immunoreactivity was detected by chemiluminescence using ECL western blotting substrate (Bio-Rad). HPRT was used for normalization. Densitometric analysis and quantification were performed using the ImageJ software (NIH, USA), as described[27].

GATA2 subcellular distribution

BOECs were seeded on immunofluorescence coverslips (50,000 cells/coverslip) and cultured for 24h in EGM-2 supplemented with 10% FBS, then culture medium was removed and cells were fixed with 4% paraformaldehyde for 20 minutes. Cells were then permeabilized with 0.1% Triton X-100 and blocked with PBS supplemented with BSA 1% for 1 hour. GATA2 protein was stained with a rabbit monoclonal antibody (Abcam, 1:200) as primary antibody and an anti-rabbit Alexa 488-conjugated as secondary antibody (Abcam, 1:1000). F-actin was stained with rhodamine phalloidin (ThermoFisher, Massachussets, USA). Nuclei were stained using the prolong Diamond Antifade Mountant with DAPI. Images were acquired with a laser scanning confocal microscope LSM 800 with Airyscan (Zeiss, Oberkochen, Germany) using a 63x oil immersion and 1.4 NA objective[28].

NO production

NO generation in BOECs and platelets was studied by flowcytometry using a specific fluorescent probe (4-amino-5-methylamino-2',7'-Difluorofluorescein diacetate, DAF-FM diacetate, Invitrogen). BOECs were resuspended in EBM2 without FBS and loaded with 30µM of DAF-FM diacetate for 45 min at room temperature (RT), then cell suspensions were centrifuged (160xg, 10 min, RT) and cell pellets resuspended in 500µl of complete EBM2 medium. BOECs were stimulated with acetylcholine 10 µM or acetylcholine 10 µM plus N^5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO) 100µM, a NOS inhibitor[29]. Cell fluorescence was measured for 3 minutes in a flow cytometer (CytoFLEX, Beckman Coulter,CA,USA). Platelet-rich plasma (PRP) was obtained from citrated blood by centrifugation at 160xg for 10 min at room temperature. 500 µl of PRP were loaded with 30 µM DAF-FM diacetate for 45 min at RT. Then suspension was centrifuged for 10 sec at 10,000xg at RT and resuspended with PBS plus Ca^{2+} 2mM and Mg^{2+} 1mM. Samples were stimulated with type I collagen (Mascia Brunelli, Milan, Italy) at increasing concentrations (1 to 10 µg/mL) and platelet fluorescence was analyzed as previously described[30].

eNOS activity assay

eNOS activity was measured by assessing the enzymatic conversion of (H^3)L-arginine to (H^3)L-citrulline. Briefly, BOEC resuspended in serum-free EBM2 at the concentration of $5x10^5$ cells/mL were incubated with 37 MBq/L of (H^3)L-arginine for 30 min at 37°C, then cell suspensions were diluted with HEPES Tyrode's to a final concentration of $2x10^5$ cells/mLprior to triggering the reaction by adding 10 µM acetylcholine and2mmol/L$CaCl_2$. After 5 min samples were centrifuged, the pellet was extracted with homogenization buffer, and the supernatant was added to an ion exchange resin which binds L-arginine but not L-citrulline. The eluate was recovered, added to scintillation tubes and counted in a beta-counter. A standard curve with increasing (H^3)L-arginine concentrations was built for each assay[31].

NOx measurement

Nitrites and nitrates (NOx) were measured in citrated plasma and in BOECs supernatant by the Griess reagent using a commercial assay (R&D system, MN, USA), as described[21]. BOECs suspended in serum-free medium were seeded in 24-well plates at a concentration of $150x10^3$ cells/well, challenged with acetylcholine 10µM for 30 minutes in a humidified atmosphere with 5% CO_2 at 37°C then the supernatant was collected, centrifuged at 10,000xg for 3 min and frozen at -80°C for later assay. Plasma was obtained by centrifugation of citrated blood at 1,000 x g for 10 min. For the measurement of NO_2/NO_3 (NOx), the stable end-products of NO metabolism, samples were first deproteinized using $ZnSO_4$. Nitrate was then reduced to nitrite by nitrate reductase in the presence of NADPH and the reaction was followed by colorimetric detection of nitrite as product of the Griess reaction. The assay was performed in a standard 96-well polystyrene microfilter plate[21]. Detection limit was 0.25 µmol/L.

e-NOS, GATA2 and c-Jun/AP-1 mRNA expression

Total RNA was isolated from BOECs using Trizol-LS (Invitrogen^{TM}). 500ng of total RNA was used as template for single-strand cDNA synthesis using the iScript kit (Biorad, Hercules, CA, USA). Specific primers were designed to amplify human GATA2 mRNA (forward: 5'-AGACGACAACCACCACCTTA-3'; reverse: 5'-TCCTGCATGCACTTTGACAG-3'), e-NOS mRNA (forward: 5'-GACGCTACGAGGAGTGGAAG-3' reverse: 5'-CCTGTATGCCAGCACAGCTA-3') and c-Jun/AP-1 mRNA (forward: 5'-TTTCAGGAGGCTGGAGGAAG-3'; reverse:5'-CTGCCACCAATTCCTGCTTT-3'). Gene expression was measured by RT-PCR using the Brilliant SYBR Green QPCR Master Mix Kit (Thermo Fisher Scientific) with the Mx3000P qPCR System (Stratagene). The reaction was carried out using 10 μM of each primer. Results, normalized to GAPDH and reported as relative expression, were calculated by the MxPro software (Stratagene). The 2-ΔΔCt method was applied for comparative quantitation[23].

siRNA GATA2 silencing

Small interfering RNA (siRNA) targeting GATA2 (siGATA2), or a scrambled siRNA as negative control, were purchased from IDT (Integrated DNA Technologies, California, USA). To silence GATA2 expression BOEC from healthy controls were transfected with siGATA2 (25nM) using the jetPRIME DNA & siRNA transfection reagent (Polyplus-transfection S.A., New York, USA) for 24, 36 and 48 hours. We used three different DsiRNAs targeting three different sequences of GATA2 mRNA (hs.Ri.GATA2.13.1: 5'-GUGUCACUGACGGAGAGCAUGAAGA-3'; hs.Ri.GATA2.13.2: 5'CUUG

GAGACUUGGUGGUCUGAGCTG-3'; hs.Ri.GATA213.3: 5'ACUAGUGUCCGCAUAAGAAAAAGAA-3'). After transfection cells were harvested and centrifuged at 500g x 5 min and pellets were lysed for RNA and protein extraction.

Coimmunoprecipitation

BOEC from healthy controls and GATA2-deficient patients were lysed in protein extraction buffer (Tris 40mM, NaCl 0.3M, EDTA 1mM, Na_3VO_4, NaF, pH 7.4) additioned with NP-40 and protease inhibitors for 30 minutes in ice, centrifuged for 10 minutes at 10,000xg and protein concentration in the supernatant was assessed by the Bradford dye-binding method, as described[26]. 250 μg proteins were used for coimmunoprecipitation. 10 μg of GATA2 rabbit monoclonal antibody (Abcam) were added to each sample and samples were incubated for 2h under gentle rotation at 4°C. Then 50 μl of protein G Sepharose beads (Sigma) were added and incubated overnight under the same conditions. Immune complexes where washed, boiled for 5 min in sample buffer, and analyzed by western blot using a rabbit Runx1 polyclonal antibody (Abcam).

***In vitro* Matrigel angiogenesisassay**

96-well plates were coated with Matrigel (Corning) polymerized for at least 30 min before use. BOEC were seeded (3×10^4 cells/well) in triplicate onto Matrigel. After 24 hours at 37°C, cells were imaged at 10x magnification with an inverted microscope. Tubule number was quantified using the software imageJ (AngioTool64). Angiogenesis was estimated by measuring total tube length

and by counting tubule number and branching points[34]. In selected experiments, the NO donor SNAP, at the concentration of 100µM, or its vehicle (DMSO) were added to the wells 1h after cell seeding. DMSO final concentration never exceeded 0.5%.

Cell viability and proliferation assays

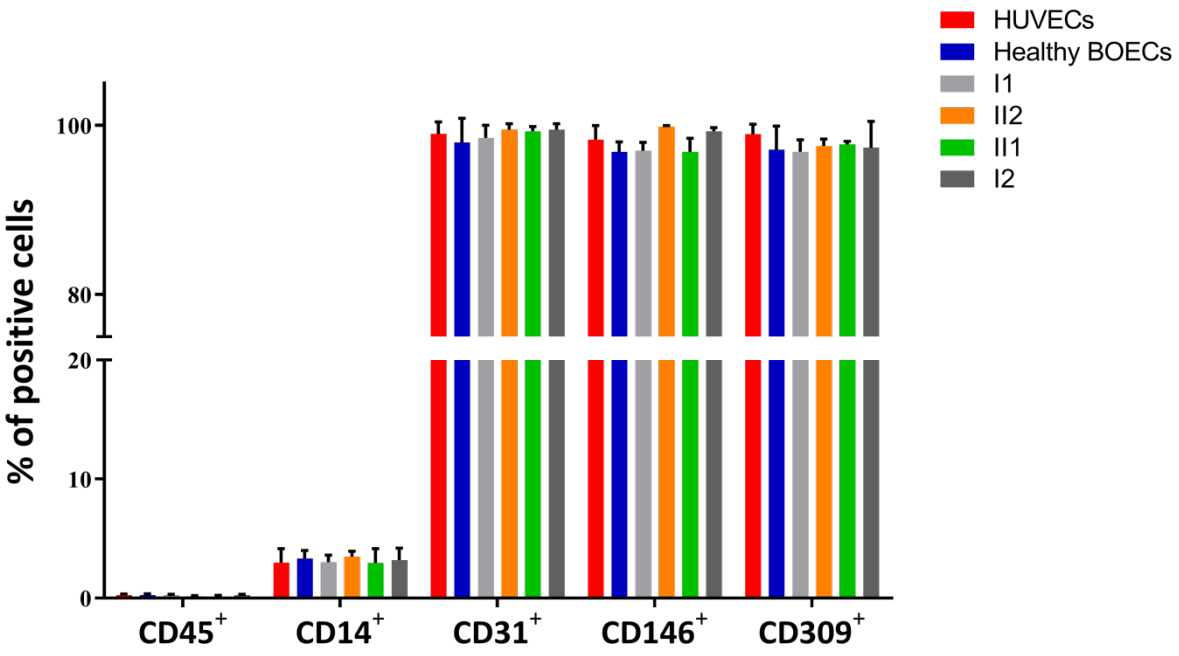
Cell viability was assessed by FDA/PI staining (Thermo Fisher) and with a FITC Annexin V apoptosis detection kit (BD Pharmigen), according to manufacturers' instructions. BOEC were treated with 5 µg/mL FDA (Fluorescein diacetate) plus 2 µg/ml PI (Propidium iodide) and incubated for 5 minutes in the dark. Cells were centrifuged at 400xg for 5 minutes, resuspended in PBS and analyzed by flow cytometry. Cell proliferation was determined in vitro using a BrdU proliferation ELISA kit (Roche) according to the manufacturer's instructions.

Statistical analysis

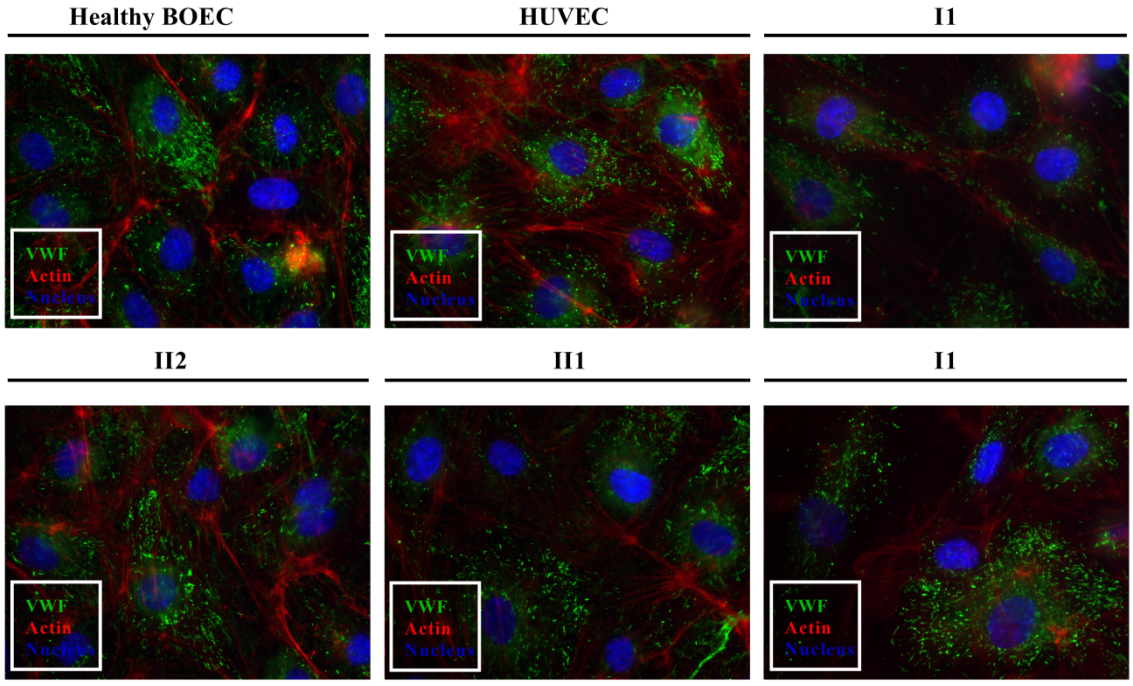
All results are expressed as means±standard error of the mean (SEM). Multiple comparisons were assessed by the 1-way ANOVA followed by the Dunnett's multiple comparison test between all groups. Comparisons between 2 groups were made using the paired Student's *t* test. All calculations were performed using the GraphPad Prism 6.0 software. A p value <0.05 was considered as statistically significant.

SUPPLEMENTARY FIGURES

A



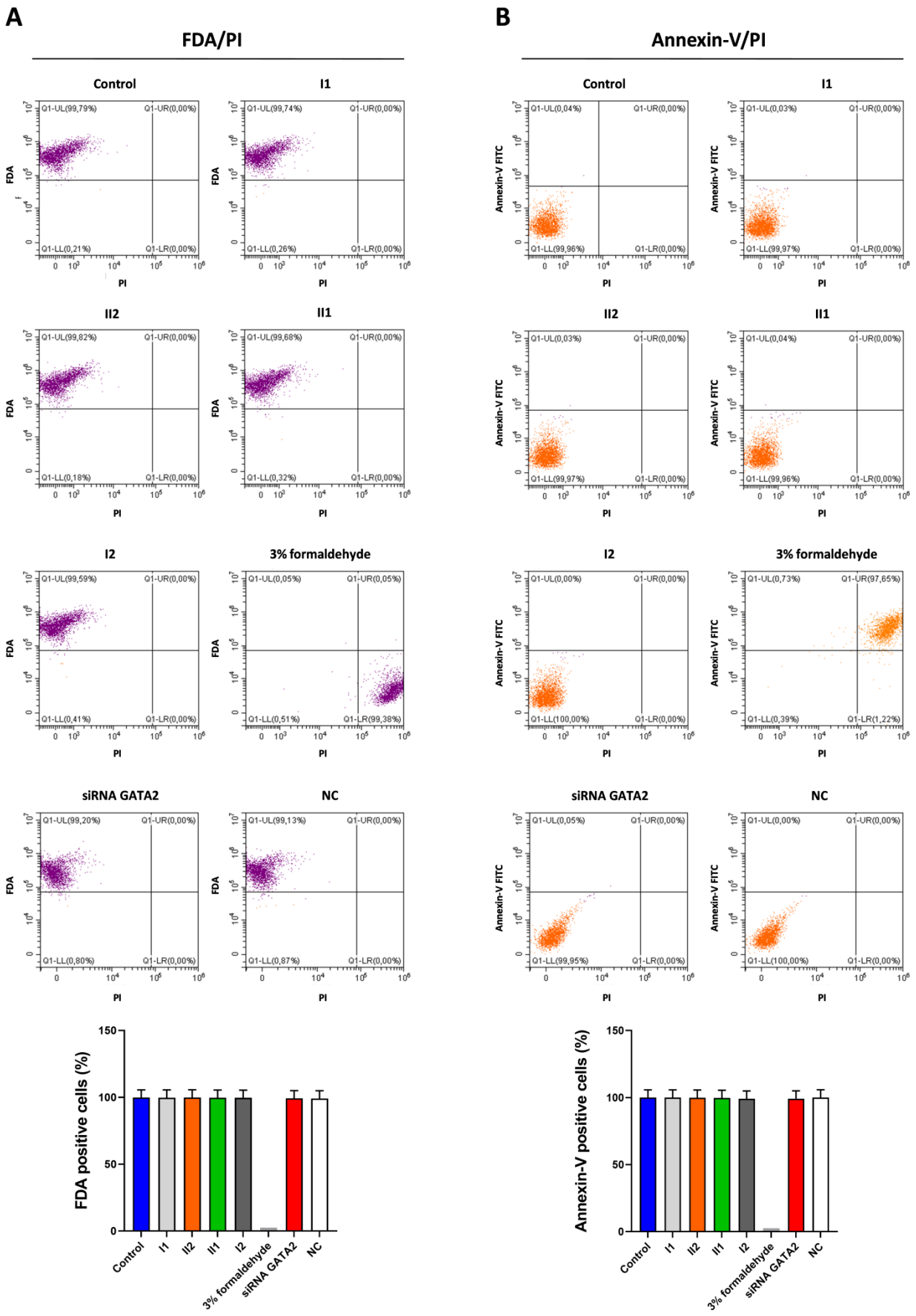
B



Supplementary Fig.1: BOEC derived from healthy donors and from GATA2-mutated patients show typical endothelial surface markers and express VWF.

A) BOEC isolated from peripheral blood of GATA2-deficiency family members and from age- and sex- matched healthy controls expressed all the typical endothelial surface markers (CD31, CD146, CD309) and were negative for leucocyte and monocyte markers (CD45, CD14), similar to Human Umbilical Vein Endothelial Cells (HUVECs), used as positive control.

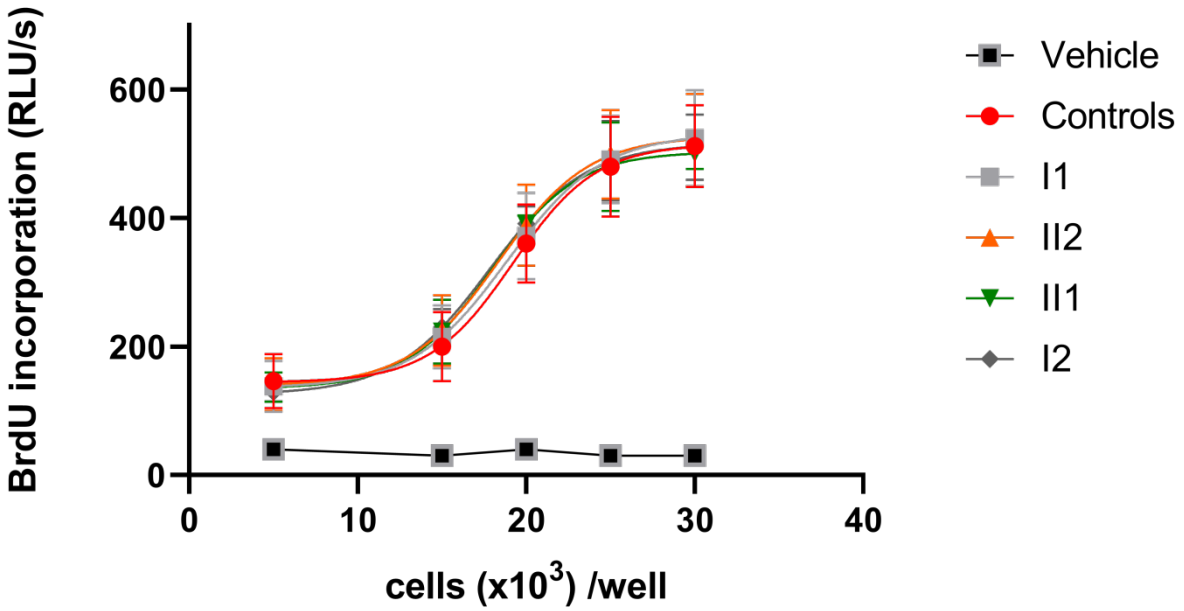
B) Representative images of VWF protein distribution analyzed by confocal microscopy. VWF is stained in green (Alexa Fluor® 488 Goat Anti-Rabbit IgG), Actin is stained in red (Rhodamine Phalloidin) and Nucleus is stained in blue (DAPI). Samples were mounted with the ProLong Antifade Mountant with DAPI. Specimens were analyzed at room temperature by a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Gmbh, Oberkochen, Germany) using a 63X oil immersion Plan-Apochromat objective and images acquired using the Zen 2.6 software (Carl Zeiss Gmbh, Oberkochen, Germany). The images are representative for 3 repeated measures for VWF.


A
FDA/PI
Control
I1
II2
II1
I2
3% formaldehyde
siRNA GATA2
NC
FDA
PI
Q1-UL(99,79%)
Q1-UR(0,00%)
Q1-LL(0,21%)
Q1-LR(0,00%)
Q1-UL(99,74%)
Q1-LL(0,26%)
Q1-UL(99,82%)
Q1-LL(0,18%)
Q1-UL(99,68%)
Q1-LL(0,32%)
Q1-UL(99,59%)
Q1-LL(0,41%)
Q1-UL(0,05%)
Q1-UR(0,05%)
Q1-LL(0,51%)
Q1-LR(99,38%)
Q1-UL(99,20%)
Q1-LL(0,80%)
Q1-UL(99,13%)
Q1-LL(0,87%)
FDA positive cells (%)
150
100
50
0
Control
I1
II2
II1
I2
3% formaldehyde
siRNA GATA2
NC
B
Annexin-V/PI
Annexin-V FITC
Q1-UL(0,04%)
Q1-LL(99,96%)
Q1-UL(0,03%)
Q1-LL(99,97%)
Q1-UL(0,03%)
Q1-LL(99,97%)
Q1-UL(0,04%)
Q1-LL(99,96%)
Q1-UL(0,00%)
Q1-LL(100,00%)
Q1-UL(0,73%)
Q1-UR(97,65%)
Q1-LL(0,39%)
Q1-LR(1,22%)
Q1-UL(0,05%)
Q1-LL(99,95%)
Q1-UL(0,00%)
Q1-LL(100,00%)
Annexin-V positive cells (%)

Supplementary Fig.2: BOEC derived from healthy controls and GATA2-deficiency family members show the same viability and siRNA GATA2 does not affect it

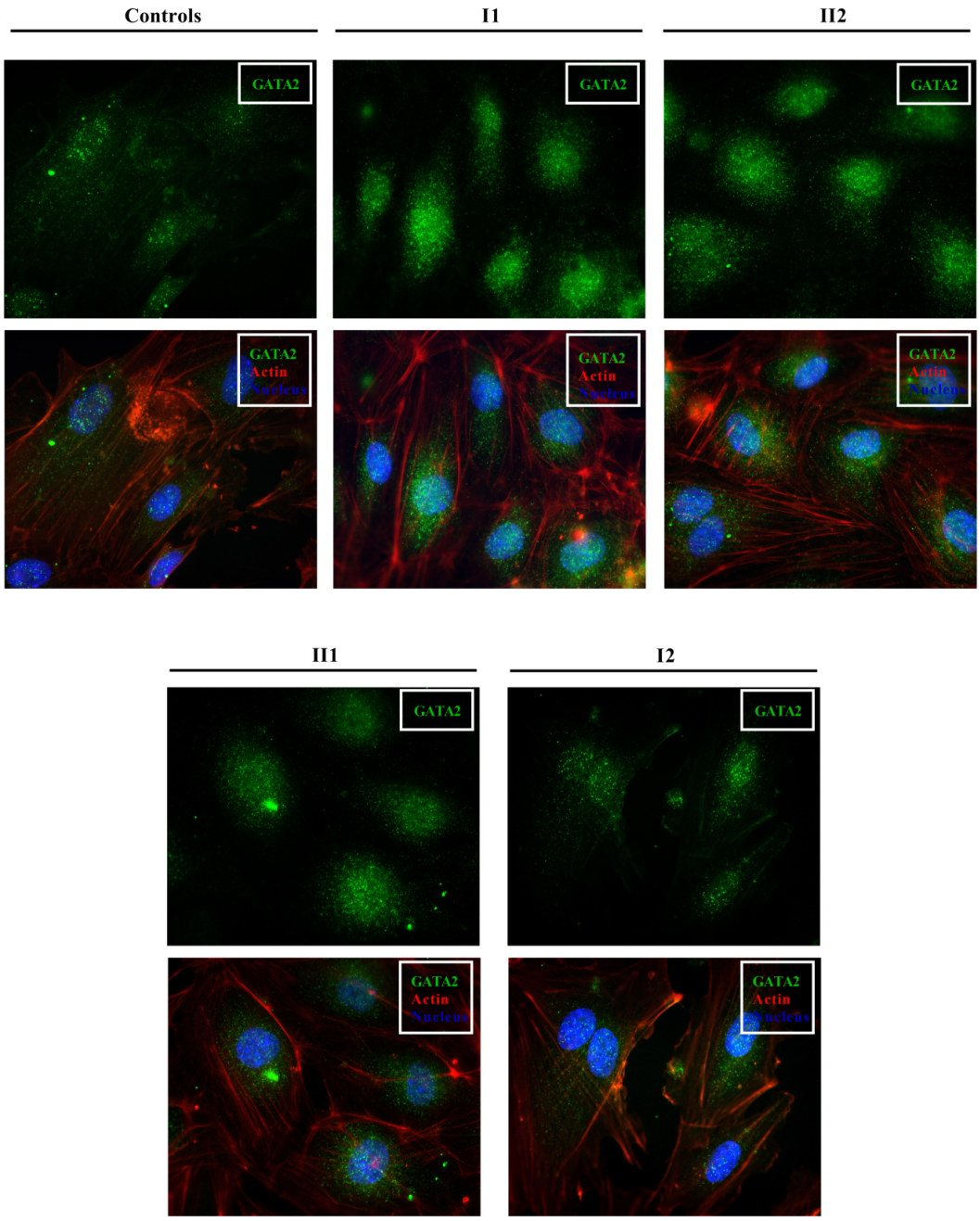
A) Flow cytometry of healthy control, GATA2-mutated BOEC and control BOEC after treatment with GATA2 siRNA, stained with FDA and PI. Cells positive for fluorescein diacetate (FDA) and negative for propidium iodide (PI) were considered as viable cells. 3% formaldehyde is used as positive control for cell death (positive for PI). Columns represent mean±SEM of 6 repeated measures.

B) Flow cytometry of healthy control, GATA2-mutated BOEC and control BOEC after treatment with GATA2 siRNA, stained with Annexin-V FITC and PI. Viability of cells is shown as Annexin-V surface positive cells and negative for PI. 3% formaldehyde is used as positive control for cell death (positive for PI). Columns represent mean±SEM of 6 repeated measures.



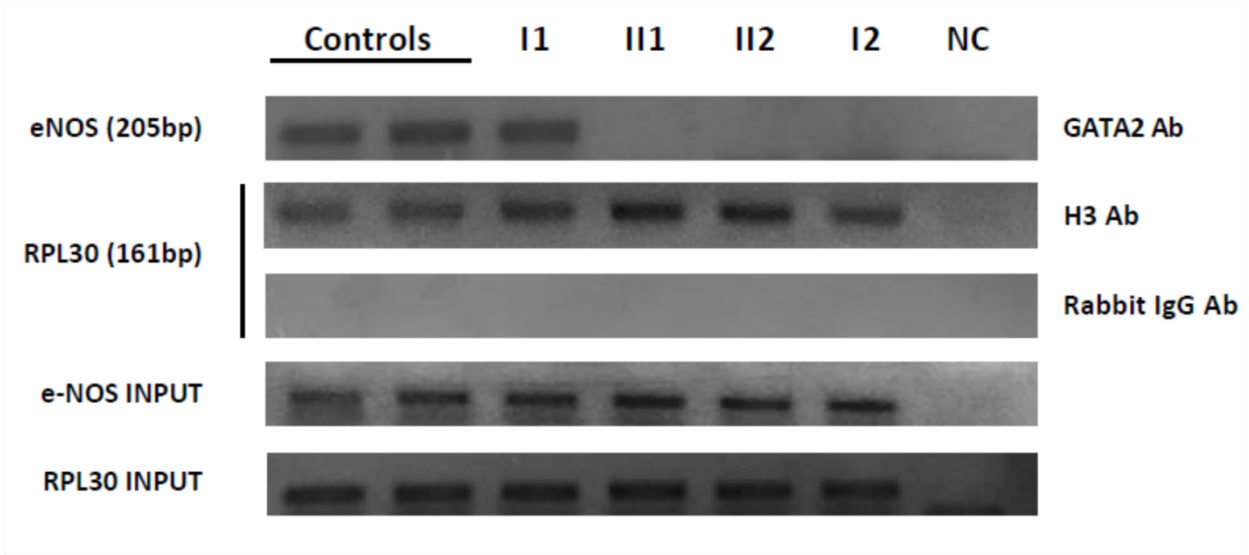
Supplementary Fig.3: BOEC derived from healthy controls and GATA2-deficiency family members show the same proliferation rate

In vitro BrdU incorporation in BOEC from healthy controls and GATA2-deficiency family members. Cells treated with vehicle (EBM2 medium) were used as negative control. The assay was performed in a standard 96-well polystyrene microfilter plate. Cells were plated at increasing concentrations in triplicate and marked with BrdU 10 µM. After 24h of incubation, cell proliferation was evaluated by a microplate luminometer with photomultiplier technology, measuring light emission. Values are means±SEM of 6 repeated measures.



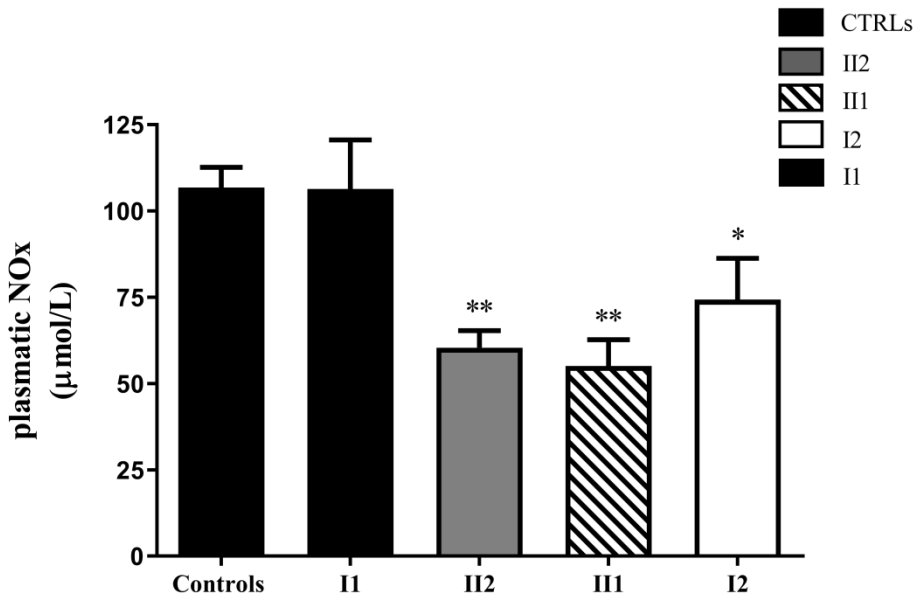
Supplementary Fig.4: BOEC from healthy controls and from GATA2-mutated patients show the same distribution of GATA2 both in the nucleus and cytoplasm

Representative images of GATA2 protein distribution analyzed by confocal microscopy. GATA2 is stained in green (Alexa Fluor® 488 Goat Anti-Rabbit IgG), Actin is stained in red (Rhodamine Phalloidin) and Nucleus is stained in blue (DAPI). Samples were mounted with the ProLong Antifade Mountant with DAPI. Specimens were analyzed at room temperature by a laser scanning confocal microscope AxioObserver.Z1 (Carl Zeiss Gmbh, Oberkochen, Germany) using a 63X oil immersion Plan-Apochromat objective and images acquired using the Zen 2.6 software (Carl Zeiss Gmbh, Oberkochen, Germany). The images are representative for 3 repeated measures for GATA2.



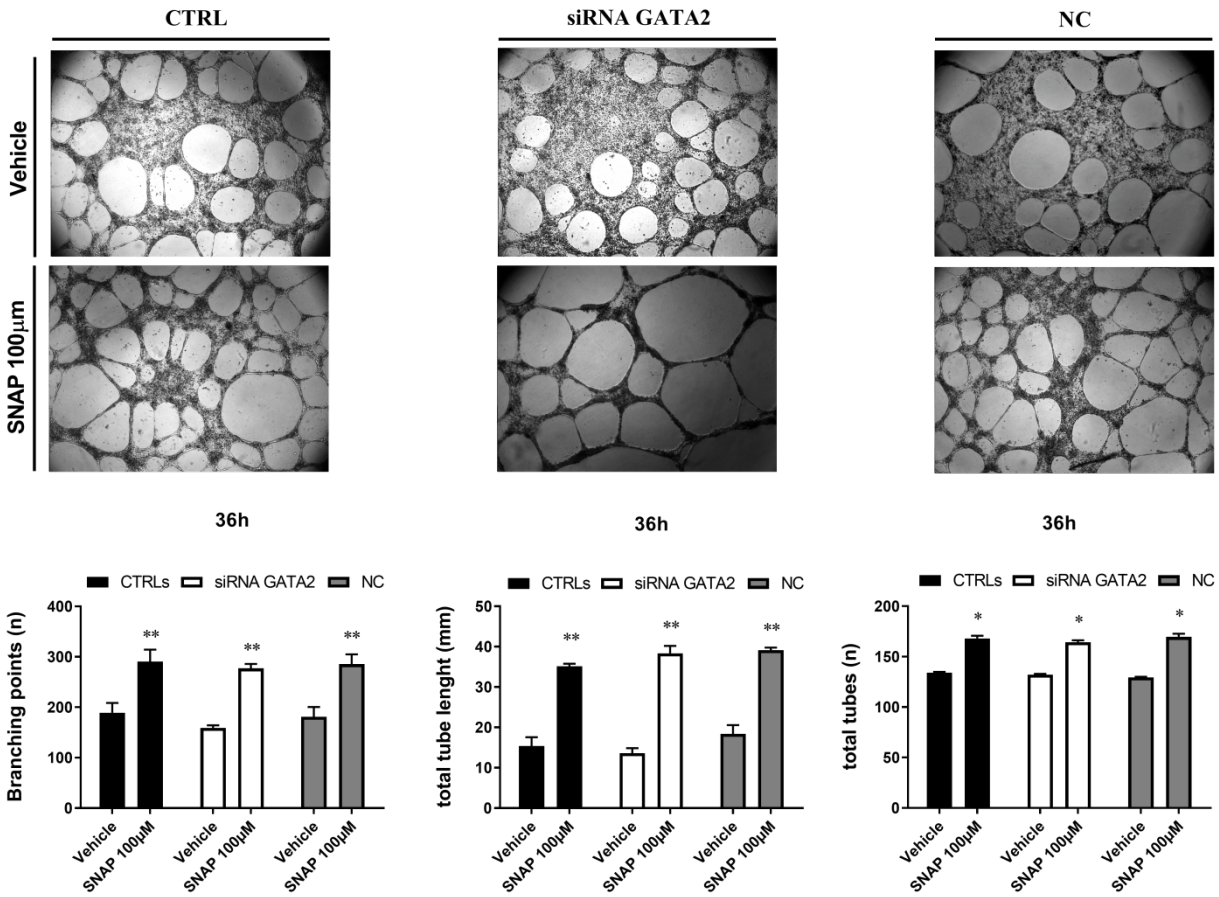
Supplementary Fig.5: Impaired GATA2 binding to DNA in BOEC from R398W variant-carrying patients

Electrophoresis agarose gel of PCR products of immunoprecipitated chromatin in BOECs from healthy controls and GATA2-deficiency family members. The figure shows the eNOS gene amplification product in GATA2-immunoprecipitated chromatin and RPL30 PCR product in histone H3-immunoprecipitated positive control and in IgG-immunoprecipitated negative control. PCR products of eNOS and RPL30 positive control were observed for each primer set in the INPUT sample. (NC: blanc; H3 Ab: Histone H3 Rabbit mAb positive control; Rabbit IgG Ab: normal rabbit IgG negative control).



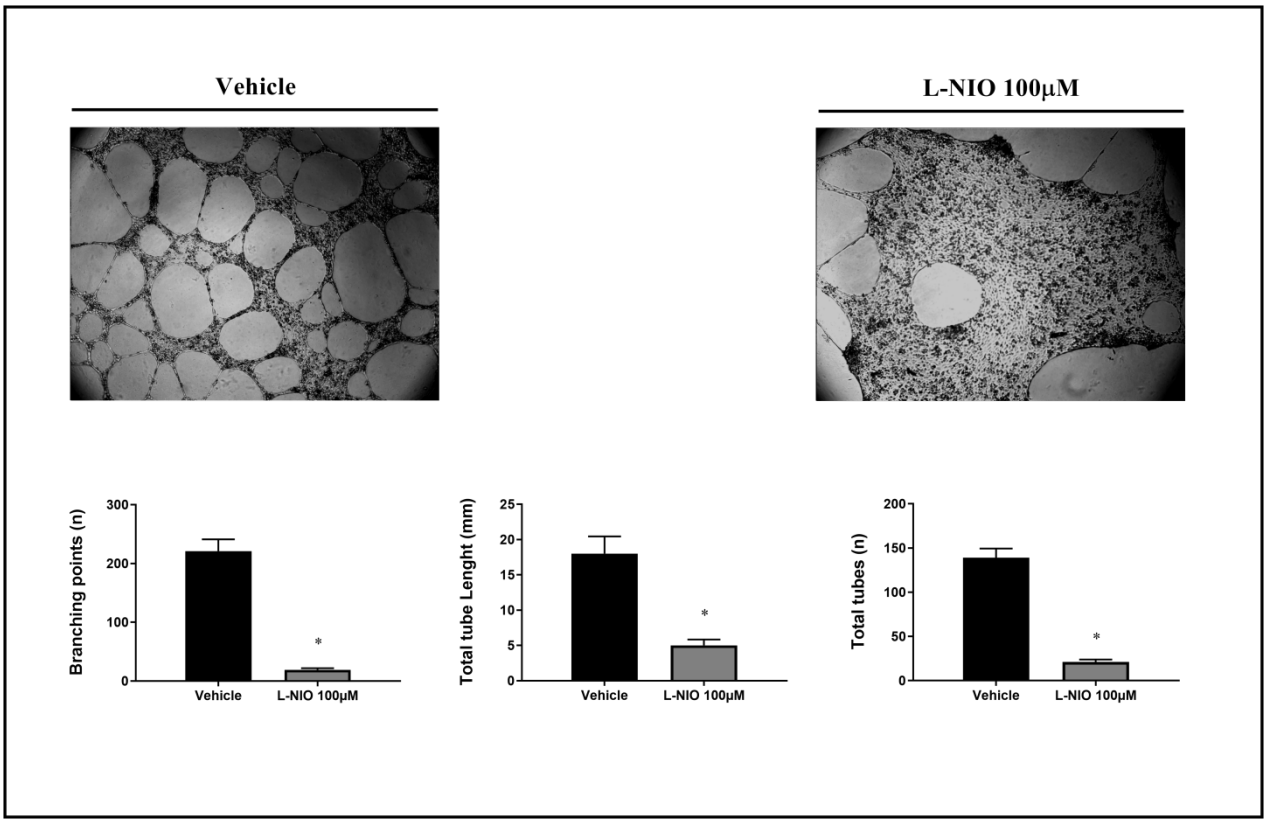
Supplementary Fig.6: Reduced NOx in citrated plasma derived from GATA2-mutated patients

Nitrite and nitrate (NOx) concentration in plasma from healthy controls and GATA2-mutated patients. Concentration is expressed in µM. Values are means ± SEM of 6 repeated measures from 6 controls and 3 different preparations from the patients (*$p < 0.005$ vs Control, one-way ANOVA followed by Dunnett's multiple comparison test).



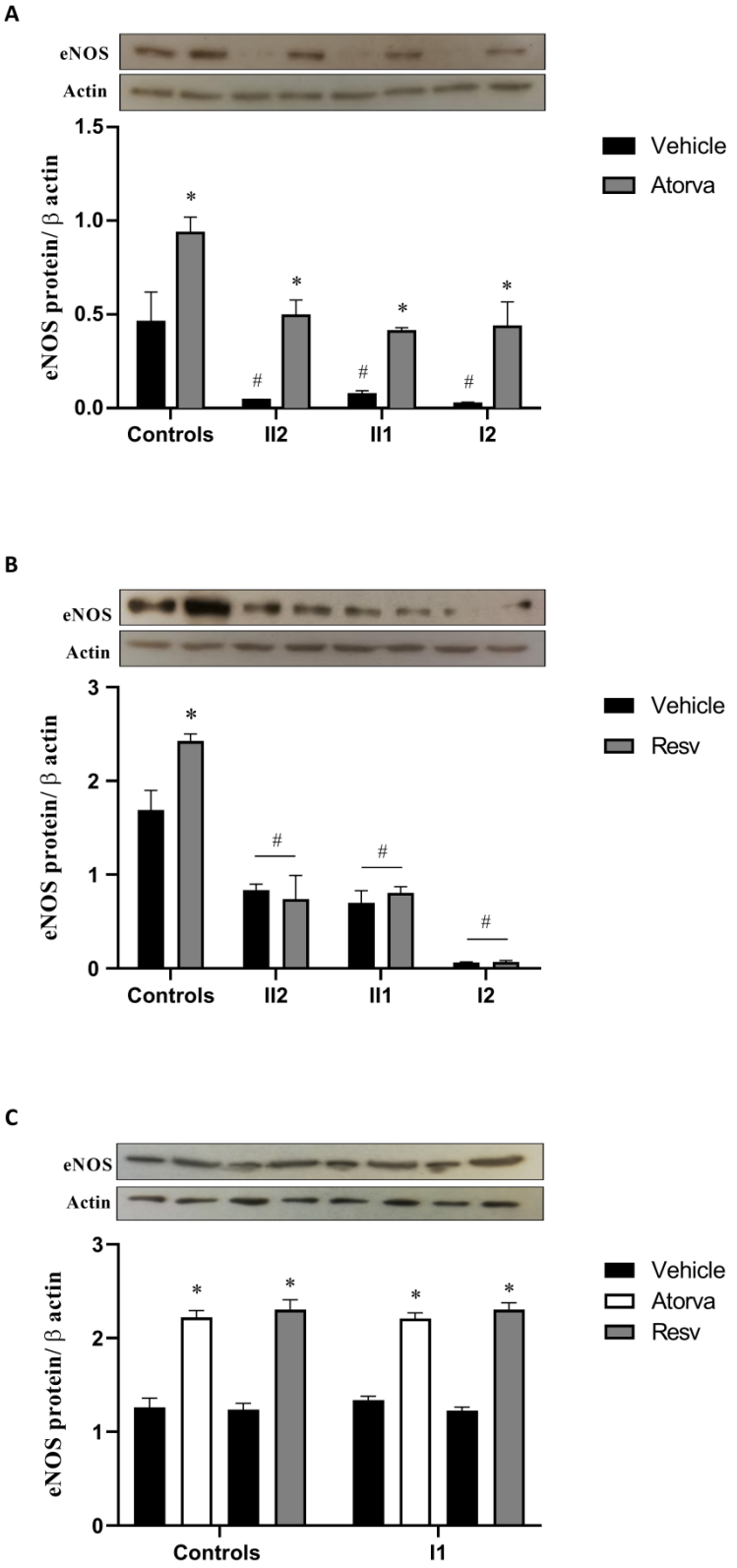
Supplementary Fig.7: Normal angiogenesis after 36h GATA2 silencing in healthy control BOEC

Capillary tube formation in GATA2-silenced (36h) BOEC from healthy controls (n=6). The NO donor SNAP 100µM for 24h increases angiogenesis that was normal 36h after GATA2 silencing, when GATA2 protein is suppressed but eNOS protein is not yet reduced. Angiogenesis was quantified with ImageJ (AngioTool64) software by measuring total tube length and counting tube number and branching points. Values are means ± SEM of 6 repeated measures (*$p < 0.0001$ vs vehicle, two-way ANOVA followed by Tukey's multiple comparison test). Specimens were analyzed at room temperature by a Carl Zeiss Axio Observer.A1 microscope (Carl Zeiss Inc, Oberkochen, Germany) using a 2.5X Plan-Apochromat objective and images acquired using the AxioVision software (Carl Zeiss Inc).



Supplementary Fig.8: eNOS inhibitor impairs angiogenesis in control BOEC

Healthy control BOEC treated with L-NIO 100 μM show impaired angiogenesis, compared with BOEC incubated with vehicle (DMSO). Angiogenesis was quantified with ImageJ (AngioTool64) software by measuring total tube length and counting tube number and branching points. Values represent mean ± SEM of 6 repeated measures (*$p < 0.001$ vs vehicle, two-way ANOVA followed by Tukey's multiple comparison test). Specimens were analyzed at room temperature by a Carl Zeiss Axio Observer.A1 microscope (Carl Zeiss Inc, Oberkochen, Germany) using a 2.5X Plan-Apochromat objective and images acquired using the AxioVision software (Carl Zeiss Inc).



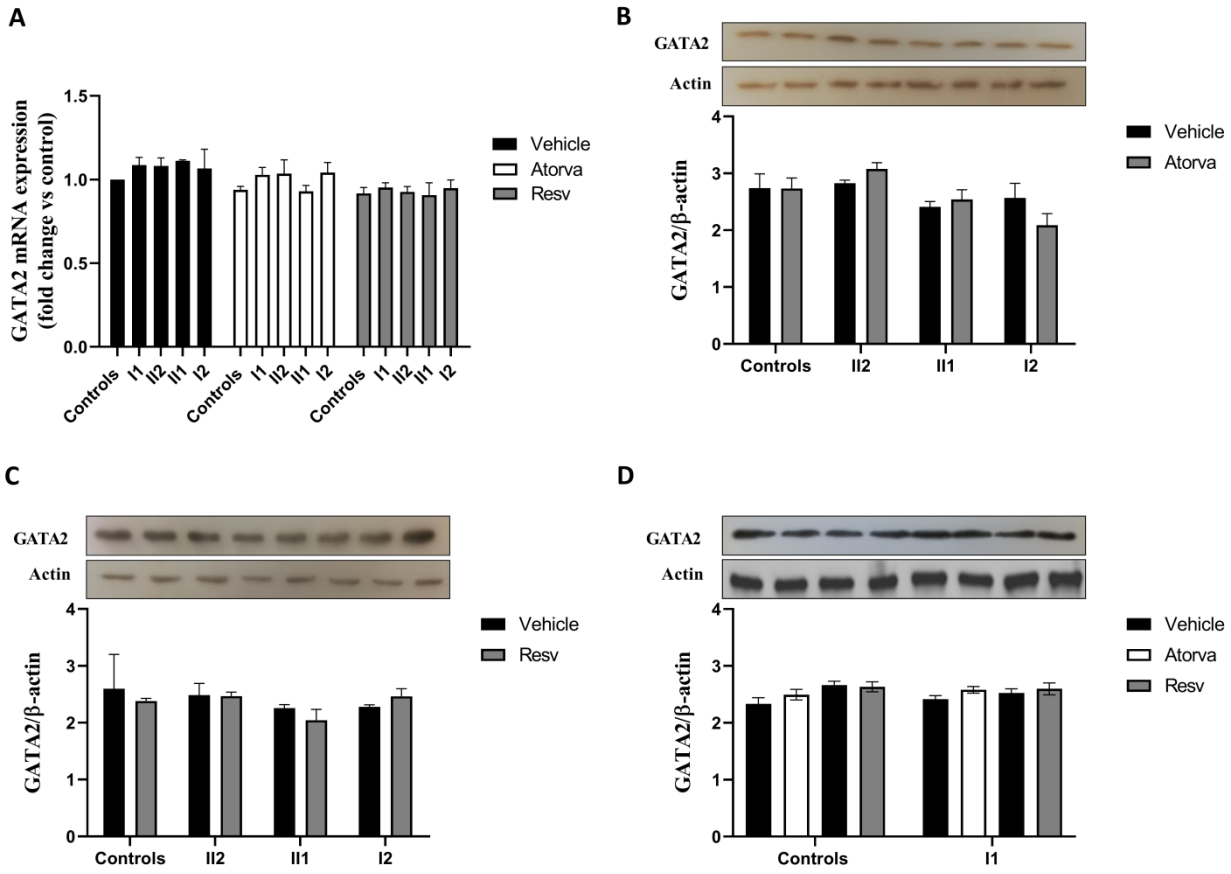
Supplementary Fig.9: Atorvastatin, but not resveratrol, increases eNOS protein expression in GATA2-mutated patients

A) Western blotting of eNOS protein in BOECs from healthy controls and from GATA2-mutated patients after stimulus with atorvastatin (50 µM) for 24h. Actin was used as loading control.

Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures from 6 controls and 3 different preparations from the patients (*$p < 0.001$ vs Vehicle $^{\#}p < 0.001$ vs Control, two-way ANOVA followed by Tukey's multiple comparison test).

B) Western blotting of eNOS protein in BOECs from healthy controls and from GATA2-mutated patients after stimulus with resveratrol (40 μM) for 24h. Actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures from 6 controls and 3 different preparations from the patients (*$p < 0.001$ vs Vehicle $^{\#}p < 0.001$ vs Control, two-way ANOVA followed by Tukey's multiple comparison test).

C) Western blotting of eNOS protein in BOECs from healthy controls and from the unaffected family member (I1) after incubation with resveratrol (40 μM) and atorvastatin (50 μM) for 24h. β-actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures (*$p<0.001$ vs Vehicle, two-way ANOVA followed by Tukey's multiple comparison test).



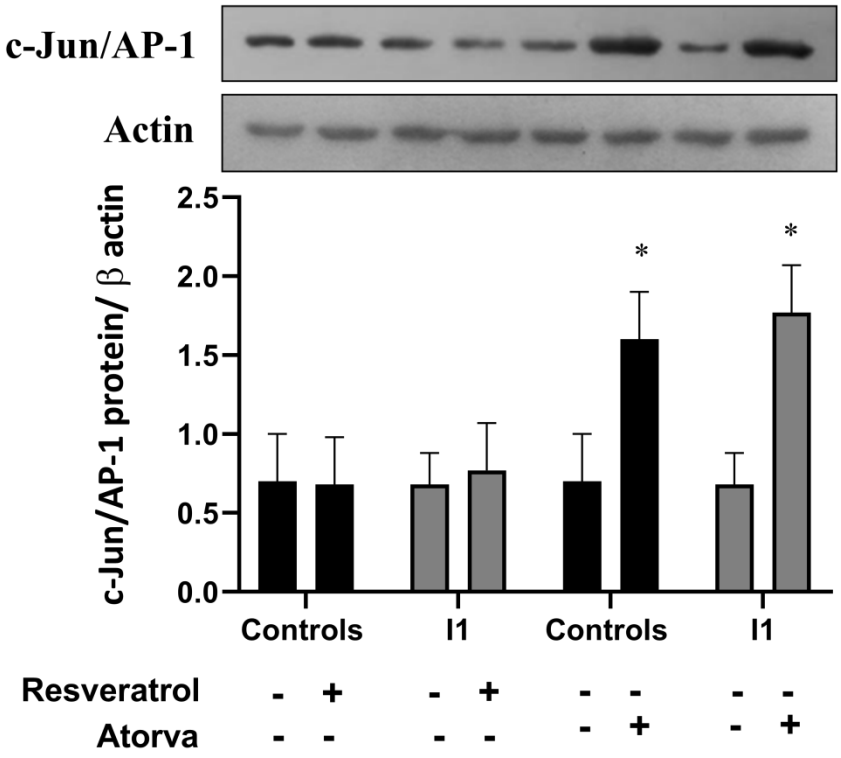
Supplementary Fig.10: GATA2 expression is not influenced by treatment with eNOS inducers

A) Real Time PCR of GATA2 mRNA of BOEC from healthy controls and GATA2-mutated patients after preincubation with atorvastatin (50 µM) or resveratrol (40 µM) for 24h. The expression of GATA2 mRNA is reported as fold change versus healthy control BOEC and normalized to a housekeeping mRNA (GAPDH). Values are means±SEM of 4 repeated measures from 4 controls and 3 different preparations from the patients (two-way ANOVA followed by Tukey's multiple comparison test).

B) Western blotting of GATA2 protein in BOECs from healthy controls and from GATA2-mutated patients after stimulus with atorvastatin (50 µM) for 24h. Actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean ± SEM of 6 repeated measures from 6 controls and 3 different preparations from the patients (two-way ANOVA followed by Tukey's multiple comparison test).

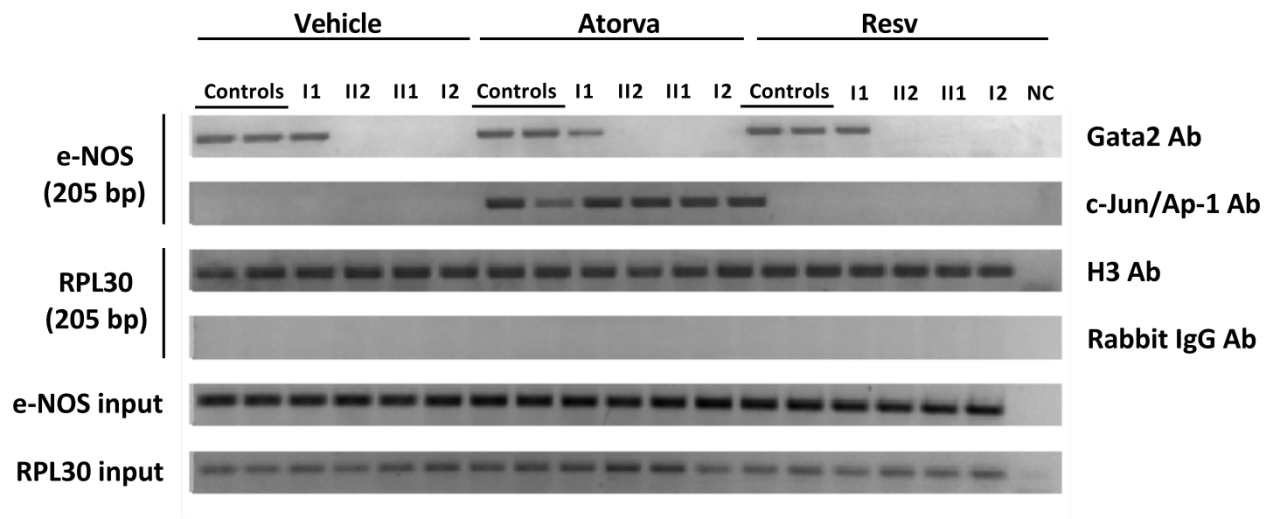
C) Western blotting of GATA2 protein in BOECs from healthy controls and from GATA2-mutated patients after incubation with resveratrol (40 µM) for 24h. Actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean ± SEM of 6 repeated measures from 6 controls and 3 different preparations from the patients (two-way ANOVA followed by Tukey's multiple comparison test).

D) Western blotting of GATA2 protein in BOECs from healthy controls and from the unaffected family member (I1) after incubation with resveratrol (40 μM) and atorvastatin (50 μM) for 24h. Actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures (*p< 0.001 vs Vehicle, two-way ANOVA followed by Tukey's multiple comparison test).



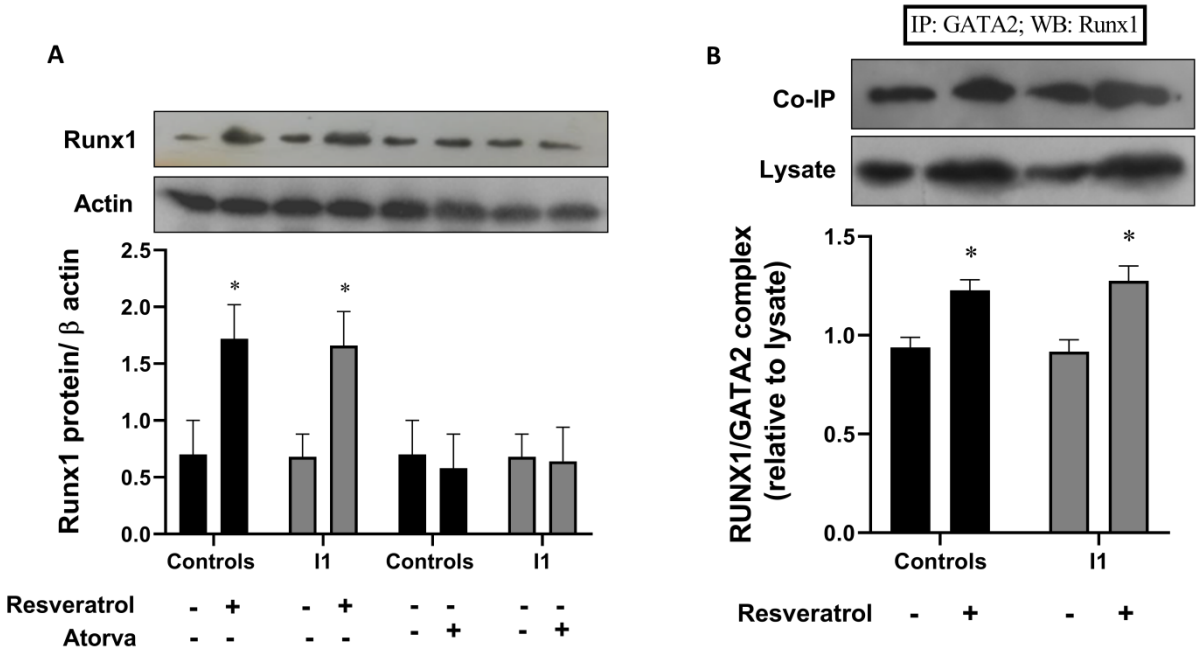
Supplementary Fig.11: BOEC derived from the unaffected family member (I1) present the same features of healthy control BOEC

Western blotting of c-Jun/AP-1 protein in BOECs from healthy controls and from the unaffected family member (I1) after incubation with resveratrol (40 μM) and atorvastatin (50 μM) for 24h. Actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures (*p<0.001 vs Vehicle, two-way ANOVA followed by Tukey's multiple comparison test).



Supplementary Fig.12: Atorvastatin, but not resveratrol, increases the efficiency of c-Jun/AP-1 transcription factor in binding the eNOS promoter

Electrophoresis agarose gel of PCR products of immunoprecipitated chromatin in BOECs from healthy controls and GATA2-deficiency family members treated with atorvastatin (50 µM) and resveratrol (40 µM) for 24h. Figure shows the eNOS gene amplification product evaluated in GATA2-immunoprecipitated chromatin and in c-Jun/AP-1-immunoprecipitated chromatin, RPL30 PCR product in histone H3-immunoprecipitated positive control and in IgG-immunoprecipitated negative control. PCR products of eNOS and RPL30 positive control were observed for each primer set in the INPUT sample.



Suppl. Fig. 13: BOEC derived from the unaffected family member (I1) present the same features of healthy control BOEC

A) Western blotting of RUNX1 protein in BOECs from healthy controls and from the unaffected family member (I1) after incubation with resveratrol (40μM) and atorvastatin (50μM) for 24h. β-actin was used as loading control. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures (*p<0.001 vs Vehicle, two-way ANOVA followed by Tukey's multiple comparison test)

B) Coimmunoprecipitation of RUNX1-GATA2 complex in BOECs from healthy controls and from the unaffected family member (I1) after incubation with resveratrol (40μM) for 24h. GATA2 protein was immunoprecipitated and western blotting was evaluated on RUNX1 protein. RUNX1 total lysate was used as INPUT for quantification. Optical densitometric analysis was performed using ImageJ software and results are expressed in arbitrary units. Values represent mean±SEM of 6 repeated measures (*p < 0.001 vs Vehicle, two-way ANOVA followed by Tukey's multiple comparison test)