The α₂-adrenergic receptor pathway modulating depression influences the risk of arterial thrombosis associated with BDNFVal66Met polymorphism

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ABSTRACT

Depression is associated with thrombotic risk and arterial events, its proper management is strongly recommended in coronary artery disease (CAD) patients. We have previously shown that the Brain-Derived Neurotrophic Factor (BDNF)Val66Met polymorphism, related to depression, is associated with arterial thrombosis in mice, and with an increased risk of acute myocardial infarction in humans. Herein, expanding the previous findings on BDNFVal66Met polymorphism, we show that desipramine, a norepinephrine reuptake-inhibitor, rescues behavioral impairments, reduces the arterial thrombosis risk, abolishes pathological coagulation and platelet hyper-reactivity, normalizes leukocyte, platelet, and bone marrow megakaryocyte number and restores physiological norepinephrine levels in homozygous knock-in BDNF Val66Met (BDNF$^{Met/Met}$) mice. The *in vitro* data confirm the enhanced procoagulant activity and the alpha$_{2A}$-adrenergic receptor (α$_{2A}$-ADR) overexpression found in BDNF$^{Met/Met}$ mice and we provide evidence that, in presence of Met variant, norepinephrine is crucial to up-regulate procoagulant activity and to enhance platelet generation. The α$_2$-ADR antagonist rauwolscine rescues the prothrombotic phenotype in BDNF$^{Met/Met}$ mice and reduces procoagulant activity and platelet generation in cells transfected with BDNF^{Met} plasmid or exposed to pro-BDNF^{Met} peptide. Finally, we show that homozygous BDNF$^{Met/Met}$ CAD patients have hyper-reactive platelets overexpressing abundant α$_{2A}$-ADR. The great proplatelet release from their megakaryocytes well reflects their higher circulating platelet number compared to BDNF$^{Val/Val}$ patients. These data reveal an unprecedented described role of Met allele in the dysregulation of norepinephrine/α$_{2A}$-ADR pathway that may explain the predisposition to arterial thrombosis. Overall, the development of α$_{2A}$-ADR inhibitors might represent a pharmacological treatment for depression-associated thrombotic conditions in this specific subgroup of CAD patients.

1. Introduction

Depression in patients with coronary artery disease (CAD) is associated with increased trombotic risk and arterial events [1,2]. In addition to the indicated treatment for cardiovascular disease, the management of depression represents an additional approach to

improve the clinical outcome of these patients [2,3]. In this context, the cardiovascular benefit of antidepressant drug treatment in patients with cardiovascular disease is being assessed, as well as which specific subgroup of patients may benefit most from antidepressant therapy [3,4]. Several factors, like efficacy, tolerability, and safety aspects need to be considered to assess the optimal treatment for CAD patients with depression comorbidity [2].

Thus, understanding the pathophysiological mechanisms underlying the comorbidity of depression and CAD is essential to develop an appropriate treatment. Reputed mechanisms to explain this interplay include endothelial dysfunction, platelet hyperreactivity, enhanced coagulation, hypofibrinolysis and increased inflammation [2,5–7].

We have recently shown that Brain-Derived Neurotrophic Factor (BDNF) Val66Met (rs6265) polymorphism, previously related to mood disorders, stress vulnerability [8], and to CAD-related depression [9–11], is associated with the increased risk of acute myocardial infarction (AMI), predicts major cardiovascular events post-MI in humans, and predisposes to arterial thrombosis in a knock-in mouse model [12–14]. In particular, we have shown that homozygous BDNF$^{Met/Met}$ mice display a hypercoagulable and hypofibrinolytic state, platelet hyper-reactivity as suggested by their enhanced aggregation, GPIIbIIIa and P-selectin expression, platelet-fibrinogen binding, platelet/leukocyte aggregates, and higher levels of tissue factor (TF) along with the well-known depressive/anxious-like phenotype [12,15], suggesting a complex interaction among genetic modifications, depression and CAD. Interestingly, desipramine, a tricyclic antidepressant that selectively blocks reuptake of norepinephrine, has antidepressant effects and rescued behavioral impairments in mutant BDNF Met mice [11,15]. Accordingly, individuals with depression carrying the BDNFVal66Met polymorphism show a better clinical response to long-term serotonin/norepinephrine reuptake inhibitors and TCA compared to selective serotonin reuptake inhibitor (SSRI) treatment [16–18]. All these findings suggest that a specific class of antidepressants may be an effective treatment option for depression in humans with this BDNF genetic variant.

Although the ability of antidepressant drugs to prevent thrombosis *in vivo* and to reduce platelet activation *in vitro* is well known [19–23] their antithrombotic effect in patients with depression and CAD comorbidity is not clearly established, as yet. In particular, whether norepinephrine reuptake inhibitors may attenuate depressive symptoms and concomitantly prevent cardiovascular events, at least in a specific subgroup of CAD patients, is still completely unknown.

In this study, we evaluated the potential involvement of norepinephrine/adrenergic receptor pathway in the regulation of coagulation, megakaryopoiesis and platelet function in homozygous BDNFVal66Met mouse and in homozygous BDNFVal66Met CAD patients. In particular, exploiting the mouse model, we investigated whether inhibition of norepinephrine reuptake rescues not only the behavioral impairment but also the arterial thrombosis predisposition.

2. Materials and methods

2.1. Ethics statement

Animal studies: All procedures were performed in wild type control (BDNF$^{Val/Val}$) or BDNF$^{Met/Met}$ mice [15], and approved by the National Ministry of Health-University of Milan Committee and Cogentech S.r.l. Committee. All procedures were performed in 10–12-weeks old male BDNF$^{Val/Val}$ or BDNF$^{Met/Met}$ mice.

Human studies: homozygous BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$, age 50–80 years at the time of enrolment, were identified within a large set of male patients with a clinical history of stable angina or acute coronary syndrome followed by coronary artery bypass graft surgery, [12]. All the patients had taken part or were still taking part in a secondary prevention program run in the Centro Cardiologico Monzino by specialists in internal medicine and nutritionists. Each homozygous BDNF$^{Met/Met}$

patient (uncommon allele) was matched for age (+/- 2 years), clinical history of stable, unstable angina or acute myocardial infarction, and pharmacological treatments with one homozygous BDNF$^{Val/Val}$ patients. Selected candidates were contacted and asked to take part in the study and to carry out a preliminary assessment of eligibility. They were then invited to a single study visit. After providing complete verbal and written information about the objectives and procedures of the study, signed consent was obtained. A detailed anamnesis and physical examination were then carried out to identify any of the following exclusion criteria: 1) recent acute cardiovascular event (throughout the last 6 months), 2) acute or chronic infections, 3) autoimmune diseases, 4) severe heart failure (NYHA class III/IV), 5) severe renal insufficiency (creatinine clearance <30 mL/min), 6) any form of cancer in the last 5 years (except skin basalioma), 7) major trauma or major surgical interventions in the last 6 months, and 8) current use of anti-inflammatory, immunosuppressive or antidepressive drugs.

Venous blood, obtained at fasting from an antecubital vein, was collected from eligible patients for study assays.

The study complies with the Declaration of Helsinki and was approved by the Ethical Committee of Centro Cardiologico Monzino IRCCS. All participants provided written informed consent. Clinical and demographical features of CAD patients are described in Supplementary Table S1. As the patients enrolled in this study were affected by coronary artery disease, they were almost all on treatment with drugs for secondary prevention of cardiovascular diseases according to the current evidence-based guidelines, including antiplatelets, statins and beta blockers, as shown in Supplementary Table S1. None of the participants in this study, including 4 patients that had referred a past history of anxiety or depression, was on a regular treatment with ansiolitic or antidepressant drugs.

Human umbilical cord blood was collected following normal pregnancies and deliveries upon informed parental consent in accordance with the Ethical Committee of the IRCCS San Matteo Foundation and with the principles of the Declaration of Helsinki.

2.2. Experimental design

The effect of desipramine or rauwolscine in BDNF$^{Va/Val}$ and BDNF$^{Met/Met}$ mice was studied separately in different set of mice. Specifically, desipramine (10 mg/Kg, Sigma-Aldrich) or saline (2 mL/kg) was administrated in drinking water for 3 weeks, an adequate period to appreciate the antidepressant effect, (N = 22 mice/group) [11,15]. Rauwolscine (200 µg/Kg, Cayman) or vehicle (DMSO) was intraperitoneally injected every day for 1 week to specifically discriminate between central and peripheral effect (N = 22 mice/group) [24], then mice were analysed.

2.3. Novelty suppressed feeding

Novelty suppressed-feeding was performed as described by Ieraci et al.[25]. Briefly, the testing apparatus consists of a large rectangular arena (60×40×15 cm) with the floor covered with wooden bedding. A regular chow pellet was placed on a white paper platform placed in the center of the arena. A single 24 h′ food-deprived mouse was positioned in a corner and the latency to eat the pellet was recorded. Immediately after the first bite, mice were transferred to their home cage and the amount of food consumed during 5 min was measured (home cage food consumption).

2.4. Arterial thrombosis model

FeCl$_3$ injury of the carotid artery was performed as described by Amadio et al.[12]. Anesthetized Male mice (75 mg/kg; ketamine chlorhydrate -Intervet- and 1 mg/kg medetomidine –Virbac). Carotid blood flow was monitored, after dissection free of the left carotid artery, employing a Doppler flow probe (model 0.7 V, Transonic System,

Transonic, Ithaca, USA) connected to a transonic flow meter (Transonic T106). Initial blood flow between the mouse genotypes was similar. When baseline blood flow was constant for 7 min at least 0.8 mL/sec, a 1 × 1 mm strip of filter paper (Whatman N°1) soaked with $FeCl_3$ (5% or 10% solution as indicated; Sigma-Aldrich, St. Louis, MO, USA) was placed over the carotid artery. After 3 min the filter paper was removed, the carotid artery was washed with phosphate-buffered saline (PBS), and the flow was recorded until 30 min. We considered a total and stable occlusion when the blood flow was reduced by > 90% of baseline within and during 5 min and, during those 5 min, it did not further increase by more than 1%.

2.5. Blood collection and biochemical analysis

Peripheral blood sample was collected from patients into vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid) disodium salt (9.3 mM; Vacutainer System, Becton Dickinson, Franklin Lakes, NJ, USA) or into vacutainer tubes containing Sodium Citrate (0.105 M; Vacutainer System, Becton Dickinson, Franklin Lakes, NJ, USA). For animal experiments, blood was collected by heart venipuncture into 3.8% sodium citrate (1:10 vol:vol) or 0.1 M EDTA from anesthetized mice. For plasma preparation, citrated/EDTA blood was centrifuged within 30 min at 3000 rpm for 20 min, which was immediately stored at −80 °C until further analysis.

ELISA kit was used to measure norepinephrine (Cloude-Clone Corp.) and epinephrine (Cusabio) in EDTA-plasma or bone-marrow lysates according to the manufacturer's instructions.

For the bone-marrow isolation, both the end of each femur were cut and the tissue was flushed using a 23-gauge needle and a 5 mL syringe filled with ice-cold HBSS. Epinephrine was stabilized with the addition of Na-metabisulfite and all the subsequent passages were performed in the dark to avoid photodestabilization. Bone-marrow was disrupted by passing the tissue five times through an insulin syringe with a 28-gauge needle. Samples were centrifuged for 20 min at 10000 g and the supernatants were collected and immediately stored at −80 °C for further analysis.

The concentration of fibrinogen [26] in plasma was measured by Clauss methods [12].

2.6. Platelet and leukocyte count

Blood from mice was collected by orbital sinus bleeding into Unopette pipettes and immediately diluted in Unopette®-System reservoirs (Becton Dickinson) containing 1% ammonium oxalate. After thorough mixing, platelets and leukocytes were counted optically, using the Brecher-Cronkite method, by an operator blinded to the experimental groups.

Human platelet and leukocyte count were performed by Sysmex automated haematology analyzer XS-1000i/XS-800i (Sysmex Corporation).

2.7. Aortae ex vivo experiment

Aortae from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice were isolated and cut into approx. 2 × 2 mm and incubated in DMEM with desipramine (DES: 1 μM, Sigma-Aldrich) for one hour and then for four hours with norepinephrine (1 μM, Cayman). Tissue was then washed with PBS and frozen for further analysis.

2.8. Mouse Fetal liver derived-megakaryocytes preparation

Mouse fetal liver derived-megakaryocytes were prepared as described by Barbieri et al. [27]. Mouse embryonic fetal livers were isolated 14.5 days postcoitus from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ female mice and homogenized in DMEM (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Gibco), and 10 ng/mL

of recombinant mouse TPO (Peprotech). Cells were cultured at 37 °C and 5% CO2 for 4 days.4 Mature megakaryocytes were enriched using a BSA density gradient on day 4. Collected megakaryocytes were used in biochemical assay and flow cytometry or incubated for an additional 24 h to induce proplatelet formation and released platelets were then quantified by flow cytometry.

Megakaryocytes differentiated from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ fetal liver cells were collected, fixed with 4% PFA, permeabilized with BD Citofix/CitopermTM fixation and permeabilization solution (BD Biosciences) and stained with CD41-PE and anti-α2A adrenoreceptor primary antibody (1:200) in 5% normal goat serum in PBS for 2 h at room temperature. Cells were centrifuged, resuspended in PBS and stained with Alexa488-conjugated secondary antibody (1:2000, Invitrogen) for one hour at room temperature. After three wash with PBS, cells were analysed. A minimum of 10000 events was collected in the CD41$^+$ gate. For ploidy analysis, bone marrow cells from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice were flushed with PBS; and erythrocytes were lysed with 0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM disodium EDTA, pH 7.4. BM cells were fixed in 70% ethanol, washed twice, and resuspended in washing buffer. Cells were incubated with FITC rat anti-CD41 antibody (clone MWReg30, Biolegend). After washing twice, cells were incubated with 0.5 mg/mL of RNase (Sigma-Aldrich), incubated with 50 μg/mL of propidium iodide, and analysed. Platelets produced *in vitro* were analysed using the same forward and side scatter pattern as human and mouse peripheral blood platelets. Platelets were identified as CD41$^+$, and their number was calculated using a TruCount bead standard by flow cytometry (BD Bioscience). All the samples were acquired with a Beckman Coulter FacsDiva flow cytometer (Beckman Coulter Inc). Non-stained samples and relative isotype controls were used to set the correct analytical gating. Off-line data analysis was performed using Beckman Coulter Kaluza® version software package. Mature megakaryocytes were analysed or incubated for 24 h to induce proplatelet formation.

2.9. Human megakaryocyte cultures

Human megakaryocytes were differentiated from cord blood CD34- or peripheral blood CD45-positive cells as described by Balduini et al. [28,29]. Specifically, CD34- or CD45-positive cells were separated from the blood of healthy subjects by immunomagnetic beads selection (Miltenyi Biotec) and cultured for two weeks in Stem Span medium (Stem Cell Technologies) supplemented with 1% L-glutamine, 100 U/mL penicillin, 100 ng/mL streptomycin, 10 ng/mL of recombinant human thrombopoietin and Interleukin-11 (PeproTech EC Ltd). In this set of experiments, cells were cultured in the presence of pro-BDNF^{Val} e pro-BDNF^{Met} propeptides (10 ng/mL, Alomone) for 13 days or norepinephrine (1 μM, Cayman) starting from day 10. At the end of cultures, CD34-derived megakaryocytes were seeded in the presence of propeptides (10 ng/mL, Alomone) for an additional 24 h to allow platelet release. CD45-derived megakaryocytes were enriched with a 3–4% BSA gradient and re-seeded for 24 h in the presence of pro-BDNF^{Val} e pro-BDNF^{Met} propeptides. For experiments on CAD patients (BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$), CD45$^+$ cells were purified from peripheral blood by immunomagnetic beads selection and cultured as above described. At day 13, BSA enriched megakaryocytes were seeded onto 12 mm glass coverslips coated with 100 μg/mL fibrinogen (Sigma) for additional 24 h in the presence of propeptides or Rawoulscine and assessed for proplatelet formation. To analyse proplatelet formation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with a primary antibody against β1–tubulin (a kind gift of Prof. Joseph Italiano, Brigham and Women's Hospital, Boston, MA, USA). Proplatelet formation was calculated as the percentage of megakaryocytes-extending proplatelets over the total megakaryocyte population adhering to the substrate. Analysis was performed at least on 20 different fields for each sample. The cell count was performed in a double-blind manner by two independent investigators.

2.10. Cell culture and aortae ex vivo treatments

Human malignant epithelial cells (HeLa) were cultured in sterile flasks and maintained in DMEM medium (Lonza) supplemented with HEPES (1 mM, Invitrogen), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 10% FBS, at 37 °C in 5% CO2/95% air atmosphere. Almost every 3 days they were detached with trypsin/EDTA (Gibco) and transferred in new flasks after 1:4 dilution. HeLa cells were transfected for 24 h with BDNF^{Val} or BDNF^{Met} plasmids (4 µg/µL per well) containing a C-terminal HA epitope and a C-terminal FLAG epitope tag, using JETprime transfection reagent (Polyplus-Euroclone)[12] Occurred transfection of plasmids was verified with western blot analysis or immunofluorescence against HA (Abcam) and Flag (Sigma-Aldrich) epitope tags. After reaching the confluence of 90–95% cells were starved overnight in DMEM medium supplemented with HEPES (1 mM, Invitrogen), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 5% FBS. The day of the experiment Hela cells were incubated in DMEM medium supplemented with HEPES (1 mM, Invitrogen), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and without FBS.

For propeptide treatment, cells were starved overnight with DMEM medium supplemented with HEPES (1 mM, Invitrogen), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 5% FBS. The day of the experiment HeLa cells were incubated in DMEM medium supplemented with HEPES (1 mM, Invitrogen), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and without FBS. pro-BDNFVal e pro-BDNFMet propeptides (10 ng/mL, Alomone) were added for 4 h.

Desipramine (1 µM, Sigma-Aldrich), rauwolscine (10 µM, Cayman) or Prazosin (10 µM, Sigma-Aldrich) were added. After one hour, norepinephrine (1 µM, Cayman) was added and after 4 h, wells were washed with ice-cold PBS and stored at − 20 °C for further analysis.

2.11. Platelet studies

Washed mouse platelets were obtained from PRP as described by Amadio et al. [12] with serial centrifugation and addition of 0.2 µM PGI$_2$ and 0.01 mg/L apyrase. Platelet pellets were resuspended in HEPES--Tyrode's buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl$_2$, 12 mmol/L NaHCO$_3$, 0.4 mmol/L Na$_2$HPO$_4$, 0.35% bovine serum albumin, 10 mmol/L HEPES, and 5.5 mmol/L glucose), pH 7.4.

Platelet activation was assessed by flow cytometry analyses. Washed mouse platelets diluted with HEPES-Tyrode's buffer to obtain 2×10^5 platelet/µL and incubated with PE-conjugated JON/A (Emfret Analytics) antibody, raised against the activated form of GPIIbIIIa (αIIbβ3 integrin), and with anti-CD62P, FITC-conjugated antibody (P-selectin; BD Biosciences). Platelets in human whole blood were analysed using anti-CD62P antibody (eBioscience) and anti-PAC-1 antibody (BD Biosciences). Samples were stimulated for 15 min, if not otherwise indicated, at room temperature with ADP or thrombin without stirring. The reaction was stopped by 400 µL ice-cold PBS, and samples were analysed within 30 min. Platelets were identified by forward and side scatter distribution, and by anti-CD61 positivity.

For *platelet-leukocyte aggregates*, undiluted mouse and human whole blood was stimulated with ADP for 5 min and red blood cells were lysed by FACS Lysing solution (BD Biosciences) and the sample centrifuged and stained with anti-CD45 (BD Biosciences) and anti-CD61 (BD Biosciences). Samples were acquired with Novocyte 3000 (ACEA Biosciences) and analysed with Novoexpress software. A minimum of 10000 events were collected in the CD45$^+$ gate by flow cytometry.

Reticulated platelets were identified by the thiazole orange method as described by Amadio et al.[12]. Briefly, 10 µL of PRP were incubated with 390 µL of thiazole orange (Retic-Count; BD Biosciences) or PBS as control, and anti-CD41 at room temperature for 10 min, in the dark. Immediately after incubation, samples were analysed by flow cytometry collecting 10000 CD41-positive events, the percentage of reticulated platelets was recorded, and the absolute number of reticulated platelets was calculated by multiplying by the platelet count.

2.12. Procoagulant activity

Before assay, samples were lysed with 15 mM n-Octyl-β-D-glucopyranoside lysis buffer at 37 °C for 10 min, sonicated at 20 kHz for 20 s and diluted with 25 mM HEPES saline. The total protein concentrations of aortae, carotid artery or HeLa cells homogenates were determined using the Bradford method. For aorta and carotid artery, 40 µL of homogenate (0.031 mg/mL) or vehicle were mixed with 40 µL citrated pooled wild-type mouse plasma and 40 µL CaCl$_2$ (final concentration 15 mM), and procoagulant activity was quantified by a one-stage plasma recalcification time assay.

For HeLa cells, 60 µL of homogenate (0.07 mg/mL) or vehicle were mixed with 60 µL citrated pooled wild-type mouse plasma and 60 µL CaCl$_2$ (final concentration 25 mM), and procoagulant activity was quantified by a one-stage plasma recalcification time assay.

Clotting times were expressed in relative Unit/µg protein based on a standard curve of serially diluted human thromboplastin preparation [12]. To assess the TF dependence of procoagulant activity, for *mouse experiments*: after preincubation (30 min at room temperature) of the samples with anti-mouse TF antibody (AF3178 from R&D); for *human cells:* HeLa cells were performed with plasma deficient of Factor VIIa and/or after preincubation (30 min at room temperature) of the samples with a specific TF-neutralizing antibody (American Diagnostica GmbH, SEKISUI).

2.13. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from cells or aorta tissue with TRIzol Reagent according to the manufacturer's instructions. 1 µg of RNA was reverse transcribed using SuperScriptTM II RNase H (Invitrogen). qRT-PCR was then carried out to assay murine Tf, Sirt1 and Adra2a [α$_{2A}$-Adr (m)] transcripts and human ADRA2A [α$_{2A}$-ADR (h)]. Gapdh and 18 S were used for normalization in mouse and human samples respectively. The primers used were listed in Table 1. Samples of cDNA (2.5 µL) were incubated in 25 µL IQ Supermix containing Tf, Sirt1, or Gapdh primers and fluorescent dye SYBR Green (Bio-Rad Laboratories). qRT-PCR was carried out in triplicate for each sample on the CFX Connect real-time System (Bio-Rad Laboratories).

Table 1
List of primer used in the study.

Gene	Forward primer (5′−3′)	Revers primer (5′−3′)
Tf (m)	CGGGTGCAGGCATTCCAGAG	CTCCGTGGGACAGAGAGGAC
Sirt1 (m)	AGCAGGTTGCAGGAATCCAA	CACGAACAGCTTCACAATCAACTT
α$_{2A}$-Adr (m)	TCGTCATCATCGCCGTGTTC	TGGAAGAGTGGGAGTTGCTGTTG
Gapdh (m)	CGTGCCGCCTGGAGAAACC	TGGAAGAGTGGGAGTTGCTGTTG
α$_{2A}$-ADR (h)	TCGTCATCATCGCCGTGTTC	AAGCCTTGCCGAAGTACCAG
18 S (h)	CGGCTACCACATCCAAGGAA	CCTGTATTGTTATTTTTCGTCACTACCT

2.14. Protein preparation and immunoblotting

Bone marrow, HeLa cells and fetal liver-derived megakaryocytes were lysed in cold RIPA buffer (62.5 mM TRIS HCl, 100 mM NaCl, EDTA 2.5 mM, 1% Tween 100, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, 10 mM Na-pyrophosphate, 10 mM NaF pH 8.0 and protease inhibitor cocktail). After sonication to remove cell debris, the protein yield was quantified using the BCA protein assay kit (Pierce) [30]. Thirty μg of sample was prepared with the Laemmli method, and equivalent amounts of protein were separated on 7 or 10% SDS-PAGE gels, transferred to nitrocellulose membrane and bands of interest detected using anti-alpha$_{2A}$-adrenergic receptor (α_{2A}-ADR) antibody (1:500, Sigma-Aldrich) or Tissue factor (1:1000, Abcam- ab189483). GAPDH (1:5000, Santa Cruz), CD41 (1:5000, Abcam) and Vinculin (1:5000, Sigma-Aldrich) served as loading controls. Subsequently, membranes were incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce).

2.15. Immunofluorescence

Cells were fixed with 4% PFA, permeabilised with 0.5% Tween20 in PBS for ten minutes, blocked with 5% normal goat serum and 0.1% Tween20 in PBS for two hours and incubated overnight at 4 °C with anti-α_{2A}-ADR primary antibody (1:1000) in 5% normal goat serum and 0.1% Tween 20 in PBS. Subsequently, coverslips were incubated with Alexafluor 555-conjugated secondary antibody (1:250, Life-Technologies) for 1 h in PBS at room temperature, nuclei were stained with DAPI, and were mounted with fluorescent mounting medium (Dako). Images were acquired with a Zeiss LSM-510 laser confocal microscope producing image stacks and analysed with Zeiss ZEN blue software [31].

Femurs from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice were embedded in OCT cryo-sectioning medium, and snap-frozen in a chilling bath. 5 μm-tissue sections were taken by using a Microm Microtome HM 250 (Bio Optica S.p.A). Frozen sections were fixed for 20 min in 4% PFA, washed with PBS, and blocked with 2% Bovine Serum Albumin (BSA) (Sigma-Aldrich) in PBS for 30 min. Unspecific binding sites were saturated with 5% goat serum, 2% BSA and 0.1% glycine in PBS for 1 h. FITC-conjugated anti CD41 (Clone MwReg 30, Biolegend) was diluted 1:100 and incubated overnight at 4 °C in washing buffer (0.2% BSA, 0.1% Tween 20 in PBS). Nuclei were counterstained using Hoechst 33258 (100 ng/mL in PBS) at room temperature. Sections were then mounted with micro-cover glasses using Fluoro-mount (Bio-optica). Images were acquired using the Olympus BX51 fluorescence microscopy (Olympus Deutschland GmbH) and a 20X/0.75 Olympus UplanF1 objectives.

2.16. Statistical analysis

Statistical analyses were performed with GraphPad Prism7.0 and SAS v. 9.4 software (SAS Institute, Cary, NC, USA). Data were analyzed by the Mann-Whitney test, or one or two way-ANOVA with or without repeated measures, followed by a Dunn's Multiple Comparison test or Bonferroni *post hoc* analysis as appropriate. The Spearman coefficient (r) was calculated to quantify the correlation between variables. Linear regression analysis was performed. Values of $p < 0.05$ were considered statistically significant. All human performed analyses were adjusted for age, drug treatments and depression state. The sample size calculation was based on a preliminary study and statistical program.

3. Results

3.1. Desipramine treatment prevents the prothrombotic phenotype and reduces the latency in the novelty-suppressed feeding in BDNF$^{Met/Met}$ mice

We tested whether 3 weeks of desipramine treatment [11,15], a norepinephrine reuptake inhibitor antidepressant already known for its positive effect on behavioral impairment in BDNF$^{Met/Met}$ mice, reverts also the propensity to arterial thrombosis associated with this BDNF polymorphism.

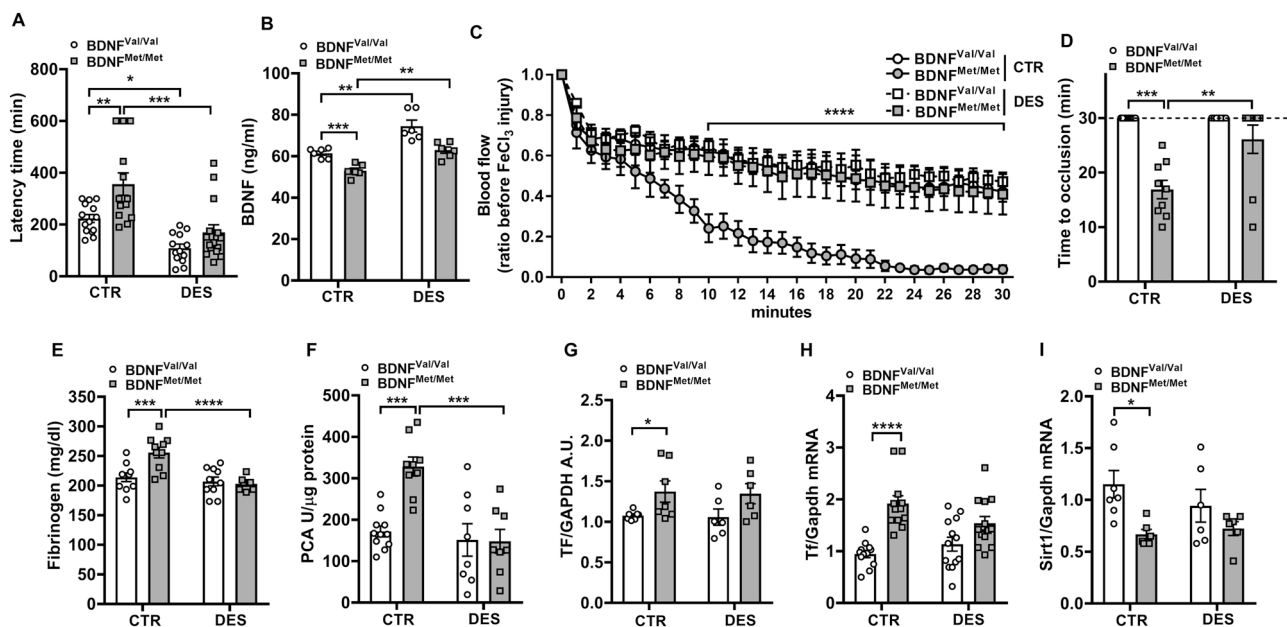Desipramine significantly reduced latency time in novelty-

Fig. 1. Effect of desipramine treatment on behavioral impairment and prothrombotic phenotype in BDNF$^{Met/Met}$ compared to BDNF$^{Val/Val}$ mice. Mice were treated with desipramine (DES; 10 mg/Kg) or with vehicle (CTR) for 3 weeks. (A) Latency time measured during the novelty-suppressed-feeding test (n = 13–15 mice/group), and (B) analysis of BDNF hippocampal levels measured by ELISA assay (n = 6 mice/group). Kinetic of blood flow in mouse carotid arteries expressed relative to the value before the injury induced by 5% $FeCl_3$ (panel C), and time to occlusion during 30 min of observation (panel D) (n = 7–9 mice/group). Detection of (E) fibrinogen by Clauss assay in plasma and (F) procoagulant activity (PCA) in aorta tissues (n = 8–11 mice/group). (G) Quantization of western blot analyses of TF protein (representative western blot shown in Supplementary Fig. S2B) (n = 6 mice/group). mRNA expression of (H) Tf (n = 12–13/mice group), and (I) Sirt1 (n = 6 mice/group) measured in aorta tissues. Data are shown as mean ± SEM. *p < 0.05, * *p < 0.01, * **p < 0.005 and * *** *p < 0.001.

suppressed-feeding test (NSF) and increased hippocampal BDNF protein in both BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice (Fig. 1A-B), as well as protected BDNF$^{Met/Met}$ mice against FeCl$_3$-induced carotid artery thrombosis (Fig. 1C-D). In particular, desipramine counteracted injury-dependent blood flow reduction, with almost 90% of treated BDNF$^{Met/Met}$ mice not developing a stable thrombus during the 30 min of recording. Interestingly, desipramine administration did not affect arterial thrombosis in BDNF$^{Val/Val}$ mice (Fig. 1C-D and Supplementary Fig. S1).

The reversal of the prothrombotic phenotype in treated BDNF$^{Met/Met}$ mice was accompanied by a decrease in fibrinogen in plasma (Fig. 1E) and in procoagulant activity in both aorta and carotid tissues to levels comparable with BDNF$^{Val/Val}$ mice (Fig. 1F and Supplementary Fig. S2A). Desipramine treatment did not significantly influence protein (Fig. 1 G and . Supplementary Fig. S2B) and mRNA expression level of Tf as well as of its modulator, Sirt1 in aortae (Fig.s 1 G-I).

In BDNF$^{Met/Met}$ mice, desipramine restored the physiological number of circulating blood cells, reducing leukocytes and platelets, and the amount of immature platelets (Fig. 2A-C), and abolished platelet hyper-activation ADP and/or thrombin mediated (Fig. 2D-F). In particular, desipramine-treated BDNF$^{Met/Met}$ mice showed a reduction in the percentage of ADP stimulated platelet/leukocyte aggregates in whole blood (Fig. 2D), as well as a lower percentage of GPIIbIIIa and P-selectin positive cells in washed platelets exposed to either ADP or thrombin (Fig. 2E-F).

BDNFVal66Met polymorphism affected also megakaryopoiesis. Indeed, BDNF$^{Met/Met}$ mice have a significant increase in bone marrow megakaryocytes when compared to BDNF$^{Val/Val}$ mice and desipramine administration restored their normal number (Fig. 3A-B). Megakaryocyte maturation profile was similar in BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice and was not influenced by desipramine treatment with no differences

detected by *ex vivo* megakaryocyte ploidy analysis (Supplementary Fig. S3). Overall, desipramine administration did not affect the hae-mostatic, thrombopoietic and megakariopoietic profile in BDNF$^{Val/Val}$ mice (Fig. 2 and Fig. 3). Moreover, BDNF$^{Met/Met}$ mice have significantly higher levels norepinephrine in bone marrow compared to BDNF$^{Val/Val}$, whereas similar norepinephrine levels in plasma were found between the two groups of mice (Fig. 3C-D). Desipramine reduced norepinephrine levels in both bone marrow and plasma of BDNF$^{Met/Met}$ without affecting those of BDNF$^{Val/Val}$ mice (Fig. 3C-D). Epinephrine levels showed no changes in all experimental conditions (Supplementary Fig. S4).

Together, these data show a critical effect of the norepinephrine-reuptake inhibitor desipramine to revert behavioral impairment and to prevent arterial thrombosis in BDNF$^{Met/Met}$ mice, suggesting a critical role of norepinephrine pathway.

3.2. BDNF Val66Met polymorphism affects procoagulant activity via the norepinephrine/ alpha$_2$-adrenergic receptor pathway

To investigate the potential contribution of norepinephrine on the activation of arterial procoagulant activity, we performed *ex-vivo* experiments on isolated aortae from both mouse groups. As previously shown, the basal procoagulant activity was greater in the aorta of BDNF$^{Met/Met}$ than in BDNF$^{Val/Val}$ [12], and the incubation of aortae with norepinephrine increased the procoagulant activity in both groups. However, norepinephrine effect was more pronounced in BDNF$^{Met/Met}$ aorta, with an almost threefold increase in procoagulant activity in BDNF$^{Met/Met}$ aortae and twofold in BDNF$^{Val/Val}$. Pre-incubation of aortae with desipramine, decreased norepinephrine -induced procoagulant activity only in aortae isolated from BDNF$^{Met/Met}$ mice (Fig. 4 A).

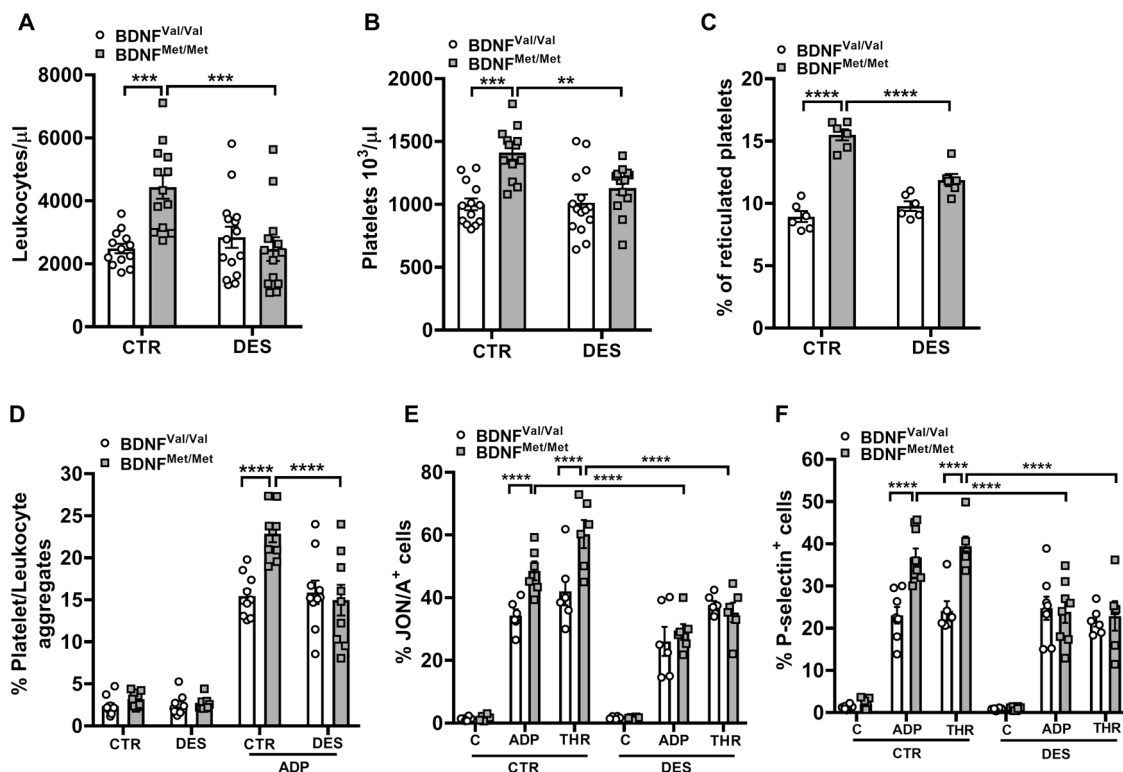To shed light on the mechanisms underlying procoagulant activation

Fig. 2. Effect of desipramine treatment on platelet reactivity and percentage of immature platelets in BDNF$^{Met/Met}$ compared to BDNF$^{Val/Val}$ mice. Desipramine (DES; 10 mg/Kg) or vehicle (CTR) were administered for 3 weeks to BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. Number of (A) leukocytes and (B) platelets in whole blood (n = 13–15 mice/group). (C) Percentage of reticulated platelets (n = 6 mice/group) detected by flow cytometry in platelet-rich plasma of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (D) Percentage of platelet/leukocyte aggregates in the whole blood in resting condition and after stimulation with ADP (n = 10 mice/group), (E) activation of GPIIbIIIa by JON/A-PE antibody and (F) expression of P-selectin (n = 6 mice/group) in washed platelets in resting condition and after stimulation with ADP (10 μM) or thombin (THR, 0.1 U/mL) detected by flow cytometry. Data are shown as mean ± SEM. * *p < 0.01, * **p < 0.005 and * ** *p < 0.001.
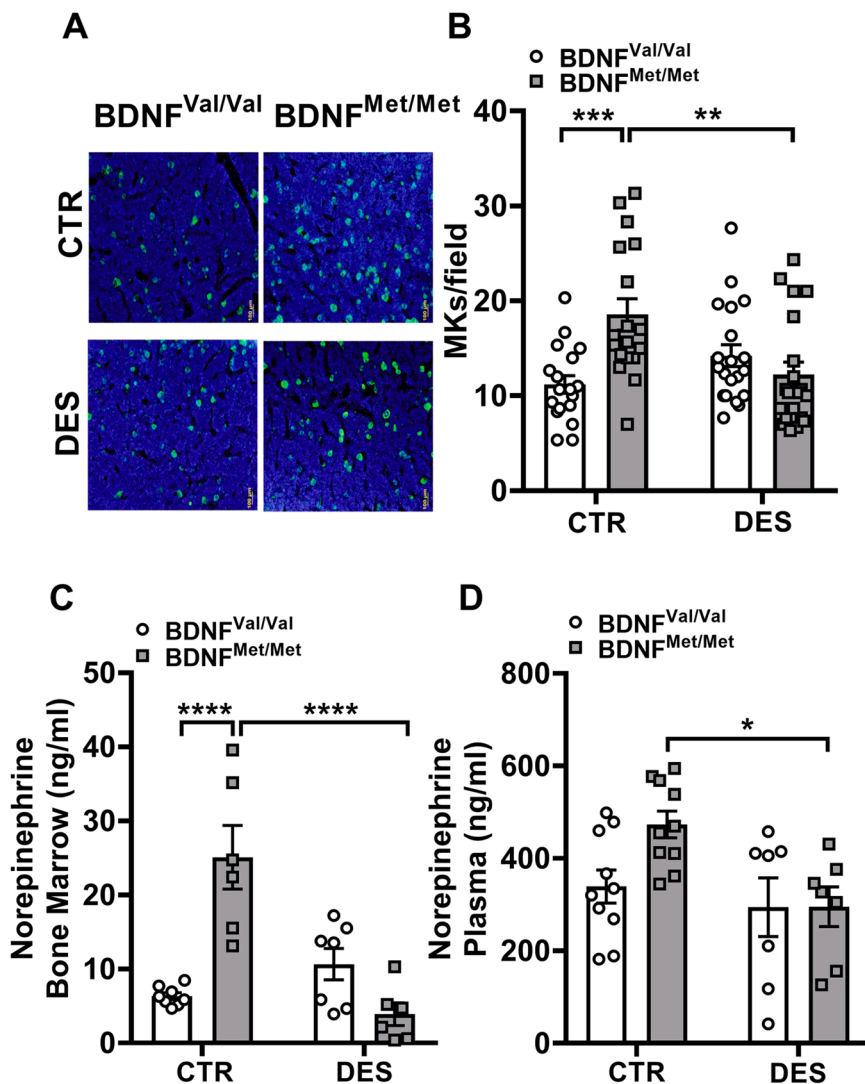
Fig. 3. Desipramine treatment affects megakaryocyte number and norepinephrine levels in BDNF$^{Met/Met}$ mice compared to BDNF$^{Val/Val}$ mice. Desipramine (DES; 10 mg/Kg) or vehicle (CTR) were administered for 3 weeks to BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (A) Immunofluorescence of bone marrow megakariocytes (scale bar 100 μm, 20 x magnification) and (B) quantification of the immunofluorescence panel expressed as megakariocytes (MKs) per field (n = 6 mice/group, 3 images for mouse). Levels of norepinephrine detected by ELISA kit in (C) bone marrow and (D) plasma of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice (n = 6–10 mice/group). Data are shown as mean ± SEM. *p < 0.05, * *p < 0.01, * **p < 0.005 and * ** *p < 0.001.

induced by norepinephrine, HeLa cells, transfected with BDNF^{Val} (HeLa^{Val}) or BDNF^{Met} (HeLa^{Met}) plasmids, were treated with various adrenergic receptors (ADR) antagonists before norepinephrine exposure.

Consistently with experiments on mouse aortic tissue, HeLa^{Met} cells showed significantly higher basal procoagulant activity compared to HeLa^{Val} cells and this difference was maintained after norepinephrine stimulation. Desipramine completely prevented procoagulant activity in both groups of norepinephrine -treated cells. Only the alpha$_2$-adrenergic receptor (α$_2$-ADR) antagonist rauwolscine (RAW) counteracted the effect of norepinephrine, whereas the α$_1$-ADR antagonist prazosin had no effect (Fig. 4B). In addition, α$_{2A}$-ADR protein (Fig. 4 C) and mRNA levels (Supplementary Fig. S5A; CTR: 1.002 ± 0.0282; HeLa^{Val}: 1.138 ± 0.0754; HeLa^{Met}: 2.638 ± 0.248; p < 0.01) were significantly more expressed in HeLa^{Met} than in HeLa^{Val} cells as well as in aortae of BDNF$^{Met/Met}$ mice than in BDNF$^{Val/Val}$ mice (Fig. 4D).

Then we assessed whether modified BDNF precursor protein (pro-BDNF^{Met}) mimics the effects induced by the Met mutation *per se*. The addition of cleavable pro-BDNF^{Met} peptide to HeLa cells induced a 50% raise of α$_{2A}$-ADR protein level (Fig. 4E-F) and of its mRNA expression (CTR: 1.010 ± 0.075; pro^{Val}: 0.936 ± 0.085; pro^{Met}: 1.974 ± 0.097; p < 0.01; Supplementary Fig. S5B). In addition, the cleavable pro-BDNF^{Met} peptide was able to increase the procoagulant activity (Fig. 4 G). Finally, both desipramine and rauwolscine treatment prevented procoagulant activity induced by norepinephrine in wild type

HeLa cells exposed to pro-BDNF^{Val} or pro-BDNF^{Met}, whereas prazosin failed to do it (Fig. 4 G).

Overall, these data show that the Met allele affects procoagulant activity through the modulation of norepinephrine /α-ADR receptor pathway.

3.3. Rauwolscine treatment prevents the prothrombotic phenotype of BDNF$^{Met/Met}$ mice

Consistent with both *in vivo* and/or *in vitro* results obtained with desipramine treatment, one-week of *in vivo* rauwolscine administration, decreased fibrinogen in plasma and procoagulant activity in the aorta and carotid tissue (Fig. 5A-B, and Supplementary Fig. S6), without affecting the mRNA expression levels of both Tf and Sirt1 in the aorta of BDNF$^{Met/Met}$ mice (Fig. 5C-D). Rauwolscine diminished leukocyte and platelet number, and the percentage of ADP-induced aggregates (Fig. 5E-G), and it prevented arterial thrombosis in BDNF$^{Met/Met}$ mice (Fig. 5H-I), but did not affect BDNF protein expression in the hippocampus (Fig. 5J). Similarly, to what observed after desipramine treatment, thrombus formation in BDNF$^{Val/Val}$ mice was not modified by rauwolscine treatment (Supplementary Fig. S7).

Interestingly, rauwolscine significantly affected megakaryopoiesis *in vivo* reducing the number of bone marrow megakariocytes (Fig. 6 A), and *in vitro* study showed that differentiated megakariocytes from BDNF$^{Met/Met}$ mouse fetal livers displayed a significantly higher
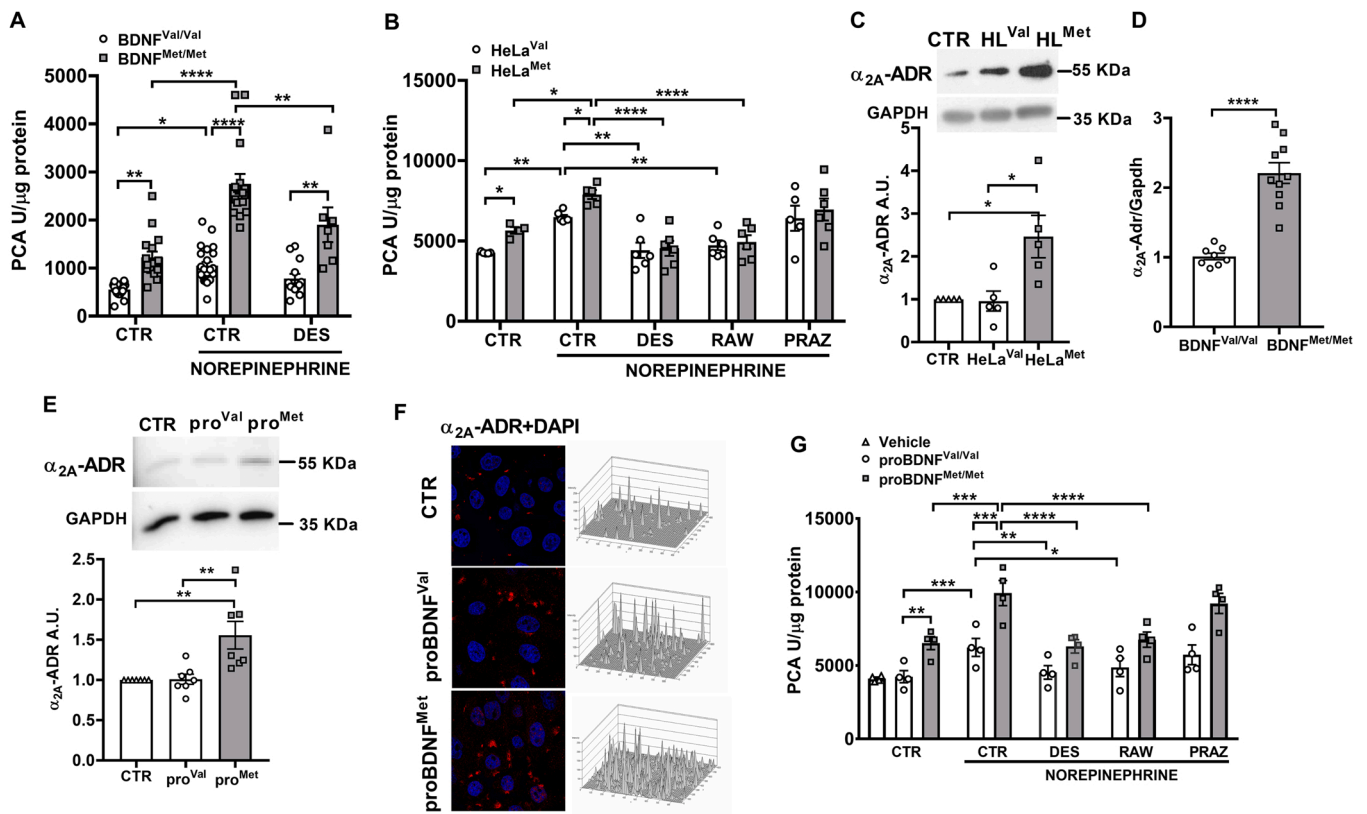
Fig. 4. Impact of Met allele on norepinephrine/α-adrenergic receptor pathway and procoagulant activity. Procoagulant activity (PCA) of (A) aorta tissue isolated from BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice (n = 8–10 aorta/group), and of (B) HeLa cells transfected with Val (HeLa^{Val}) or Met (HeLa^{Met}) sequence pre-incubated for 1 h with vehicle (CTR), desipramine (DES; 1 μM), rauwolscine (RAW; 10 μM), or prazosin (PRAZ; 10 μM) and then stimulated with 1 μM norepinephrine for 4 h (n = 5–6 independent experiments, each sample is in triplicate). (C) Representative western blot images and relative densitometric bar graphs of α$_{2A}$-ADR in HeLa cells transfected with Val (HeLa^{Val}) or Met (HeLa^{Met}) sequence. GAPDH was used as protein loading control (n = 5 independent experiments). (D) α$_{2A}$-Adr mRNA levels in aortae of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice, Gapdh was used as mRNA loading control. (7–10 mice/group). (E-G) HeLa cells were treated with pro-BDNF^{Val} or pro-BDNF^{Met} synthetic peptides for 6 h and then incubated with vehicle (CTR), desipramine (DES; 1 μM), rauwolscine (RAW; 10 μM), or prazosin (PRAZ; 10 μM) before stimulation with 1 μM norepinephrine for 4 h as indicated. Representative image and quantization of (E) western blotting of α$_{2A}$-ADR, GAPDH was used as protein loading control, and of (**F**) immunofluorescence of cells stained with α$_{2A}$-ADR antibody (red) (Nuclei were counterstained using Hoechst 33258 (blue); scale bar 20 μm, magnification 63X). (**G**) Procoagulant activity (PCA) evaluated by recalcification clotting time assay. (n = 4/6 independent experiments). Data are shown as mean ± SEM. *p < 0.05; * *p < 0.01, * **p < 0.005 and * ** *p < 0.001.

expression of α$_{2A}$-ADR than those obtained from BDNF$^{Val/Val}$ mice (Fig. 6B-C).

According to *in vitro* data, these findings show that inhibition of norepinephrine /α-ADR receptor pathway reduces procoagulant activity and prevents arterial thrombosis in BDNF$^{Met/Met}$ mice, affecting furthermore platelet function.

3.4. Characterization of homozygous BDNFVal66Met patients with coronary artery disease

In a retrospective analysis of 971 patients with CAD, we recently showed that the BDNF^{Met} homozygosis (AA) was associated with an increased risk to develop myocardial infarction.[12] Here, we explored, in a subset from the same patient cohort, the association between Met allele and platelet generation and reactivity in relation to α$_2$-ADR receptor expression.

Thus, we verified whether the above-described effects of BDNF Val66Met polymorphism on coagulation, platelet activation, and thrombopoiesis through the activation of norepinephrine /α-ADR pathway could be translated to human.

BDNF$^{Met/Met}$ patients have a greater number of circulating platelets and leukocytes, a higher expression of P-selectin and activation of GPIIb/IIIa complex in platelets, as well as an increased percentage of platelet/leukocyte aggregates than GG patients (BDNF$^{Val/Val}$) (Fig. 7A-

E). In addition, α$_{2A}$-ADR expression was higher in platelets isolated from AA than from GG patients (Fig. 7F). The percentage of megakariocytes differentiated from CD45$^+$ cells was comparable between the two groups of patients (Fig. 7G), but the percentage of proplatelets released from megakariocyte from AA patients was almost two-fold higher than in GG-bearing subjects (Fig. 7H). A positive correlation between the *in vitro* proplatelet formation rate and peripheral blood platelet count was found (r = 0.562; p = 0.0485; Supplementary Fig. S8), further confirming the key role of BDNF Val66Met polymorphism on megakaryocyte-platelet behavior.

To dissect the impact of BDNF^{Met} allele on megakario-thrombopoiesis, we differentiated human megakariocytes from peripheral blood CD34$^+$ and CD45$^+$ cells of healthy subjects in the presence of pro-BDNF^{Val} or pro-BDNF^{Met} peptides.

The expression of α$_{2A}$-ADR, according to what shown in bone marrow megakaryocytes of BDNF$^{Met/Met}$ mice (Fig. 6B-C), was significantly higher in CD34$^+$-derived human megakaryocytes exposed to pro-BDNF^{Met} (Fig. 7I). However, pro-BDNF^{Met} *per se* was not able to increase the rate of platelet released by CD34$^+$-derived megakaryocytes, while an enhanced platelet release was detected when the differentiation was induced in the presence of pro-BDNF^{Met} plus norepinephrine (Fig. 7J-K). Of note, the number of platelets released from CD45$^+$-derived megakariocytes differentiated in the presence of the pro-BDNF^{Met} peptide alone was greater compared to the treatment with pro-BDNF^{Val} (Fig. 7L).
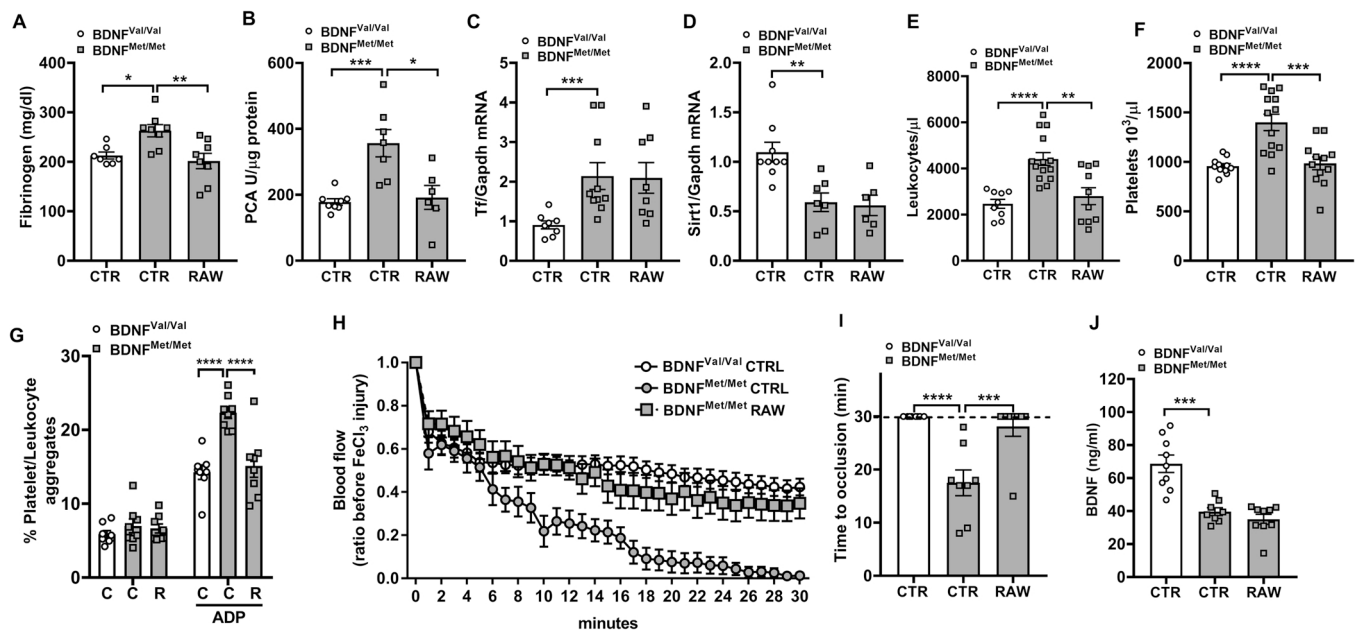
Fig. 5. Effect of rauwolscine treatment on prothrombotic phenotype in BDNF$^{Met/Met}$ compared to BDNF$^{Val/Val}$ mice. Rauwolscine (RAW; 200 μg/Kg) or vehicle (CTR) have been administered for 1 week to BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (A and B) Detection of (A) fibrinogen by Clauss assay in plasma (n = 6–10 mice/group) and (B) procoagulant activity (PCA) in aorta tissues (n = 6 mice/group). Expression of (C) Tf and (D) Sirt1 mRNA in aorta tissue, Gapdh was used as mRNA loading control. (n = 7–9/group). Number of circulating (E) leukocytes and (F) platelets in whole blood (n = 10–14 mice/group). (G) Percentage of platelet/leukocyte aggregates in the whole blood in resting condition and after stimulation with ADP (C: vehicle and R: rauwolscine) (n = 7/8 mice/group). Kinetic of blood flow in mouse carotid arteries expressed relative to the value before the injury induced by 5% FeCl$_3$ (panel H) and time to occlusion during 30 min of observation (panel I) (n = 8 mice/group). (J) Hippocampal BDNF levels (n = 8–9 mice/group). Data are shown as mean ± SEM. *p < 0.05 and * *p < 0.01, * **p < 0.005 and * *** *p < 0.001.
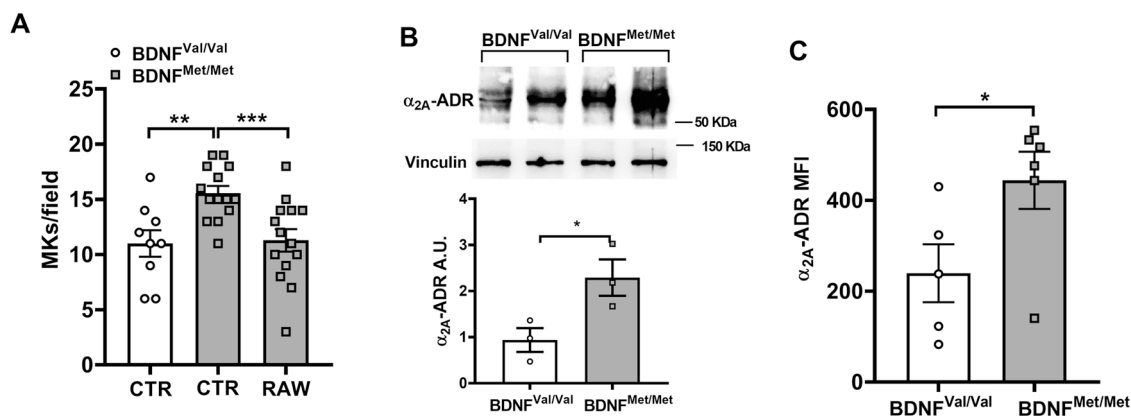

Fig. 6. Effect of rauwolscine treatment on megakariopoiesis in BDNF$^{Met/Met}$ compared to BDNF$^{Val/Val}$ mice. Rauwolscine (RAW; 200 μg/Kg) or vehicle (CTR) have been administered for 1 week to BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (**A**) Number of bone marrow megakariocytes (MKs) per high power field (n = 3–5 mice/group, 3 images for mouse). (**B** and **C**) Expression of (**B**) α$_{2A}$-adrenergic receptor (α$_{2A}$-ADR) in megakariocytes differentiated from hematopoietic progenitors of fetal livers of BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice. (**B**) Representative western blot images and relative densitometric bar graphs of α$_{2A}$-ADR. Vinculin was used as protein loading control. (n = 3 independent preparation/group), and (**C**) Graphical rappresentation of mean fluorescence intensity (MFI) of α$_{2A}$-ADR (n = 5–6 independent preparation/group). Data are shown as mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.005.

Consistently, rauwolscine treatment prevented platelet release sustained by pro-BDNF^{Met} (Fig. 7L).

Overall, these results showed that pro-BDNFMet peptide increases platelet production synergistic with norepinephrine through the up-regulation of α$_{2A}$-ADR expression.

4. Discussion

In the present study we report that homozygous knock-in BDNF$^{Met/Met}$ mouse well recapitulates the human phenotype, sharing not only depressive and anxious phenotypes, but also the high number and the

hyper-reactivity of circulating platelets, as well as the greater expression of α$_{2A}$-ADR. We can hypothesize that the pathway here identified might help to reduce the risk of depression-associated thrombosis and cardiovascular events. In particular, we show that BDNF$^{Met/Met}$ patients display an abnormal thrombopoietic profile, which may predispose to arterial thrombosis, and that BDNFVal66Met polymorphism is associated with enhanced activation of the norepinephrine/α$_{2A}$-ADR pathway, leading to increased thrombopoiesis and procoagulant activity. Finally, we provide evidence that desipramine administration reverts the depressive/anxious-like behavior and concomitantly the prothrombotic phenotype in BDNF$^{Met/Met}$ mice. Altogether, these data suggest a new
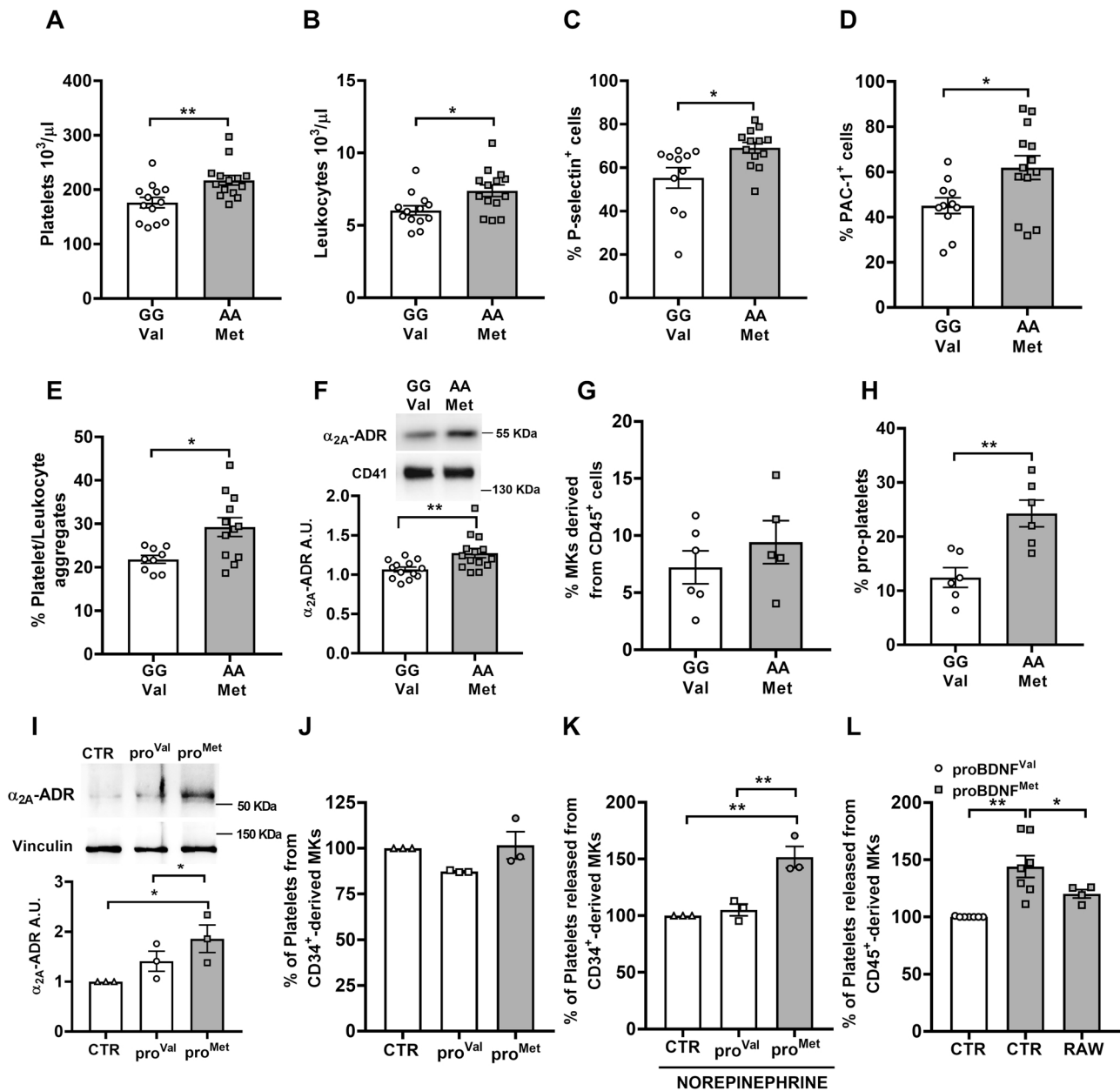
Fig. 7. Characterization of homozygous BDNFVal66Met patients with CAD, and effect of BDNFVal66Met polymorphism on thrombopoiesis. Number of (A) platelets and (B) leukocytes, (C) percentage of P-selectin^{+} (CD62P^{+}), (D) PAC-1^{+} platelets, and (E) platelet/leukocyte aggregates after ADP stimulation in whole blood in BDNF[Val/Val] (GG) and BDNF[Met/Met] (AA) CAD patients. (F) Representative western blot images and relative densitometric bar graphs of α_{2A}-ADR in human platelets. CD41 was used as protein loading control. (n = 13–14 patient/group). (G) Percentage of megakaryocyte (MK) differentiated from peripheral blood CD45^{+} cells, and (H) platelet formation from megakariocytes derived from CD45^{+} cells isolated from CAD patients (n = 6 patients/group). (I) Representative western blot images and relative densitometric bar graphs of α_{2A}-ADR of megakariocytes differentiated from CD34^{+} cells isolated from human cord-blood. Vinculin was used as protein loading control. (n = 3 undependent experiments). (J and K) Released platelets were quantified by flow cytometry using anti-CD41 antibody and counting beads in megakariocytes (MKs) differentiated from CD34^{+} cells isolated from cord blood, with or without pro-BDNF[Val] or pro-BDNF[Met] synthetic peptides (J), and concomitantly stimulated with norepinephrine as indicated (K) (n = 3 independent experiments). (L) Platelet generation from megakariocytes (MKs) differentiated from whole blood CD45^{+} cells with or without pro-BDNF[Val] or pro-BDNF[Met] synthetic peptides in the presence or absence of rauwolscine (RAW; 10 μM) as indicated (n = 3 independent experiments). Data are shown as mean ± SEM. *p < 0.05 and **p < 0.01.

mechanism explaining the platelet hyper-reactivity and the predisposition to arterial thrombosis observed in BDNF[Met/Met] subjects.

The BDNF[Met] phenotypic profile well summarizes the dysregulation in the norepinephrine/α_{2A}-ADR pathway reported in depressive disorders and subjects under stressful conditions with associated cardiovascular vulnerability. Dysregulation of the sympathetic nervous system is one of the pathways underlying the relationship among depression, anxiety, and CAD. Elevated levels of norepinephrine in plasma and urine have been related to depressive symptoms [32], measured in CAD

patients with depression [33], and associated with cardiovascular events and poor outcomes in cardiovascular patients [5,34]. Catecholamines potentiate the effect of other agonists enhancing platelet response and generation. In particular, norepinephrine administration promotes thrombopoiesis [35] and induces blood coagulation and platelet activation [34,36].

In addition to changes in catecholamine availability, a dysfunction of α_2-ADR density and activity has been shown in depressive disorders. An up-regulation in both absolute expression and super-sensitivity of α_2-

ADRs has been found in the brain tissue of depressed patients who committed suicide, as well as in platelets of subjects with depression [5, 37]. Interestingly, chronic antidepressant therapies downregulate α_2-ADR density and high-affinity conformational state in both brain and platelets [37,38]. Desipramine has a higher selectivity for inhibition of norepinephrine reuptake than serotonin and modulates expression levels and activity of α_{2A}-ADR, which is the ADR subtype upregulated in depressive conditions [39,40].

Here, we show that the BDNF$^{Met/Met}$ homozygosity increases the norepinephrine levels in the bone marrow and the α_{2A}-ADR expression in platelets, megakaryocytes, aorta tissue and HeLa cells and that this effect is related to pro-BDNF^{Met} function. Modifications in the α_{2A}-ADR play a key role in thrombosis. The higher abundance of α_2-ADR in bigger platelets than in smaller ones may explain the increased α_2-ADR binding capacity detected under pathological conditions, including myocardial infarction, where platelet turnover is increased and larger platelets enter into the circulation [41]. α_2-ADR activation sustains platelet aggregation and increases residual platelet reactivity in CAD patients on dual antiplatelet therapy [42]. α_{2A}-ADR polymorphism has been associated with enhanced platelet reactivity, whereas the deletion of this receptor was related to low platelet activation and reduced thrombus stability [43–45]. Interestingly, inhibition of α_2-ADRs prevented platelet aggregation and platelet formation induced by norepinephrine *in vitro* [35], whereas antagonists of α_1-ADRs determined an increase of circulating platelets [46], suggesting a critical role for the α_2-ADRs blockade in the inhibition of thrombopoiesis.

In line with this evidence, the BDNFVal66Met polymorphism, as well as pro-BDNF^{Met} *per se,* failed to modulate *in vitro* platelet generation from megakariocytes. The concomitant presence of norepinephrine, exogenously added to the culture medium of CD34$^+$ cells or stored in platelets [41] isolated with CD45$^+$ cells, was necessary to boost this process, emphasizing the importance of norepinephrine/α_2-ADR pathway activation in this regulation. Consistently, BDNF$^{Met/Met}$ mice have an elevated number of circulating and reticulated platelets, associated with a greater number of megakariocytes and higher bone marrow levels of norepinephrine, compared to BDNF$^{Val/Val}$ mice.

The association between the elevated number of leukocytes and of platelets and the risk of CAD reported in several large prospective studies [47,48], especially in CAD and depression co-morbidity [6], underlies the clinical importance of increased blood counts detected in BDNF$^{Met/Met}$ subjects. BDNF^{Met} carriers are less resilient to stressful events, depression and anxiety [11,49,50]. Interestingly, the higher levels of norepinephrine measured in bone marrow of BDNF$^{Met/Met}$ mouse, potentlyally related to its anxious and depressive phenotype, may explain the higher number of leukocytes and platelets here measured, as well as its predisposition to cardiovascular complications [12,35,51].

In addition, the abundance of immature reticulated platelets might contribute in predisposing BDNF$^{Met/Met}$ subjects to cardiovascular complications. Indeed, reticulated platelets are a highly active pro-thrombotic platelet subpopulation, and have a greater propensity to participate in the early stages of thrombus formation. Reticulated platelets are newly-released platelets, they are larger and contain more dense granule than older platelets. They express abundant GPIIbIIIa and P-selectin and aggregate more robustly upon stimulation with several platelet agonists compared to non-reticulated ones [52,53]. All these findings support the hypothesis that alterations in platelet phenotype may be an important link between depression and arterial thrombosis [5,6].

The arterial thrombosis model used here allow to study both the role of the platelets in thrombogenesis and the contribution of TF expression in leukocytes, extracellular vesicles or derived from the arterial wall. Indeed, after FeCl$_3$ application, TF-positive vesicles and red blood cells adhere to the intact endothelial surface, and contribute to the thrombus growth promoting platelet recruitment and supporting thrombin generation [54,55].

Interestingly, we show that pro-BDNF^{Met} treatment is sufficient *per se* to increase procoagulant activity which is enhanced by the norepinephrine. Both desipramine and rauwolscine normalized procoagunat activity induced by norepinephrine without modifying TF expression as well as its modulator, Sirt1. BDNFVal66Met polymorphism may then increase activity enhancing the TF expression through the up-regulation of SIRT1, as previuolsy shown [12,56], or with a post-trascriptional mechanism through the α_2-ADR over-expression.

We can also hypothesize that desipramine may modulate procoagulant activity affecting TF decryption in BDNF$^{Met/Met}$ mice. Indeed, desipramine prevented the disruption of membrane rafts in a mouse model of endotoxemia by inducing rapid degradation of mature acid sphingomyelinase [57], thus blocking both procoagulant TF and thrombin generation [58]. However, no effect was observed in untreated control mice [58]. Since treatment with desipramine *in vivo* was unable to prevent the arterial thrombosis in BDNF$^{Val/Val}$ mice, we assume that FeCl$_3$ damage is not sufficient itself to trigger the TF decryption, and that inflammation may be an important modulator of this pathway. Of note, BDNF$^{Met/Met}$ mice not only are less resilient and more prone to develop deressive-and anxious-like phenotypes, but they also have an enahnched proinflammatory phenotype [12,14,59] that might explain the effect of desipramine on procoagulant activity observed here.

In addition, desipramine treatment, through a ceramide dependent mechanism, might promote the internalization and the down-regulation of α_2-ADRs [40,60]. This double effect of desipramine, reduction in procoagulant activity and α_2-ADR expression, might also explain the marked improvement in the prothrombotic phenotype herein observed *in vivo*. On the other hand, changes in susceptibility to arterial thrombosis, procoagulant activity, platelet reactivity, and thrombo- and megakaryopoiesis induced by short term rauwolscine treatment in BDNF$^{Met/Met}$ mice, associated to unchanged BDNF brain levels, strongly suggest a relationship with a direct peripheral cellular inactivation of the α_2-ADR-pathway.

We are aware of the limitations of our study. The sample size of patients is very small for an adequate statistical analysis to appreciate difference in body mass index, and depression incidence. However, evidence supporting an influence of the BDNF$^{Met/Met}$ genotype on the co-morbid incidence of depression and CAD, as well as protective effects of norepinephrine/α_{2A}-ADR pathway inhibitor drugs against thrombosis is still lacking in humans. Our data need to be confirmed in a bigger cohort of patients carrying the BDNFVal66Met polymorphism and in a clinical study using α_2-ADR inhibitors, for instance, mirtazapine that is a relatively safe and effective medication for patients suffering from both depression and CAD [41].

5. Conclusion

Taking advantage of a mouse model that allows studying the pathophysiological relationship between brain and heart, we have shed light on some of the intricate mechanisms by which depression may predispose to thrombosis, showing an unprecedented role of BDNF$^{Met/Met}$ in the activation of α_2-ADR pathway in both mice and in patients with cardiovascular disease. The α_2-ADR pathway might represent a potential pharmacological target to concomitantly treat depression and/or reduce platelet hyperactivation and thrombosis in this specific subgroup of CAD patients.

Author contributions

SSB conceived the study; SSB, LS, AI designed all experiments; LS, PA, AI, AM, PMS, MZ performed experiment, and LS, AI, AM, AB, SSB analyzed the data. JPW and GIC recruited patients, AB and FV performed statistical analysis. LS, AI, and SSB wrote the first version of the manuscript. All authors discussed the results and edited the manuscript.

CRediT authorship contribution statement

SSB conceived the study; SSB, LS, AI designed all experiments; LS, PA, AI, AM, PMS, MZ performed experiment, and LS, AI, AM, AB, SSB analyzed the data. JPW and GIC recruited patients, Ab and FV performed statistical analysis. LS, AI, and SSB wrote the first version of the manuscript. All authors discussed the results and edited the manuscript.

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Conflict of interest statement

The authors declare no competing financial interests.

Data Availability

All the data that support the conclusions are presented in the main manuscript and supplementary data. They also can be requested by contacting the corresponding author.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112557.

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