Deficiency of the long pentraxin PTX3 promotes vascular inflammation and atherosclerosis.

Giuseppe Danilo Norata PhD 1,2, Patrizia Marchesi MSc 1, Vivek Krishna Pulakazhi Venu MSc 1, Fabio Pasqualini BS 3, Achille Anselmo PhD 3, Federica Moalli MSc 3, Irene Pizzitola MSc 1, Cecilia Garlanda VetScD 3, Alberto Mantovani MD, PhD 3,4, Alberico Luigi Catapano PhD 1,2.

1Department of Pharmacological Sciences, University of Milan, Italy.
2Center for the Study of Atherosclerosis, Società Italiana Studio Aterosclerosi, Ospedale Bassini, Cinisello Balsamo, Italy
3Istituto Clinico Humanitas, IRCCS, Rozzano, Milan, Italy
4Department of Translational Medicine, University of Milan, Italy.

SUPPLEMENTAL MATERIAL

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Additional material and methods.

Animals and treatment

Double knock out mice (PTX3 -/-; Apo E -/- mice) were generated by crossbreeding mice deficient in PTX3 ¹ with mice lacking apolipoprotein E (purchased from the Jackson Laboratories, Bar Harbour, Maine, USA). Both animals were on C57Bl/6J background; after two generations, three groups were selected: PTX3 -/-; Apo E -/- mice, PTX3 +/-; Apo E -/- mice and PTX3 +/-; Apo E -/- mice used for the control group. Each genotype was evaluated following PCR amplification for specific DNA sequences, for PTX3 as previously described ¹ and for Apo E as indicated here:


At 9 weeks of age, animals were fed ad libitum with western type diet (21% fat, 0.15% cholesterol and 19.5% casein, Piccioni Italy) for 16 weeks. All experimental procedures were in accordance with the institutional guidelines for animal research. The mice were sacrificed with an injection of Avertin 2.5% (Aldrich Chemical Co. USA). The heart and the arterial tree were perfused with saline solution under physiological pressure. Then the aortas and the hearts were isolated, placed in a storing solution (RNA laterICEtm, Ambion, Germany) at –20°C or paraffin embedded ². For RNA isolation the samples were homogenized in a dismembrator (B. Braun, Melsungen AG, Germany) then processed as described ³.

Plasma lipid analysis

Blood samples were collected in EDTA tubes immediately before death by retro-orbital bleeding and plasma was separated by low-speed centrifugation at 4°C. The measurement of plasma lipids
was performed by standard enzymatic techniques (ABX for Cobas Mira Plus, Montpellier, France); HDL-C was determined after precipitation of apoB-containing lipoproteins.

**Quantification of atherosclerosis**

Cross sequential sections 5 µm thick were prepared from the aortic root up to 150µm after the valve leaflets were no longer detectable. For the aortic sinus and the ascending aorta the slides were stained with hematoxilin-eosin, for each tract of the aorta 15 sections were analyzed. Images of the aortas were captured with Axiovert 200 microscope and the atherosclerotic lesion area was quantified by computer image analysis, using Axiovision LE rel 4.4 software by two blinded observer. Data are expressed as µm² and are calculated as the differences between the area of the media + intima subtracted of the media area. The quantification of the collagen content was performed following picro-sirius red staining, images of the aortas were captured with Axiovert 200 microscope (ZEISS, Milan, Italy) under regular and polarized light, then fibrillar collagen, detected by yellow birefringence, was quantified using Image J analysis software, capable of colours segmentation and automation by programmable macros, by two blinded observer. Data are expressed as the percentage of the total atherosclerotic lesion area covered by fibrillar collagen ± standard error.

**cDNA microrray analysis**

A comprehensive gene expression analysis was performed in the tract of the aorta between the aortic sinus and the ascending aorta where the morphometric analysis was performed. For this purpose 10µm section were prepared. Non aortic tissue was removed from each section under the
microscope for a total of 150µm for each mouse. Aortas were removed from the glass and the samples from mice of the same group were pooled. RNA was extracted using the kit RNAeasy Formalin-fixed, paraffin-embedded (FFPE) tissue sections from Qiagen (Milan, Italy). The quality of the RNA was tested with standard procedures. Five hundred µg of RNA were then used for RNA amplification using the SuperScript RNA Amplification System (Invitrogen, Italy) according to the manufacturer instructions. Two µg of amplified RNA were then processed for microarray experiments while the remaining was used for validation with Q-PCR as described below. For microarray experiments Dual Chip Mouse Inflammation microarray was used (Eppendorf, Italy). Biotin 11-dATP and biotin 11-cCTP cDNA was synthesized according to the protocol from the manufacturer and hybridized to the microarrays. For these experiments the gene expression pattern in male and female were investigated separately. The arrays were developed with the Silverquant Detection Kit (Eppendorf, Italy), the images were acquired with the Silverquant Detection Software and analyzed with the Silverquant Analysis Software (Eppendorf, Italy). Data are expressed as fold of induction of PTX3−/−/ApoE−/−; or PTX3+/−/ApoE−/− mice vs PTX3+/+/ApoE−/− mice. For each group, two replicates, each resulting from pooling the mRNA of four animals were analyzed. (The cDNA microarray experiments were performed according to the MIAME guidelines, the raw data for each hybridization and the final processed files are accessible at the following address: http://www.sisalombardia.it/dati3/dati3.htm).

Real time quantitative polymerase chain reaction

Total RNA was reverse transcribed as described 5, 6. Three µL of cDNA were amplified by real-time quantitative PCR with 1X Syber green universal PCR mastermix (BioRad, Italy). The specificity of the Syber green fluorescence was tested as described 5. The primers used have been
described previously \(^4\). Each sample was analyzed in duplicate using the IQ\(^\text{TM}\)-Cycler (BioRad). The PCR amplification was related to a standard curve ranging from 10-11 mol/L to 10-14 mol/L and data were normalized for the housekeeping gene ribosomal protein L13a (RLP13a) (NM_009438).

**Immunohistochemistry**

Eight-mm consecutive frozen sections were cut, mounted on Superfrost slides (Bio-Optica, Milan, Italy) and fixed for 15 min in 4% PFA at room temperature. Sections were hydrated with PBS twice for 10 min, incubated for 5 minutes with PBS-0.03% H2O2-1%BSA and with PBS-1%BSA for 10 minutes to block endogenous peroxidase and unspecific sites, respectively. Immunohistochemistry (IHC) was performed using a Rabbit IgG affinity purified against human PTX3; after 2 hours of incubation at room temperature, the slides were washed in PBS pH 7(3x5 minutes each).

The reactions were revealed by means of anti rabbit HRP-Envision System Labelled Polymer (Dako Cytomation). The chromogen was 3,3' diaminobenzidine free base (DAB). Double immunofluorescence was performed with rat anti mouse CD68 (Hycult Biotechnology, for macrophages) and anti human PTX3 \(^7\).

PTX3 was developed in green (anti Rabbit 488 as secondary antibody), while CD68 in red (anti Rat 594 as secondary antibody). Nuclei were stained with DAPI (Invitrogen). The slides were mounted with FluorSave (Calbiochem) and analysed with fluorescence microscopy.

For macrophage, smooth muscle cell and T-lymphocyte detection, rat anti mouse F4/80 (antigen, AbD Serotec, Oxford, UK, dilution 1:100), rabbit polyclonal to CD3 and rabbit polyclonal to smooth muscle actin, (Abcam, Cambridge UK, dilution 1:50 and 1:100 respectively), or non-
immune IgG as a control were used (4°C overnight) followed by incubation with a secondary biotinylated antibody (anti-rabbit IgG or anti-rat IgG, Vector Laboratories, Burlingame, CA, USA, diluted 1:200 in 1% normal goat serum and PBS) 1h at room temperature. Immunoreactivity was visualized using avidin-biotin-peroxidase method (Vectastain ABC kit from Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine substrate (Sigma, Italy) as the chromogen. Dehydrated sections were observed using Zeiss Axioskop microscope (Zeiss) and analysed with color-video image analysis system linked to the microscope, for the quantification of the immunoreactive area the Image J analysis software, capable of colours deconvolution and segmentation by programmable macros, was used.

**Statistics**

Data were analyzed using SPSS 16.0 for Windows (SPSS, Chicago, IL). The effects of PTX3 deficiency were analyzed in the all group and for female and male separately. Data in Figure 1 were analyzed using one-way ANOVA. Data in table 1, figure 2, 3, 5 and 6 were analyzed with two way ANOVA followed by Sheffe contrast. When histological data were compared between groups, parametric t-tests setting the significance level at p<0.05 were used and to compensate for a potential lack of normal distribution, nonparametric Wilcoxon tests were applied. Data in figure 7 were analysed following bivariate correlation test.

**References**


Figure I on line supplement:
PTX3 expression in aging ApoE -/- mice.
Serial sections of ascending aorta from ApoE -/- mice were immunostained with an anti PTX3 specific antibody followed by alexa555 conjugated secondary antibody (red signal), and counterstained with Hoestch 33258 (blue) the autofluorescence of the laminas is shown in green. Control slides were immunostained with non-immune IgG. A representative panel is shown. Scale bar: 20μm.

For immunofluorescence studies, mice aorta specimens (5μm thick) were incubated with rabbit-polyclonal anti-PTX3 (1:20) overnight at 4°C, followed by incubation with anti-rabbit IgG Alexa 555 or 633-conjugated or (1:100, Invitrogen, Molecular Probes) for 1h then propidium iodide or Hoescht 55 was added for 20’ to stain the nuclei. The coverslips were analyzed with confocal microscope as described 28.
Figure II on line supplement:
Aortic sinus and ascending aorta atherosclerosis in male PTX3 -/-;ApoE -/-, PTX3+/-;ApoE -/- and PTX3 +/+;ApoE -/- mice fed with the western type diet.
A representative panel of the aortic sinus of thoracic aorta (upper panels) and of abdominal aorta (lower panels) of PTX3 -/-;ApoE -/-, PTX3+/-;ApoE -/- and PTX3 +/+;ApoE -/- mice is shown. At two months of age, mice were fed a western type diet for 4 months than animals were sacrificed and aortic sinus analysed as described in the material and methods section.
Female thoracic aorta

PTX3 +/+ / APOE -/-
PTX3 +/- / APOE -/-
PTX3 -/- / APOE -/-

Female abdominal aorta

PTX3 +/+ / APOE -/-
PTX3 +/- / APOE -/-
PTX3 -/- / APOE -/-

Figure III on line supplement:
Thoracic and abdominal atherosclerosis in male PTX3 -/-;ApoE -/-, PTX3+/-;Apo E -/- and PTX3 +/-;ApoE/- mice fed with the western type diet.
A representative panel of the aortic sinus of thoracic aorta (upper panels) and of abdominal aorta (lower panels) of PTX3 -/-;ApoE -/-, PTX3+/-;Apo E -/- and PTX3 +/-;ApoE/- mice is shown. At two months of age, mice were fed a western type diet for 4 months than animals were sacrificed and aortic sinus analysed as described in the material and methods section.
Figure IV on line supplement:
Aortic sinus atherosclerosis in PTX3 -/-; ApoE -/-; or PTX3 +/-; ApoE-/- mice and PTX3 +/+; ApoE-/- mice fed chow diet.

The extent of the atherosclerotic lesion in PTX3 -/-;ApoE -/- mice, PTX3+/-;Apo E -/- mice and PTX3 +/+;ApoE/-/- mice for aortic sinus in female is shown for 6, 12 and 18 months old mice (n=6 for each group), and is expressed as mean ± S.D (* p<0,05 vs PTX3 +/+; ApoE -/-).
Female aortic sinus

PTX3 +/+  
PTX3 +/-  
PTX3 -/-

Male aortic sinus

PTX3 +/+  
PTX3 +/-  
PTX3 -/-

Figure V on line supplement:
Aortic sinus atherosclerosis in PTX3 -/-; PTX3 +/- and PTX3 +/-mice fed western type diet.
A representative panel of the aortic sinus of female (upper panels) and male (lower panels) PTX3 -/-, PTX3 +/- and PTX3 +/- mice is shown. At two months of age, mice were fed a western type diet for 4 months than animals were sacrificed and aortic sinus analysed as described in the material and methods section.
Supplemental Figure VI: Relative expression of inflammatory mediators and their receptors in peripheral blood mononuclear cells by quantitative real-time PCR.

Relative mRNA expression of CCL2, CCR2, CCR5, CX3CR1, IL6, MMP-9, is shown for PTX3 -/-; ApoE -/- mice, PTX3 +/-; Apo E -/- and for PTX3 +/+; ApoE -/- mice (male and female pooled, n=6 for each group). Results, normalised for the RLP13a expression, are shown as fold of induction vs. PTX3 +/+; ApoE -/- mice (data are expressed as mean ± S.D., * p<0.05 vs. PTX3 +/+; ApoE -/- mice).
Supplemental Figure VII:
Analysis of the fibrillar collagen content within the atherosclerotic lesion in female mice.
Panel A. Relative mRNA expression of collagen 1A1, 1A2 and 3A1 is shown for PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice. The results, normalised for the RLP13a expression, are shown as fold of induction vs. PTX3+/+/ApoE−/− (data are expressed as mean ± S.D.) (* p<0.05 vs. PTX3+/+/ApoE−/−). Panel B. Representative sections of aortic sinus stained with sirius red from female PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice are shown. The data are presented as the percentage of atherosclerotic lesion covered by Sirius Red positive fibrillar collagen, and are expressed as mean ± S.E, scale bar: 100μm. The percentage of the area positive for fibrillar collagen for aortic sinus is shown on the right part the panel (data are expressed as mean ± S.D).
Supplemental Figure VIII

Analysis of the fibrillar collagen content within the atherosclerotic lesion in male mice.

Panel A. Relative mRNA expression of collagen 1A1, 1A2 and 3A1 is shown for PTX3+/+/ApoE-/-, PTX3+/−/ApoE-/- and PTX3−/−/ApoE-/- mice. The results, normalised for the RLP13a expression, are shown as fold of induction vs. PTX3+/+/ApoE-/- (data are expressed as mean ± S.D.) (* p<0.05 vs. PTX3+/+/ApoE-/-). Panel B. Representative sections of aortic sinus stained with sirius red from female PTX3+/+/ApoE-/-, PTX3+/−/ApoE-/- and PTX3−/−/ApoE-/- mice are shown. The data are presented as the percentage of atherosclerotic lesion covered by Sirius Red positive fibrillar collagen, and are expressed as mean ± S.E, scale bar: 100μm. The percentage of the area positive for fibrillar collagen for aortic sinus is shown on the right part the panel (data are expressed as mean ± S.D.).