Overexpression of Gremlin-1 in patients with Loeys-Dietz syndrome: Implications on pathophysiology and early disease detection

Wellbrock, J; Sheikhzadeh, S; Oliveira-Ferrer, L; Stamm, H; Hillebrand, M; Keyser, B; Klokow, M; Vohwinkel, G; Bonk, V; Otto, B; Streichert, T; Balabanov, S; Hagel, C; Rybczynski, M; Bentzien, F; Bokemeyer, C; von Kodolitsch, Y; Fiedler, W

Abstract: BACKGROUND: The Loeys-Dietz syndrome (LDS) is an inherited connective tissue disorder caused by mutations in the transforming growth factor (TGF-) receptors TGFBR1 or TGFBR2. Most patients with LDS develop severe aortic aneurysms resulting in early need of surgical intervention. In order to gain further insight into the pathophysiology of the disorder, we investigated circulating outgrowth endothelial cells (OEC) from the peripheral blood of LDS patients from a cohort of 23 patients including 6 patients with novel TGF- receptor mutations. METHODS AND RESULTS: We performed gene expression profiling of OECs using microarray analysis followed by quantitative PCR for verification of gene expression. Compared to OECs of age- and sex-matched healthy controls, OECs isolated from three LDS patients displayed altered expression of several genes belonging to the TGF- pathway, especially those affecting bone morphogenic protein (BMP) signalling including BMP2, BMP4 and BMPR1A. Gene expression of BMP antagonist Gremlin-1 (GREM1) showed the most prominent up-regulation. This increase was confirmed at the protein level by immunoblotting of LDS-OECs. In immunohistochemistry, abundant Gremlin-1 protein expression could be verified in endothelial cells as well as smooth muscle cells within the arterial media. Furthermore, Gremlin-1 plasma levels of LDS patients were significantly elevated compared to healthy control subjects. CONCLUSIONS: These findings open new avenues in the understanding of the pathogenesis of Loeys-Dietz syndrome and the development of new diagnostic serological methods for early disease detection.

DOI: https://doi.org/10.1371/journal.pone.0104742

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-104783
Journal Article
Published Version

Originally published at:
Wellbrock, J; Sheikhzadeh, S; Oliveira-Ferrer, L; Stamm, H; Hillebrand, M; Keyser, B; Klokow, M; Vohwinkel, G; Bonk, V; Otto, B; Streichert, T; Balabanov, S; Hagel, C; Rybczynski, M; Bentzien, F; Bokemeyer, C; von Kodolitsch, Y; Fiedler, W (2014). Overexpression of Gremlin-1 in patients with Loeys-Dietz syndrome: Implications on pathophysiology and early disease detection. PLoS ONE, 9(8):e104742. DOI: https://doi.org/10.1371/journal.pone.0104742
Overexpression of Gremlin-1 in Patients with Loeys-Dietz Syndrome: Implications on Pathophysiology and Early Disease Detection

Jasmin Wellbrock1, Sara Sheikhzadeh2, Leticia Oliveira-Ferrer1, Hauke Stamm1, Mathias Hillebrand2, Britta Keyser3, Marianne Klokow1, Gabi Vohwinkel1, Veronika Bonk1, Benjamin Otto4, Thomas Streichert4, Stefan Balabanov1,5, Christian Hagel6, Meike Rybczynski5, Frank Bentzien7, Carsten Bokemeyer1, Yskert von Kodolitsch2, Walter Fiedler1

1 Hubertus Wald University Cancer Centre, Department of Oncology, Hematology and Bone Marrow Transplantation with section Pneumology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany, 2 Center of Cardiology and Cardiovascular Surgery, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany, 3 Institute of Human Genetics, Hannover Medical School, Hannover, Germany, 4 Department of Clinical Chemistry/Central Laboratories, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany, 5 Division of Hematology, University Hospital Zurich, Zurich, Switzerland, 6 Institute for Neuropathology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany, 7 Department of Transfusion Medicine, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany

Abstract

Backgrounds: The Loeys-Dietz syndrome (LDS) is an inherited connective tissue disorder caused by mutations in the transforming growth factor β (TGF-β) receptors TGFBR1 or TGFBR2. Most patients with LDS develop severe aortic aneurysms resulting in early need of surgical intervention. In order to gain further insight into the pathophysiology of the disorder, we investigated circulating outgrowth endothelial cells (OEC) from the peripheral blood of LDS patients from a cohort of 23 patients including 6 patients with novel TGF-β receptor mutations.

Methods and Results: We performed gene expression profiling of OECs using microarray analysis followed by quantitative PCR for verification of gene expression. Compared to OECs of age- and sex-matched healthy controls, OECs isolated from three LDS patients displayed altered expression of several genes belonging to the TGF-β pathway, especially those affecting bone morphogenic protein (BMP) signalling including BMP2, BMP4 and BMPRIA. Gene expression of BMP antagonist Gremlin-1 (GREM1) showed the most prominent up-regulation. This increase was confirmed at the protein level by immunoblotting of LDS-OECs. In immunohistochemistry, abundant Gremlin-1 protein expression could be verified in endothelial cells as well as smooth muscle cells within the arterial media. Furthermore, Gremlin-1 plasma levels of LDS patients were significantly elevated compared to healthy control subjects.

Conclusions: These findings open new avenues in the understanding of the pathogenesis of Loeys-Dietz syndrome and the development of new diagnostic serological methods for early disease detection.

Background

The Loeys-Dietz syndrome (LDS) is an inherited autosomal dominant connective tissue disorder described first in 2005 by Bart Loeys and Harry Dietz [1]. Most common characteristics of LDS patients are 1) craniofacial features such as hypertelorism, cleft palate with bifid uvula, 2) skeletal manifestations including joint laxity, scoliosis and arachnodactyly, 3) cutaneous findings such as translucent skin or easy bruising and 4) vascular manifestations affecting the aorta and other arterial branches resulting in early need of surgical intervention [1,2].

The Loeys-Dietz syndrome is caused by a mutation in the transforming growth factor β (TGF-β) type II receptor TGFBR2 or type I receptor TGFBR1. More than 50 different mutations in TGFBR2 or TGFBR1 have been described in LDS patients. The great majority of those mutations represent missense mutations which are located within the kinase domain of the receptor probably resulting in impaired receptor signalling [1–3]. Recently, mutations in the gene for TGFBR2 and the TGF-β pathway downstream mediator SMAD3 have also been associated with the pathogenesis of Loeys-Dietz syndrome [4–6].

The TGF-β superfamily consists of several isoforms of TGF-β, activin and bone morphogenic proteins (BMP). Signalling is mediated through two related transmembrane type I and type II serine/threonine kinase receptors, which form heteromeric complexes upon ligand binding and propagate the downstream
signal by phosphorylation of intracellular SMAD proteins which transduce the signal to the nucleus [7].

The vascular wall is a complex construct composed of different cell types including the inner layer of endothelial cells (EC), surrounded by smooth muscle cells (SMC) within the media and finally the adventitia composed of fibroblasts [8]. For function of the vascular wall, interdependency between endothelial cells and mural cells is required. Communication can take place via direct cellular or via paracrine interactions induced by secretion of molecules such as platelet-derived growth factor [9]. With regard to the endothelium’s crucial role for the maintenance of the integrity of the vascular wall, we decided to concentrate our studies on endothelial cells to elucidate the pathogenesis of Loeys-Dietz syndrome. A population of circulating endothelial cells, so called outgrowth endothelial cells (OEC), can be easily isolated from peripheral blood for this purpose [10]. Moreover, OECs isolated from patients with hereditary haemorrhagic telangiectasia (HHT) carrying mutations in TGF-β receptors ACVRL1 (ALK-1, activin receptor-like kinase 1) or ENG (endoglin) displayed abnormalities comparable to the vascular lesions observed in HHT patients [11].

Therefore, we isolated outgrowth endothelial cells from the peripheral blood of LDS patients and healthy donors and performed gene expression profiling in order to study aberrant gene regulation caused by mutated TGF-β receptors. The aim of our study was to identify candidate genes contributing to the disease pattern of Loeys-Dietz syndrome.

Methods
Generation of outgrowth endothelial cells
The investigation conforms with the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from individuals participating in the study after the study had been approved by the local ethical committee [PV3893, Ärztekammer Hamburg]. Mononuclear cells (MNC) were isolated from peripheral blood of LDS patients and healthy donors, plated in collagen-coated 12-well tissue culture plates and cultured in endothelial growth medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). In order to remove non-adherent cells and debris, cultures were rinsed daily with fresh medium for one week followed by medium replacement every other day. On day 30, cultures were screened for outgrowth of endothelial colonies. Endothelial character of OEC clones was confirmed in PCR analysis and flow cytometry based on expression of a panel of endothelial-specific markers including CD31, CD144 and vascular endothelial growth factor receptors and non-expression of haematological markers CD45 and CD14 (see methods and table S1 in file S1).

Mutation analysis and prediction of the functional impact of nucleotide or amino acid substitutions
DNA was extracted from EDTA-blood using standard procedures. The entire coding sequence of TGFBR1 (NM_004612.2) and TGFBR2 (NM_003242.5) was sequenced as well as the 20 bases of the flanking intronic sequences. The amplified PCR products were sequenced and analysed with the following bioinformatics tools for prediction of impact on protein function: Mutation Taster (http://www.mutationtaster.org/), PMut (http://mmb2.pcbi.ub.es:8080/PMut/), PolyPhen (http://genetics.bwh.harvard.edu/pph/), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/). Presumptive splice site changes caused by silent or intronic mutations were analysed with the Human Splicing Finder tool, [12] Berkeley Drosophila Genome Project “Splice Site Prediction” (http://www.fruitfly.org/seq_tools/splice.html) and NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/). The non-mutation carrying chromosomes of 400 Marfan and Loeys-Dietz syndrome patients were used as control chromosomes.

RNA isolation and microarray analysis
RNA was extracted using RNeasy Mini Kit (including RNase-free DNase Set, Qiagen, Hilden, Germany). For microarray analysis, quality and concentration of isolated RNA was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Loveland, CO). Procedures for cDNA synthesis, labelling and hybridization were carried out according to 3′ IVT Express Kit and Hybridization, Wash and Stain Kit (Affymetrix, Santa Clara, CA) using 100 ng total RNA. All experiments were performed using Human GeneChip U133 Plus 2.0 Array (Affymetrix). Microarrays were scanned with the GeneChip Scanner 3000 7G. The signals were processed with GeneChip Operating Software (version 1.4, Affymetrix). Signal quality control and data normalization via gcrma procedure was performed using the webserver www.arrayanalysis.org. Differentially expressed genes were determined by filtering out genes that were increased or decreased at least 1.74 fold (Signal Log Ratio ≥ 0.8) in each sample pair and exhibited a permutation p-value below 0.05. Gene expression data are available at GEO Accession No. GSE38961.

cDNA synthesis and Real-Time quantitative PCR
RNA was reverse transcribed using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Fairfield, CT) and Random Primers (Invitrogen). Primers were designed with Primer 5 software (Whitehead Institute for Biomedical Research, Boston, MA). Quantitative Real-Time PCR analysis was carried out on the capillary-based Light Cycler (Roche, Basel, Switzerland) using the FAST Start DNA Master Sybr Green Kit (Roche). Relative expression of cDNA of the target gene in comparison to a reference gene was calculated using a mathematical model proposed by Pfaffl [13]. Samples were analysed in duplicate and averaged. Calculated cDNA amounts of the target genes were normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data are represented as ratio of the target gene/GAPDH. Primers are shown in table S2 in file S1.

Immunohistochemistry
For immunohistochemical labelling, formalin-fixed paraffin-embedded aortic tissue sections of LDS patients or healthy donors were pre-treated in citrate buffer and incubated with antibodies against Gremlin-1 (bs-1475R, Bioss, Woburn, MA) in an automated stainer (Ventana Medical Systems, Tucson, AZ) according to a standard protocol (CC1st). For double labelling with Gremlin-1 and muscle actin (ENZ-30931, Enzo Life Sciences GmbH, Loerrach, Germany) or Gremlin-1 and CD34 (M7165, Dako, Hamburg, Germany), incubation with Gremlin-1 antibodies was followed by a short denaturing step and incubation with the secondary antibody. Bound antibodies were detected by the peroxidase method using diaminobenzidine as chromogen (760–500, Ultraview DAB, Ventana). For double labelling studies, bound Gremlin-1 antibodies were visualized with DAB as described above and expression of muscle actin or CD34 was demonstrated by alkaline phosphastase linked secondary antibodies using fast red as chromogen (760–501, Ultraview Universal Detection Kit, Ventana).
Immunoblotting

Protein extracts were prepared with RIPA lysis buffer solution (Sigma-Aldrich, St. Louis, MO) supplemented with 1× protease inhibitor cocktail (Roche) and 1 mM sodium orthovanadate. Protein lysates were boiled for 5 min in SDS-sample buffer before being applied into a 4–20% SDS-PAGE (Thermo Fisher Scientific, Rockford, IL). After electrotransfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and blocking in TBS-T buffer containing 5% non-fat milk for 1 h, blots were incubated with Gremlin-1 primary antibody (Santa Cruz, Santa Cruz, CA) overnight. The subsequent incubation with the peroxidase-conjugated secondary antibody was followed by detection using ECL Western blotting detection reagents (GE Healthcare) and the FusionSL 4 3500 WL detection system (Vilber Lourmat, Sud Torcy, France). Membranes were incubated with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) followed by incubation with an α-Tubulin antibody (Sigma-Aldrich) as reference protein. Quantification of protein amount was determined using Bio-1D software (Vilber Lourmat).

Collection of human plasma samples and anti-human Gremlin-1 ELISA analysis

For analysis of Gremlin-1 plasma levels, peripheral blood samples of LDS patients and healthy donors were collected and centrifuged for 10 min at 2,000 g in order to separate the plasma from the blood cells. Gremlin-1 protein levels were measured using the enzyme-linked immunosorbent assay (ELISA) Kit for Gremlin-1 (Uscn Life Science Inc., Missouri City, TX) following the instructor’s manual. Absorbance of color change was quantified with the Sunrise ELISA plate reader and Magellan software (Tecan, Maenndorf, Switzerland).

Statistical analysis

All statistical analyses were performed with SPSS 16 (SPSS Inc, Chicago, IL). OEC frequency was analysed using the Mann-Whitney-U test. Differences in Gremlin-1 plasma levels between LDS patients and healthy controls or between gender were accessed by Welch’s t-test. Correlation of Gremlin-1 plasma levels with age was analysed by Pearson’s correlation. P-value ≤0.05 was considered as statistically significant. For the microarray data, a permutation procedure was performed to obtain adjusted p-values. Differentially expressed genes were identified by filtering out genes with a permutation p-value below 0.05 and a minimum absolute signal-log-ratio of 0.8 in each of the three sample pairs.

Results

LDS patients and novel mutations in TGFBR2 and TGFBR1

By evaluation of clinical presentation and mutation analysis of TGFBR1 and TGFBR2, 23 patients were diagnosed with Loeys-Dietz syndrome at the University Medical Centre Hamburg-Eppendorf. These 23 patients belong to thirteen families. Sixteen patients carry a heterozygous mutation in the TGFBR2 gene and seven patients harbour a TGFBR1 mutation. We identified four novel mutations in TGFBR2 and two in TGFBR1 whereas the other mutations have already been described before (table 1) [1–3]. Two of the novel TGFBR2 mutations are located within the kinase domain, both of them representing missense mutations (p.N384K and p.A414T). Analysis with bioinformatic tools Mutation Taster, PMut, Polyphen and PolyPhen2 predicted both mutations TGFBR2 p.N384K and p.A414T to be “disease causing” (data not shown). Furthermore, a silent mutation located in the region between the transmembrane and kinase domain of TGFBR2 was found (p.A232A). In addition, one patient carried a nucleotide substitution within intron 1 of the TGFBR2 gene (c.94+7G>C). In order to determine if these nucleotide changes might lead to alternative splicing sites, data analysis was performed for the silent mutation and the intronic variant within intron 1 using bioinformatic tools. Nucleotide change c.94+7G>C in TGFBR2 was predicted to cause an alternative splicing site in one of the analyses whereas no RNA splicing variants were predicted for TGFBR2 p.A232A (data not shown).

One of the novel TGFBR1 mutations occurred at amino acid position 241 in exon 4 leading to substitution of serine with proline (p.S241P) which was predicted to be “disease causing” using bioinformatic tools (data not shown). The second novel TGFBR1 mutation represented a duplication of 13 base pairs within intron 1 (c.97+25_+99dup15). The altered sequence was subjected to splice site analysis but no indications of alternative splice sites could be found (data not shown). All nucleotide changes detected within our cohort of LDS patients are summarized in table 1.

Generation of outgrowth endothelial cells from LDS patients

Peripheral blood from only nine patients with Loeys-Dietz syndrome was available for generation of outgrowth endothelial cells. Six patients had a mutation in the TGFBR type II receptor TGFBR2 and 3 patients carried a mutated type I receptor TGFBR1. Patient’s characteristics are shown in table 2. The mean number of mononuclear cells obtained from the patients was 5.6×10^5±1.6×10^5. After a cultivation period of 30 days, seven OEC clones could be isolated from four patients resulting in a frequency of 1.94 clones per 10^6 mononuclear cells. This compares to OECs isolated from healthy donors occurring with a frequency of 0.75 clones per 10^6 mononuclear cells (n = 500; p = 0.257 by Mann-Whitney-U test). To confirm their endothelial character, OEC clones were analysed by flow cytometry between passages 3–6. Expression of endothelial-specific markers such as CD31 or CD144 and non-expression of haematopoietic markers CD45 and CD14 was comparable in LDS-OECs and OECs isolated from healthy donors (table S1 in file S1).

Gene profiling of outgrowth endothelial cells from LDS patients

Due to insufficient in vitro proliferation capacity, not all OEC clones were available for microarray analysis. OEC clones from patients LDS1, LDS3 and LDS11 were used for gene profiling. LDS1 and LDS5 both carried the p.R357C mutation in the TGFBR2 gene whereas LDS11 harboured the TGFBR1 mutation p.R487Q. Detailed patient characteristics are provided in table 3. OEC clones from sex- and age-matched healthy donors served as reference. Furthermore, all OEC clones were harvested at passage 4–5 when nearly reaching confluence to reduce culture-induced variability. Expression ratios were considered as altered if the signal log ratio was above 0.8 (increased expression) and below −0.8 (decreased expression). In addition, only genes with consistently altered expression in all three analysed OEC clones were used for further analysis resulting in 163 genes with increased and 210 genes with decreased expression. In order to identify affected signalling cascades, the set of involved genes was analysed using the Ingenuity Pathways Analysis algorithm (IPA Build 308060M, Ingenuity Systems, www.ingenuity.com). Data analysis ranked the topics “Cardiovascular Disease” and “Haematological System and Cardiovascular System Development and Function” within the top five of affected biological functions and networks reflecting the actual type of genetic disorder (data not shown).
In all three LDS-OEC clones, gene expression of BMP antagonist Gremlin-1 (GREM1, also known as Drm) showed the most prominent up-regulation. Verification of expression using quantitative RT-PCR analysis and normalization to reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) revealed a 1,136-, 164- and 22,145-fold higher expression in LDS-OECs compared to healthy controls (table 4).

In addition to GREM1, several other genes belonging to the TGF-β superfamily displayed altered expression levels in LDS-OECs, especially those affecting bone morphogenic protein signalling. Expression of BMP type I receptor BMPR1A was increased in all three LDS-OEC clones, whereas BMPR1A ligands BMP2 and BMP4 showed decreased mRNA expression. Furthermore, expression of the latent TGF-β binding protein 1 (LTBP1) was increased in LDS-OECs (table 4). In support of our findings, gene expression data were analysed calculating a permutation-derived adjusted p-value. This analysis revealed that all genes we have chosen for our analysis, namely GREM1, BMP2, BMP4, BMPR1A and LTBP1, yielded a permutation p-value of 0.0 (table S3 in file S2).

Increased Gremlin-1 protein expression in LDS-OECs

Western blot analysis of LDS-OECs and sex- and age-matched control OECs confirmed a higher Gremlin-1 protein expression in LDS-OECs by 2.2-, 3.8- and 1.8-fold in LDS1, LDS5 and LDS11 respectively, compared to control OECs (figure 1).

Gremlin-1 expression in aortic tissue of LDS patients

Aortic tissue specimen of LDS patients which had experienced aortic root replacement (n = 3; 2×TGFBR1 p.R487Q and 1×TGFBR2 p.R537C, the latter was corresponding to LDS1) and healthy donors (n = 3) were analysed for protein expression of Gremlin-1 in immunohistochemistry. Endothelial cells throughout the vessel wall layers stained positively for Gremlin-1 including ECs of the intimal layer as well as ECs of the vessels within the media and adventitia (figure 2). Furthermore, medial and vessel-surrounding smooth muscle cells were positive for Gremlin-1 (figure 2). Immunohistochemically, no gross differences of staining intensity or pattern between LDS patients and healthy controls could be observed probably because expression levels are difficult to quantify by immunohistochemistry (figure 2).

### Table 1. Heterozygous TGFBR2 and TGFBR1 mutations identified in LDS patients.

| Gene     | Location | Nucleotide change | Amino acid change | Type                     | Affected individuals | Mutation referenced in |
|----------|----------|-------------------|-------------------|--------------------------|----------------------|-------------------------|
| TGFBR2   | Intron 1 | c.94+7G>C         | Not known         | Nucleotide substitution  | 1                    | Novel mutation          |
| TGFBR2   | Exon 4   | c.696C>T          | p.A232A           | Silent mutation          | 1                    | Novel mutation          |
| TGFBR2   | Exon 4   | c.1152T>G         | p.N384K           | Missense                 | 3                    | Novel mutation          |
| TGFBR2   | Exon 4   | c.1159G>A         | p.V387M           | Missense                 | 1                    | Stheneur et al., Matyas et al. [3,34] |
| TGFBR2   | Exon 4   | c.1167C>T         | p.N389N           | Silent mutation          | 4                    | Stheneur et al. [3]     |
| TGFBR2   | Exon 4   | c.1240G>A         | p.A414T           | Missense                 | 1                    | Novel mutation          |
| TGFBR2   | Exon 7   | c.1583G>A         | p.R528H           | Missense                 | 1                    | Loeys et al., Stheneur et al. [1,3] |
| TGFBR2   | Exon 7   | c.1609C>T         | p.R537C           | Missense                 | 4*                   | Loeys et al., Stheneur et al. [2,3] |
| TGFBR1   | Intronic | c.97+25, +39dup15 | Not known         | Duplication              | 1                    | Novel mutation          |
| TGFBR1   | Exon 4   | c.721T>C          | p.S241P           | Missense                 | 1                    | Novel mutation          |
| TGFBR1   | Exon 9   | c.1433A>G         | p.N478S           | Missense                 | 1                    | Loeys et al. [2]        |
| TGFBR1   | Exon 9   | c.1460G>A         | p.R487Q           | Missense                 | 4                    | Loeys et al., Matyas et al. [2,34] |

*representing two unrelated families.

doi:10.1371/journal.pone.0104742.t001

### Table 2. LDS patients analysed for OEC generation.

| Patient | Sex | Age | Mutation | LDS-OEC* |
|---------|-----|-----|----------|----------|
| LDS1/LDS9 | Female | 54  | TGFBR2 p.R537C | 2        |
| LDS2    | Male   | 24  | TGFBR2 p.R537C | 0        |
| LDS3    | Male   | 50  | TGFBR2 p.A414T | 0        |
| LDS4/LDS10/LDS13 | Male | 26  | TGFBR1 duplication | 3        |
| LDS5    | Male   | 27  | TGFBR2 p.R537C | 1        |
| LDS6    | Male   | 55  | TGFBR2 p.N389N | 0        |
| LDS7    | Male   | 64  | TGFBR1 p.R487Q | 0        |
| LDS8    | Male   | 26  | TGFBR2 p.R537C | 0        |
| LDS11/LDS12 | Female | 28  | TGFBR1 p.R487Q | 1        |

* number of generated OEC clones.

doi:10.1371/journal.pone.0104742.t002
### Table 3. Patient characteristics of LDS1, LDS5 and LDS11.

| LDS patient | LDS1 | LDS5 | LDS11 |
|-------------|------|------|-------|
| Sex         | Female | Male | Female |
| Age         | 54 years | 27 years | 28 years |
| Mutation    | TGFBR2 p.R537C | TGFBR2 p.R537C | TGFBR1 p.R487Q |
| Family history for sudden cardiac death | Yes | No | Yes |
| Aortic root diameter (±95th)º | 4.4 cm (±0.7 cm) | 3.0 cm (±0.8 cm) | 4.0 cm (±0.5 cm) |
| Further aneurysms | Yes | No | No |
| Arterial tortuosity | Yes | No | No |
| Mitral valve prolapse | Yes | No | Yes |
| Dural ectasia | Yes | No | Yes |
| Cranio-facial features | Bifid uvula, hypertelorism | Bifid uvula | Bifid uvula, hypertelorism |
| Skeletal features | Pectus carinatum | Protusio acetabuli, pes planus, hypermobile joints | Scoliosis, pectus excavatum, protusio acetabuli, pes planus, hypermobile joints |
| Skin features | No | No | No |

º 95th identifies the difference of diameters obtained in study patients at baseline minus diameter (cm) at 95th percentile as assessed according to Biaggi et al. [35]. doi:10.1371/journal.pone.0104742.t003

### Table 4. Members of the TGF-β superfamily with altered mRNA expression levels in LDS-OECs compared to healthy controls.

#### GREM1

|        | Signal log ratio* | Fold Change¹ | Relative expression² |
|--------|-------------------|--------------|-----------------------|
| LDS1/BC248 | 6.3               | 80           | 1136                  |
| LDS5/BC14  | 9.3               | 617          | 164                   |
| LDS11/BC401 | 12.5              | 5873         | 22145                 |

#### BMPR1A

|        | Signal log ratio* | Fold Change¹ | Relative expression² |
|--------|-------------------|--------------|-----------------------|
| LDS1/BC248 | 5.2               | 35           | 2919                  |
| LDS5/BC14  | 1.5               | 2.7          | 15                    |
| LDS11/BC401 | 6.4               | 82           | 704                   |

#### LTBP1

|        | Signal log ratio* | Fold Change¹ | Relative expression² |
|--------|-------------------|--------------|-----------------------|
| LDS1/BC248 | 0.9               | 1.8          | 1.9                   |
| LDS5/BC14  | 2.0               | 3.9          | 1.9                   |
| LDS11/BC401 | 2.5               | 5.6          | 4.8                   |

#### BMP2

|        | Signal log ratio* | Fold Change¹ | Relative expression² |
|--------|-------------------|--------------|-----------------------|
| LDS1/BC248 | −1.8              | −3.4         | 0.7                   |
| LDS5/BC14  | −2.6              | −6.0         | 0.2                   |
| LDS11/BC401 | −1.0              | −1.9         | 0.4                   |

#### BMP4

|        | Signal log ratio* | Fold Change¹ | Relative expression² |
|--------|-------------------|--------------|-----------------------|
| LDS1/BC248 | −1.9              | −3.6         | 0.6                   |
| LDS5/BC14  | −1.8              | −3.5         | 0.1                   |
| LDS11/BC401 | −9.2              | −605         | <0.1                  |

* Signal log ratio of LDS-OEC compared to healthy control, determined in microarray analysis; ¹ expression fold change of LDS-OEC compared to healthy control, converted from microarray data; ² relative gene expression in LDS-OEC compared to healthy control, determined in quantitative PCR analysis and normalized to GAPDH expression. doi:10.1371/journal.pone.0104742.t004
Elevated Gremlin-1 plasma levels in LDS patients

Since Gremlin-1 was the gene with the most prominent increase in LDS-OECs, we wondered if an elevated Gremlin-1 protein expression could also be detected systemically. Therefore, we analysed plasma samples from LDS patients and healthy controls (n = 9 and n = 15, respectively) using a commercial human Gremlin-1 ELISA. The mean Gremlin-1 plasma level of LDS patients was 2.5-fold increased with 858 ± 622 ng/ml, compared to those of healthy donors with 349 ± 107 ng/ml (Welch’s t-test p < 0.001; figure 3). The differences of Gremlin-1 levels were independent of donor’s age (Pearson-Rho = 0.125, p = 0.560) or sex (t-test p = 0.514).

Discussion

Twenty-three LDS patients belonging to 13 different families who are cared for at the University Medical Centre Hamburg-Eppendorf were molecularly characterised. In addition to mutations previously reported in LDS patients [2,3], we identified six novel mutations. Although the great majority of mutations in Loeys-Dietz syndrome are missense mutations located within the kinase domain of TGFBR2 or TGFBR1, only three of our six novel mutations fit within this category: p.N384K and p.A414T in TGFBR2 and p.S241P in TGFBR1 which were all predicted to be probably disease causing in bioinformatic analysis.

Mutation p.A232A in TGFBR2 represented a synonymous mutation not leading to an amino acid change. This mutation could not be detected in any of the 400 control chromosomes therefore probably not representing a single nucleotide polymorphism. Furthermore, we identified two novel mutations which were located in non-coding DNA regions: a nucleotide substitution within intron 1 of the TGFBR2 gene (c.94+7G>C) and a duplication of 15 base pairs within intron 1 of the TGFBR1 gene (c.97+23_+39dup15). Nevertheless, mutations not affecting the protein sequence may account for disease manifestations in LDS as they do in cystic fibrosis, infantile spinal muscular atrophy or Crohn’s disease [14].
OEC clones could be compared to age- and sex-matched healthy controls in microarray analysis. Although LDS11 carried a mutation in the type I receptor TGFBR1 whereas LDS1 and LDS5 harboured the mutation p.R337C in type II receptor TGFBR2, more than 250 genes could be identified that displayed significant alterations in gene expression in all three analysed LDS-OEC clones. Genes with altered expression included several members of the TGF-β superfamily. Strikingly, most of them affected bone morphogenetic protein signalling, namely BMPRIA, BMP2, BMP4 and GREM1 which displayed the most prominent up-regulation.

Recently, several studies revealed a direct link between TGF-β and Gremlin-1 signalling. Interestingly, although the majority of TGF-β receptor mutations in LDS patients results in non-functional receptor kinase activity, increased phosphorylation levels of the TGF-β downstream mediator proteins SMAD2 and SMAD3 have been observed in the aortic tissue of LDS patients [1,15]. In concordance with these observations, the expression of a kinase-deficient TGFBR2 variant in a transgenic mouse model resulted in TGF-β overactivity including increased SMAD2/3 phosphorylation and induced development of fibrosis [16]. Several recently published studies could directly link TGF-β-induced phosphorylation of SMAD2/3 to increased Gremlin-1 expression [17–19]. Hence, elevated Gremlin-1 expression levels might represent a direct consequence of the dysregulated TGF-β signalling in LDS-OECs.

The drastic increase of Gremlin-1 was not only confirmed by Western Blotting of LDS-OECs, we also observed significantly elevated Gremlin-1 plasma levels in LDS patients compared to healthy subjects, suggesting that up-regulation of Gremlin-1 was a systemic phenomenon.

Although larger studies are needed to confirm increased plasma levels of Gremlin-1 in LDS, our observation may have many practical implications. Determination of Gremlin-1 concentration in peripheral blood may serve as a quick screening assay in patients with vascular abnormalities and direct more detailed molecular analysis. Since the median life expectancy in a large study of LDS was only 26 years and many patients with LDS are unrecognized, such a screening assay would permit early disease detection and timely surgical intervention [2]. Furthermore, a future molecularly targeted therapy may be followed by serial detection and timely surgical intervention [2].

Gremlin-1 is a highly conserved 184 amino acid, secreted glycoprotein protein belonging to the cysteine knot superfamily. Gremlin-1 can bind and therefore antagonize bone morphogenetic proteins, namely BMP2, BMP4 and BMP7 [20,21]. During embryogenesis, Gremlin-1 is indispensable since mice with a homozygous deletion of the Gremlin-1 gene die shortly after birth due to complete renal agenesis and lung septation defects [22]. Gremlin-1 plays a role in several vascular diseases such as diabetic nephropathy or retinopathy [23,24].

Contribution of Gremlin-1 to the pathogenesis of Loeys-Dietz syndrome may be explained by effects on vascular cells [25]. By binding to BMP2 and BMP4, Gremlin-1 should antagonize the proangiogenic BMP effects on endothelial cells therefore exhibiting antiangiogenic properties. But to the contrary, recently published data revealed that Gremlin-1 can mediate strong angiogenic effects via direct binding to the vascular endothelial growth factor receptor 2 (VEGFR2). Proangiogenic properties such as in vitro induction of proliferation, migration and vascular sprouting of endothelial cells were comparable to those achieved upon stimulation with vascular endothelial growth factor A (VEGF-A) [26,27]. Hence in endothelial cells, Gremlin’s proangiogenic effects via binding to VEGFR2 seem to predominate the antagonizing effects on bone morphogenetic proteins. Therefore we suppose that Gremlin-1 mediates predominantly proangiogenic properties in LDS-OECs. This presumption is supported by the fact that expression of proangiogenic BMP2 and BMP4 is consequently down-regulated in LDS-OECs.

Since Gremlin-1 is a secreted factor, it might not serve as an autocrine regulator of endothelial cells but may also mediate paracrine effects on other cell types. This assumption is strengthened by the fact that the Gremlin-1 plasma levels were significantly increased in LDS patients compared to healthy controls. The aortic media of LDS patients is characterized by a disorganized wall and diffuse medial degeneration with marked excess of collagen and loss of elastic fiber architecture [1,15]. Gremlin-1 might account for some of these characteristics since it has been described to play a role in extracellular matrix modulation [24,28]. In a diabetic nephropathy mouse model, a first therapeutic approach with Gremlin-1 siRNA was conducted. Inhibition of Gremlin-1 decelerated diabetic nephropathy through decrease of proteinuria, renal collagen accumulation and renal cell proliferation and apoptosis [29].

Maciel et al. investigated whether Gremlin-1 had an impact on vascular smooth muscle cells. SMCs overexpressing Gremlin-1 showed markedly increased proliferation and migration capacities compared to empty-vector transfected cells. In contrast, both proliferation and migration were reduced after gene silencing with shRNA against Gremlin-1 mRNA [30]. Our immunohistological data support these findings since Gremlin-1 expression was mainly observed in the endothelial layer of the intima or in small vessels in the adventitia and in smooth muscle cells of the media.

Recently, Cahill et al. reported that Gremlin-1 plays a key role in pulmonary arterial hypertension (PAH) [31]. The majority of patients with the heritable form of PAH harbour a mutation in the TGF-β type II receptor BMPR2 [32]. PAH shares some phenotypic features with aortic aneurysm syndromes since it is characterized by increased medial and adventitial thickness due to enhanced vascular smooth muscle cell or endothelial cell proliferation which can result in lumen loss [33].

Conclusions

In conclusion, outgrowth endothelial cells may serve as a model to analyse alterations in gene expression in Loeys-Dietz syndrome. Gene expression profiling performed on LDS-OECs demonstrated pronounced up-regulation of bone morphogenetic protein antagonist Gremlin-1 which may contribute to the vascular pathology of Loeys-Dietz syndrome. Furthermore, elevated Gremlin-1 plasma levels in LDS patients may serve as a new serological marker for early detection and diagnosis of Loeys-Dietz syndrome and as a potential follow up marker under a future targeted therapy.

Supporting Information

File S1 Supplemental methods (Flow cytometric analysis of endothelial cells) and supplemental tables S1 (Flow cytometric analysis of endothelial cells) and S2 (Primers and PCR conditions).

(DOC)

File S2 Supplemental table S3 (Expression values and permutation p-values).

(XLS)

Acknowledgments

The authors would like to thank Kristin Klaetschke of the Department of Clinical Chemistry/Central Laboratories, for having performed the
References

1. Loeys BL, Chen J, Neurope EN, Judge DP, Podowski M, et al. (2005) A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. Nat Genet 37:273–281.

2. Loeys BL, Schwarze U, Holm T, Callewaert BL, Thomas GH, et al. (2006) Aneurysm syndromes caused by mutations in the TGF-beta receptor. N Engl J Med 355:789–798.

3. Sheneur C, Collod-Beroud G, Fairet I, Gouny L, Santan G, et al. (2008) Identification of 23 TGFBR2 and 6 TGFBR1 gene mutations and genotype-phenotype investigations in 457 patients with Marfan syndrome type I and II, Loeys-Dietz syndrome and related disorders. Hum Mutat 29:E284–295.

4. Lindsay ME, Schepers D, Bolar NA, Doyle JJ, Gallo E, et al. (2012) Loss-of-function mutations in TGFBR2 cause a syndromic presentation of thoracic aortic aneurysm. Nat Genet 44:922–927.

5. van de Laar DM, Oldenburg RA,党总 G, Roos-Hesselink JW, de Graaf BM, et al. (2010) Tgf-Beta superfamily receptors-targets. Adv Exp Med Biol 664:95–105.

6. Otten J, Bokemeyer C, Fießler W (2010) Tgif-Beta superfamily receptors-targets for antiangiogenic therapy. J Oncol 2010:71068.

7. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 37:e67.

8. Pugsley MK, Tabrizchi R (2000) The vascular system. An overview of structure and function. J Pharmacol Toxicol Methods 44:333–340.

9. Hirschi KK, Kolesky SA, Beck LH, Smith SR, D’Amore PA (1999) Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 84:298–303.

10. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, et al. (2004) Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Cardiovasc Res 68:235–248.

11. Fernandez LA, Saiz-Rodriguez F, Zarate-Restitutoria R, Perez-Molino A, Hebel RP, et al. (2005) Blood outgrowth endothelial cells from Hereditary Hemorrhagic Telangiectasia patients reveal abnormalities compatible with vascular lesions. Cardiovasc Res 68:366–375.

12. Dorier FO, Harroun D, Lalande M, Collod-Beroud G, Claustrès M, et al. (2009) Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 37:e67.

13. Piaff MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e5.

14. Sauna ZE, Kimchi-Sarfaty C (2011) Understanding the contribution of synonymous mutations to human disease. Nat Rev Genet 12:683–691.

15. Małeśzewski JJ, Miller DV, Lu J, Dietz HC, Halańska MK (2009) Histopathologic findings in ascending aorta from individuals with Loeys-Dietz syndrome (LD). Am J Surg Pathol 33:194–201.

16. Denton CP, Zheng B, Evans LA, Shi-wen X, Ong VH, et al. (2003) Fibroblast-specific expression of a kinasedeficient type II transforming growth factor beta (TGFbeta) receptor leads to paradoxical activation of TGFbeta signaling pathways with fibrosis in transgenic mice. J Biol Chem 278:25109–25119.

17. Graham JR, Williams CM, Yang Z (2014) MicroRNA-27b Targets Gremlin 1 to Modulate Fibrotic Responses in Pulmonary Cells. J Cell Biochem Res Commun 447:689–695.

18. O’Reilly S, Carmichael M, Cant R, van Laar JM (2014) Interleukin-6 (IL-6) trans signaling drives a STAT3-dependent pathway that leads to hyperactive transforming growth factor-beta (TGF-beta) signaling promoting SMA3D activation and fibrosis via Gremlin protein. J Biol Chem 289:9952–9960.

19. Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM (1998) The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. Mol Cell 1:673–683.

20. Topol LZ, Marx M, Laugier D, Bogdanova NN, Boubnov NV, et al. (1997) Identification of drm, a novel gene whose expression is suppressed in transformed cells and which can inhibit growth of normal but not transformed cells in culture. Mol Cell Biol 17:4801–4810.

21. Michos O, Papann L, Vintersten K, Beier K, Zeller R, et al. (2004) Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metastatic kidney and lung organogenesis. Development 131:3401–3410.

22. McMahon R, Murphy M, Clarkson M, Tao M, Mackenzie HS, et al. (2000) HGF-2, a mesangial cell gene induced by high glucose, is human gremlin. Regulation by extracellular glucose concentration, cyclic mechanical strain, and transforming growth factor-beta1. J Biol Chem 275:9061–9064.

23. Zode GS, Clark AF, Watering R (2009) Bone morphogenetic protein 4 inhibits TGF-beta2 stimulation of extracellular matrix proteins in optic nerve head cells: role of gremlin in LCM expression. Glia 57:753–766.

24. Smadja DM, Beiche I, Silvestre JS, Germain S, Soret A, et al. (2008) Bone morphogenetic proteins 2 and 4 are selectively upregulated by late outgrowth endothelial progenitor cells and promote neangiogenesis. Arterioscler Thromb Vasc Biol 28:2137–2143.

25. Mitola S, Ravigli G, Moroni E, Salv V, Irali D, et al. (2010) Gremlin-1 is a novel agonist of the major proangiogenic receptor VEGFR2. Blood 116:3677–3680.

26. Stabile H, Mitola S, Moroni E, Belleri M, Nicolli S, et al. (2007) Bone morphogenetic protein antagonist Drm/gremlin is a novel proangiogenic factor. Blood 109:1834–1840.

27. Watering RJ, Fleener DL, Hebbel PE, Pang JH, Tovar TO, et al. (2007) Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. Invest Ophthalmol Vis Sci 48:1191–1200.

28. Zhang Q, Shi Y, Wada J, Malatakassinou SM, Liu M, et al. (2010) In vivo delivery of Gremlin siRNA plasmid reveals therapeutic potential against diabetic nephropathy by recovering bone morphogenetic protein-7. PLoS One 5:e11709.

29. Maciel TJ, Molo RS, Schor N, Campos AH (2008) Gremlin promotes vascular smooth muscle cell proliferation and migration. J Mol Cell Cardiol 47:407–417.

30. Cahill E, Costello CM, Rowan SC, Harkin S, Howell K, et al. (2012) Gremlin plays a key role in the pathogenesis of pulmonary hypertension. Circulation 125:920–930.

31. Rabinovich M (2008) Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest 118:2372–2379.

32. Mandegar M, Fung YC, Huang W, Remillard CV, Rubin LJ, et al. (2004) Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension. Microvasc Res 68:75–103.

33. Matyas G, Arnold E, Carrel T, Baumgartner D, Boileau C, et al. (2006) Identification and in silico analyses of novel TGFBR1 and TGFBR2 mutations in Marfan syndrome-related disorders. Hum Mutat 27:760–769.

34. Biaggi P, Matthews F, Braun J, Rousson V, Kaufmann PA, et al. (2009) Gender, age, and body surface area are the major determinants of ascending aorta dimensions in subjects with apparently normal echocardiograms. J Am Soc Echocardiogr 22:720–725.

Author Contributions

Conceived and designed the experiments: JW SS LOF YvK WF. Performed the experiments: JW HS BK MK GV VB CH. Analyzed the data: JW SS LOF HS MH BK MK GV VB BO TS SB CH MR YvK WF. Contributed reagents/materials/analysis tools: SS MH TS SB MR FB. Wrote the paper: JW SS CB YvK WF.