Beneficial Outcome of Losartan Therapy Depends on Type of FBN1 Mutation in Marfan Syndrome

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Background—It has been shown that losartan reduces aortic dilatation in patients with Marfan syndrome. However, treatment response is highly variable. This study investigates losartan effectiveness in genetically classified subgroups.

Methods and Results—In this predefined substudy of COMPARE, Marfan patients were randomized to daily receive losartan 100 mg or no losartan. Aortic root dimensions were measured by MRI at baseline and after 3 years. FBN1 mutations were classified based on fibrillin-1 protein effect into (1) haploinsufficiency, decreased amount of normal fibrillin-1, or (2) dominant negative, normal fibrillin-1 abundance with mutant fibrillin-1 incorporated in the matrix. A pathogenic FBN1 mutation was found in 117 patients, of whom 79 patients were positive for a dominant negative mutation (67.5%) and 38 for a mutation causing haploinsufficiency (32.5%). Baseline characteristics between treatment groups were similar. Overall, losartan significantly reduced aortic root dilatation rate (no losartan, 1.3±1.5 mm/3 years, n=59 versus losartan, 0.8±1.4 mm/3 years, n=58; P=0.009). However, losartan reduced only aortic root dilatation rate in haploinsufficient patients (no losartan, 1.8±1.5 mm/3 years, n=21 versus losartan 0.5±0.8 mm/3 years, n=17; P=0.001) and not in dominant negative patients (no losartan, 1.2±1.7 mm/3 years, n=38 versus losartan 0.8±1.3 mm/3 years, n=41; P=0.197).

Conclusions—Marfan patients with haploinsufficient FBN1 mutations seem to be more responsive to losartan therapy for inhibition of aortic root dilatation rate compared with dominant negative patients. Additional treatment strategies are needed in Marfan patients with dominant negative FBN1 mutations.

Clinical Trial Registration—http://www.trialregister.nl/trialreg/index.asp; Unique Identifier: NTR1423. (Circ Cardiovasc Genet. 2015;8:383-388. DOI: 10.1161/CIRCGENETICS.114.000950.)

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Marfan syndrome is a progressive disorder caused by mutations in FBN1 encoding the protein fibrillin-1.1 Recently, losartan has been found to reduce aortic root dilatation rate in patients with Marfan syndrome.2 However, there appeared to be large interindividual variability in response. The aim of this study was therefore to identify patients with a more enhanced effect of losartan therapy.

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Currently, over 2000 mutations have been described in the International Database.3,4 FBN1 mutations can be classified as dominant negative or haploinsufficient depending on the effect of the FBN1 mutation on the fibrillin-1 protein.5 Dominant negative mutations lead to disturbed function or folding of the protein, causing disturbed interactions with fibrillin-1 and other proteins; hence, a disorganized extracellular matrix.6 Both the strength of the fibrillin-1 matrix may be changed, as well as the release of binding proteins, such as transforming growth factor-ß (TGF-ß), which is (indirectly) captured in the fibrillin-1 network. Indeed, plasma TGF-ß levels are elevated in Marfan patients and are correlated to aortic dissection and aortic root dilatation.7,8 On the other hand, a lower production of normal fibrillin-1 protein by haploinsufficient mutations will also lead to reduced binding of TGF-ß,9,10 either as a result of whole gene deletions of FBN1,11 or degradation of the mutant protein,12 or nonsense-mediated decay by degradation of fibrillin-1 encoding messenger RNA (mRNA).13 The latter type of mutations...
leads to a homogeneous phenotype because there is no mutant protein. The reduced level of normal fibrillin-1 protein presumably results in a thinner fibrillin-1 matrix in the vasculature and thus in reduced aortic wall strength.

The objective of this study is to investigate the losartan effect on aortic root dilatation rate between Marfan patients with an \textit{FBN1} mutation leading to haploinsufficiency and Marfan patients with an \textit{FBN1} mutation leading to a dominant negative fibrillin-1 effect.

**Methods**

**Patient Population**
To investigate the losartan effect on aortic root dilatation rate between haploinsufficient and dominant negative \textit{FBN1} mutations, we included all patients with a pathogenic \textit{FBN1} mutation and a native aortic root at time of inclusion and exclusion scan of the COMPARE study. In short, the COMPARE trial investigated the effects of losartan (100 mg) on aortic dimensions in a multicenter randomized, controlled clinical trial on top of prescribed cardiovascular medication. Inclusion criteria were diagnosis of Marfan syndrome and age $\geq 18$ years. Exclusion criteria were angiotensin-converting enzyme inhibitor usage and previous replacement of $>1$ part of the aorta. The trial complied with the Declaration of Helsinki and was conducted with approval of the Medical Ethical Committees of all participating hospitals. Written informed consent was obtained from all participants. This trial is registered at the Netherlands Trial Register (number NTR1423).

**Baseline Examination**
As part of the COMPARE trial, during the baseline visit, medical specialists extensively examined all patients on clinical features of Marfan syndrome in cardiovascular, ocular, and skeletal systems according to the Ghent criteria of 1996. Punch skin biopsies were taken from the upper thigh of 55 patients at the onset of the trial after local anesthesia with ethyl chloride spray. Aortic root diameter was measured at end diastole by ECG-triggered MRI in short axis views at the level of the sinus of Valsalva during end-expiration. After a mean follow-up of 3 years, patients underwent a second MRI, and subsequently, aortic root dilatation rate was calculated. Clinical events, including aortic root surgery, aortic dissection type A and B, mitral valve surgery, and cardiovascular death, were also evaluated at the end of the study. Sanger sequencing of the 65 coding \textit{FBN1} exons in genomic DNA (gDNA) from blood cells was used to detect mutations. Large deletions were tested with multiplex ligation-dependent probe amplification (MRC-Holland, MLPA kit P065 and P066).

**Mutation Classification**
Mutations were classified as dominant negative in the case of (1) missense mutations leading to stable mutant fibrillin-1 protein with altered structure; (2) mutations leading to exon skipping or deletion resulting in in-frame events and consequently a shorter stable protein; (3) Premature Termination Codon or frameshift mutations leading to a shorter fibrillin-1 protein without causing nonsense mediated decay.

Mutations were classified as ‘haploinsufficient’ in case of (1) deletion of the whole \textit{FBN1} gene; (2) deletion of at least the first (exon 1) or the last exon (exon 65) of the \textit{FBN1} gene, which prevents transcription or translation of the gene; (3) Premature Termination Codon or frameshift mutations leading to a null-allele as a consequence of NMD; (4) missense mutations leading to degradation of the mutant protein; and (5) mutations leading to a very short truncated protein (translation of less than the first 10 exons of the \textit{FBN1} gene), which will not participate in fibril formation.

Effects of the mutations were predicted by Alamut® software (Interactive Biosoftware, Rouen, France). To confirm the mutation classification, fibrillin-1 mRNA production was measured in fibroblast cultures from available skin biopsies. In 55 patients, the effect of the mutation was tested on mRNA expression. mRNA expression in cultured fibroblasts was studied by mRNA isolation, complementary DNA synthesis, and

Figure 1. Flow chart of study population. In the COMPARE trial, 233 patients were included. For this additive analysis, we excluded 47 patients not having a pathogenic \textit{FBN1} mutation and 69 patients because of replacement of the aortic root before or during study. The red boxes include patients with a haploinsufficient mutation; the blue boxes comprise patients with a dominant negative mutation. AoRR indicates aortic root replacement.
QPCR on a Lightcycler LC480 (Roche), using UPL probe n078 (Roche) with primers: agcggggatctcacttgat (forward) and cctcacactcgtccacgtc (reverse), with the GAPD UPL reference gene kit (Roche).

Statistical Analysis
Data are presented as mean value±standard deviation or as number of patients (percent). Comparisons between continuous variables were made by Mann–Whitney U test. Comparisons between categorical variables were made by Fisher’s exact tests. A 2-way analysis of variance was used after ranking of the aortic root dilatation rates to calculate the difference in effect size of losartan between both groups. The spearman’s rank correlation coefficient was used to analyze the correlation between mean arterial blood pressure and change in aortic root dilatation rate. All statistical tests were 2-sided and differences were considered statistically significant at *P*<0.05. Data analysis was performed using the SPSS statistical package (19.0 for windows; SPSS Inc., Chicago, Illinois, USA).

Results
Classification Into Dominant Negative and Haploinsufficient FBN1 Mutations
All patients in our cohort were diagnosed with Marfan syndrome based on the Ghent criteria of 1996 and 2010 and did not have distinguishing features of other connective tissue disorders. In our COMPARE cohort, we included 233 patients, and in 186 patients, a pathogenic *FBN1* mutation was found (Figure 1). In the remainder 47 patients, we did not perform a genetic analysis because of refusal of the patient (n=1), we did not find a mutation even after sequencing and MLPA analysis (n=37), and in 9 patients, we found a pathogenic mutation in another connective tissue disorder gene: *TGFBR2* (n=2), *TGFBR1* (n=1), *MYH11* (n=2), *TGFBR2* (n=3), and *MYLK1* gene (n=1). Of the 186 patients with a pathogenic *FBN1* mutation, 69 patients were excluded for our current analysis because they already had an aortic root replacement at the start of the study (n=53) or during the study (n=16). (Figure 1) Thus, we included 117 patients with a pathogenic *FBN1* mutation and a native aortic root at the time of the exclusion scan (mean age, 35.3 years [range 18–71 years]). Classification of mutations revealed that 79 patients were positive for a dominant negative mutation (67.5%), and 38 patients were positive for a mutation causing haploinsufficiency (32.5%).

To validate the predicted mutation effect by Alamut, we compared gDNA with complementary DNA, synthesized from mRNA.
of cultured fibroblasts from skin biopsies. In total, we tested the effect of the mutation on complementary DNA sequence analysis of 55 Marfan patients. Examples are shown in Figure 2.

**Clinical Features Between Dominant Negative and Haploinsufficient Patients**

Medical specialists completely mapped our Marfan patients, resulting in a sample rate of clinical features of 100% (Table 1).

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Medical specialists completely mapped our Marfan patients, resulting in a sample rate of clinical features of 100% (Table 1).
We recently showed that losartan significantly reduces aortic root dilatation rate in Marfan patients. Here, in our selected cohort of patients with a pathogenic FBN1 mutation, patients treated with losartan also showed significant reduction in aortic root dilatation rate compared with patients without losartan therapy (no losartan, 1.3±1.5 mm/3 years, n=59 versus losartan, 0.8±1.4 mm/3 years, n=58; P=0.009).

Noteworthy, losartan therapy was highly beneficial for patients with a haploinsufficient mutation because these patients showed a prominent and significant reduction in aortic root dilatation rate (no losartan, 1.8±1.5 mm/3 year, n=21 versus losartan, 0.5±0.8 mm/3 year, P=0.01; Figure 4). In contrast, in patients with a dominant negative mutation, the effect of losartan was not significant (no losartan, 1.2±1.7 mm/3 year, n=38 versus losartan, 0.8±1.3 mm/3 year, n=41, P=0.197; Figure 4). In absolute terms, the percentage of haploinsufficient patients with a stable aortic root (defined as a dilatation rate ≤0 mm/3 years) was 58.8% in the losartan group and 19.0% in the control group (P=0.014). The percentage of dominant negative patients with a stable aortic root was 51.2% in the losartan group and 60.5% in the control group (P=0.498). When a 2-way analysis of variance was used to calculate the difference in effect size of losartan between both groups, no statistical significance in this relatively small cohort (P=0.147) was shown.

Discussion

This is the first study to demonstrate the value of classification of FBN1 mutations based on their effect on fibrillin-1 protein level. Our study shows that classification into haploinsufficient (32.5%) and dominant negative (67.5%) patients is feasible and that both groups have similar cardiovascular pathology. Interestingly, losartan therapy significantly reduced the aortic root dilatation rate in the haploinsufficient patients, whereas only a modest insignificant reduction was found in dominant negative patients.

Losartan, an angiotensin II receptor type 1 inhibitor, is known to reduce TGF-β signaling in a well-known mouse model with Marfan syndrome. Therefore, it was hypothesized that losartan has a beneficial effect in Marfan patients by lowering blood pressure and by reducing TGF-β signaling. However, we demonstrated that mean arterial blood pressure did not correlate with aortic root dilatation rate in dominant negative, as well as haploinsufficient patients treated with losartan. Therefore, the effect of losartan on the aortic root dilatation rate is not merely blood pressure–related. There is a multitude of signaling cascades downstream of angiotensin II receptor type 1 activation. It seems that one of the other pathways has a detrimental effect on the vessel wall upon chronic angiotensin II receptor type 1 activation.17–20

A possible explanation for the more enhanced beneficial effect of losartan in haploinsufficient patients may be that their aortic wall suffers from hyperextension because of a thinner fibrillin-1 network. The fibrillin-1 network is connected to the elastin and collagen network to limit excessive stretch, which may be hampered in these patients.21 Hypertension of the aorta or aortic stretch may damage the aortic wall directly, causing angiotensin II receptor type 1 activation for a rapid damage response by producing TGF-β.22 Furthermore, local angiotensin II has been associated with aneurysm formation.19 Altogether, haploinsufficient patients may have more locally produced angiotensin II and thus a more beneficial effect of losartan on local aneurysm forming.

Traditionally, most FBN1 mutations were thought to cause a dominant negative effect on the fibrillin-1 protein, resulting in Marfan syndrome.6,10 However, multiple studies, including this study, have revealed that FBN1 haploinsufficiency leads to the full spectrum of Marfan syndrome and is present in a substantial part (32.5%) of the adult Marfan population.11,13 Haploinsufficient patients have a homogeneous phenotype because of similar mutation effect on fibrillin-1: reduced but functionally normal fibrillin-1 protein. In contrast, patients with dominant negative mutations display a broad spectrum dysfunctional fibrillin-1 proteins. This probably explains the highly variable response to losartan in this patient group. In addition, we demonstrated a different effect of losartan therapy, based on this phenotypic classification, underlining the relevance of categorization of Marfan patients genetically.

In conclusion, our results show that the beneficial effect of losartan therapy on aortic root dilatation rate is more pronounced in patients with a mutation causing FBN1 haploinsufficiency. We recommend to analyze and categorize the
FBN1 mutations in all Marfan patients by genetic specialists to predict individual losartan effectiveness. Moreover, enhanced effort should be made to further characterize the Marfan patients with dominant negative FBN1 mutations to find novel therapeutics reducing aortic dilatation rate for this patient population.

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Disclosures
None.

References

CLINICAL PERSPECTIVE
Marfan syndrome leads to aortic dilatation and subsequently to potentially fatal aortic dissections. It has been shown that losartan on top of β-blockers reduces aortic dilatation in adults with Marfan syndrome. However, there is a large interindividual variability in treatment response. We classified FBN1 mutations based on the effect on fibrillin-1 protein production into dominant negative mutations (resulting in abnormal fibrillin-1 protein) and haploinsufficiency mutations (resulting in normal but deficient fibrillin-1 protein). Losartan therapy significantly reduced the aortic root dilatation rate in the haploinsufficient patients (33%), whereas only a modest insignificant reduction in aortic root dilatation rate was observed in dominant negative patients (67%). Analysis of the FBN1 mutations in Marfan patients may help analyze severity of aortic phenotype and also assist in predicting response to losartan therapy. Moreover, other novel therapeutic agents are likely needed requiring reduced aortic dilatation rate in Marfan patients with a dominant negative mutation.
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