Predictors of severe aortic involvement in Marfan syndrome

Ph.D. thesis

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1. Introduction

Marfan syndrome is a systemic connective tissue disorder, caused by mutations of the fibrillin-1 gene (*FBN1*) and inherited in an autosomal dominant manner. Aortic dilation and consequent aortic dissection, mainly affecting the ascending aorta are the major contributors to the morbidity and mortality of the syndrome. As aortic dissection is associated with a high mortality rate even when treated surgically, life expectancy of patients could be normalized by preventing acute aortic events through the means of prophylactic aortic surgery. The indications of a prophylactic surgery are mainly based on aortic diameter, however, aortic dissection may develop under the diameter threshold stated in the guidelines. Thus, predictors of more severe aortic involvement need to be identified to aid the risk stratification of patients with Marfan syndrome, thereby to optimize the timing and indications of a prophylactic surgery.

Associations between the genetic background and the aortic phenotype have been described, however, more investigations are required to further evaluate the potential predictive role of genotype-phenotype correlations. Importantly, Marfan syndrome and other hereditary aortopathies such as Loeys-Dietz syndrome (LDS) may present with overlapping clinical features, but require different disease management. Genetic testing approach needs to be optimized to aid differential diagnosis.

Arterial tortuosity, meaning increased amplitude or frequency of curvatures, has been described as a potential predictor of more severe aortic manifestations in case of the vertebral arteries and the aorta in Marfan syndrome. However, the tortuosity of these vessels may be influenced by the frequent skeletal manifestations of the disease, making them less reliable prediction parameters.

2. Objectives

We aimed to identify genetic variants with more severe aortic manifestations in patients with Marfan syndrome, which could contribute to a better risk stratification system, thereby to improved indications of a prophylactic surgery.

We also aimed to optimize the genetic testing approach of patients with Marfan syndrome and other aortopathies. This included demonstrating the relevance of copy number variation (CNV) screening.

As visceral arteries are not influenced by the skeletal deformities, a further goal of our study was to assess the tortuosity of the splenic and both renal arteries in Marfan syndrome patients and to investigate their association to the severity of aortic manifestations. The change of arterial tortuosity throughout the years is presented through a case with several years of follow-up.

3. Methods

3.1. Genetic testing

3.1.1 The importance of copy number variation (CNV) detection

The design of our study was influenced by demonstrating the relevance of copy number variation (CNV) detection in Marfan syndrome through a case of a clinically diagnosed Marfan syndrome patient and family members. Next-generation sequencing (NGS)-based targeted gene panel test including the most relevant hereditary aortopathy genes was carried out. MiSeq Personal Sequencer (Illumina, San Diego, CA, USA) was used to analyze amplicons. As deletions and duplications larger than 20 bp cannot be detected by this technique, 60x PE150 PCR-free WGS on a HiSeq X Ten platform (Illumina, San Diego, CA, USA) was performed subsequently. Nexus Copy Number (BioDiscovery, El Segundo, CA, USA) software was used to analyze the WGS data for CNVs. MLPA (P065/P066,

MRC-Holland, Amsterdam, the Netherlands) as well as standard PCR with a 407-bp amplicon spanning the deletion breakpoints followed by Sanger sequencing were applied to confirm the findings.

3.1.2 Study design

Altogether 136 patients were involved in our retrospective cross-sectional study. Phenotypic evaluation was carried out at the Marfan outpatient clinic at the Heart and Vascular Center of Semmelweis University in Budapest. Among the 136 patients, 18 were first-degree relatives, thus the investigated population covered 118 families.

3.1.3 First phase of genetic testing

3.1.3.1 Study population

Inclusion criterion was the clinical diagnosis of Marfan syndrome in the first phase of genetic testing, resulting in the enrollment of 57 patients.

3.1.3.2 Single-gene analysis

NGS with a Roche GS Junior platform was used to screen the *FBN1* gene. Sanger sequencing using ABI Prism 310 Genetic Analyser (Applied Biosystems) was applied to investigate homopolymer regions and to confirm detected variants throughout the study.

3.1.4 Second phase of genetic testing

3.1.4.1 Study population

Patients with negative results from the first phase, and further 79, altogether 96 patients were enrolled in the second phase. Inclusion criterion were the (suspected) clinical diagnosis of Marfan syndrome or Marfanoid habitus. Marfanoid habitus was defined as having a systemic score of at least 5 points.

3.1.4.2 Multi-gene panel analysis
NGS-based multi-gene panel involving the *ACTA2*, *COL3A1*, *FBN1*, *KCNN1*, *MYH11*, *SMAD3*, *TGFB2*, *TGFBR1* and *TGFBR2* genes was carried out with Illumina MiSeq platform (Illumina, San Diego, USA). Genomic libraries were prepared by QIAseq targeted DNA custom panel (QIAGEN, USA).

3.1.5 Multiplex ligation-dependent probe amplification (MLPA)
CNVs were screened by multiplex ligation-dependent probe amplification (MLPA) method (MRCHolland, Amsterdam, the Netherlands) in the *FBN1* and *TGFBR2* genes in samples where no (likely) pathogenic mutations were identified after the above detailed sequencing steps.

3.1.6 Relevance of detected variants
Varsome, Human Gene Mutation Database, Universal Mutation Database, dbSNP and gnomADv2.1 non-Finnish population databases were used to evaluate the pathogenicity of a detected variant. American College of Medical Genetics and Genomics (ACMG) guidelines were followed for variant classification. Pathogenic and likely pathogenic variants were considered disease-causing. Missense variants were classified as dominant negative (DN), while haploinsufficient (HI) variants included nonsense, splice-site and frameshift mutations, as well as CNVs.

3.1.7 Investigations of genotype-phenotype correlations
The association between ascending aortic involvement (dilation and dissection) and the type of disease-causing variant was investigated. Aortic involvement was compared between mutation positive and negative patients, between HI and DN mutations of the *FBN1* gene and between Marfan syndrome and Loeys-Dietz syndrome patients. Due to the important role of cysteine in the structure of fibrillin-1, DN mutations were divided into variants that resulted in the elimination of a cysteine

(DN Cys) and DN variants that did not substitute this amino acid (DN non-Cys).

3.1.8 Statistical analysis

Two-sample t-test and chi-squared test were used for the comparison of certain groups, the results being considered significant at $p<0.05$. The general characteristics of the examined population were described by the mean and 95% confidence interval, while the systemic score was characterized by median with first and third interquartile ranges.

3.2. Arterial tortuosity

3.2.1 Patient selection

Altogether 37 Marfan syndrome patients with available arterial phase abdominal computed tomography angiography (CTA) images were enrolled to the study. The diagnosis of Marfan syndrome was based on the revised Ghent nosology. Age and sex matched controls from our clinical imaging database were also included with a control-to-case ratio of 2:1.

3.2.2 Severity groups

Patients with Marfan syndrome were categorized into the following severity groups:

Group A (n=5) - no aortic involvement requiring surgery at the time of CT scan.

Group B (n=12) - elective surgery carried out on the ascending aorta due to mild aortic involvement before the CT scan.

Group C (n=20) – operation of patients with more severe aortic manifestations including acute type A aortic dissection.

3.2.3 CT angiography

Arterial phase CT images of the abdominal aorta, made with 256-slice CTA (Philips Brilliance iCT) were analyzed with a slice thickness of 1-2.5 mm and were reconstructed with

traditional filtered back projection (FBP) or hybrid-type iterative reconstruction (IR).

3.2.4 Image segmentation and centerline export

Image analysis was done with Medis QAngio CT (v.3.1.0.1) by a single leader blinded to the patients' clinical data. Manual segmentation of the splenic and renal arteries was carried out. The segmented arteries were saved and the centerlines were exported with the Medis QAngio CT 3D Workbench (v 0.8) in a text format with the 3 dimensional vessel coordinates.

3.2.5 Tortuosity metrics

Distance metric (DM) gives us the ratio of the actual path length to the distance of the linear end points. DM is insensitive to the frequency of the curves, thus inflection count metric (ICM) is applied, which is calculated by multiplying DM by the number of inflection points. DM and ICM mainly detect high amplitude, low frequency curves. Sum of angles metric (SOAM) is sensitive to high frequency curves. The ICM/SOAM metric assesses the contribution of amplitude and frequency to the tortuosity.

3.2.6 Statistical analysis

Tortuosity metrics are reported as medians with interquartile ranges, further analyzed by non-parametric tests. Accordingly, Mann-Whitney U-test was used for the comparison of two groups, while Kruskal-Wallis test was applied to compare multiple groups. Pairwise Mann-Whitney U-test with Benjamin-Hochberg adjustment for multiple comparisons was the applied post-hoc test.

3.2.7 Follow-up of tortuosity through a case

A patient with severe aortic involvement and bilateral internal mammary artery (IMA) aneurysms was followed-up with CT

images done in 2013, 2015, 2018, 2019 and 2020. The tortuosity of the aorta and the internal mammary arteries was assessed in addition to the monitoring of the aneurysms.

4. Results

4.1 Genetic testing

4.1.1 The importance of copy number variation (CNV) detection

Three members of the investigated family had the clinical diagnosis of Marfan syndrome. Multi-gene panel did not reveal any disease-causing variant by the index patient. A 60× PE150 PCR-free WGS was carried out, and no pathogenic SNVs or small indels were identified in the relevant HTAD genes. After analyzing the WGS data for CNVs, a 31,956-bp deletion of the *FBN1* gene (NM_000138.4:c.164+13846_442+1334del) was revealed. With the use of MLPA the detected variant was identified in the patient's mother and sister, who also fulfilled the criteria for the clinical diagnosis of Marfan syndrome.

4.1.2 First phase of genetic testing

4.1.2.1 Study population

Of the 57 clinically diagnosed Marfan syndrome patients, 19 (33%) were men and 38 (67%) were women. The average age at the time of genetic testing was 33 (30–37) years, and the median systemic score was 8 points (ranging from 7 to 10).

4.1.2.2 Identified genetic variants

Altogether 34 (likely) pathogenic variants of *FBN1* were identified with NGS and Sanger sequencing, which gives a 60% mutation detection rate. Subsequent MLPA on the negative samples revealed CNVs in 3 patients.

4.1.3 Second phase of genetic testing

4.1.3.1 Study population

In the second phase 52 (54%) men and 44 (46%) women, with the average age of 35 (21–49) years at the time of genetic screening were investigated. The median systemic score was 8 points (ranging from 7 to 9).

4.1.3.2 Identified genetic variants

Of the 30 detected pathogenic variants, 27 (90%) affected the *FBN1* gene. One pathogenic mutation was detected in the *TGFBR2* (3.3%) gene, while 2 pathogenic mutations affected the *TGFB2* (6.7%) gene, both of which are associated with LDS. Furthermore, 13 likely pathogenic variants were identified in *FBN1*, while the LDS-associated *TGFBR1* was affected by 2, and the *SMAD3* by another 2 likely pathogenic variants (*Figure 1*).

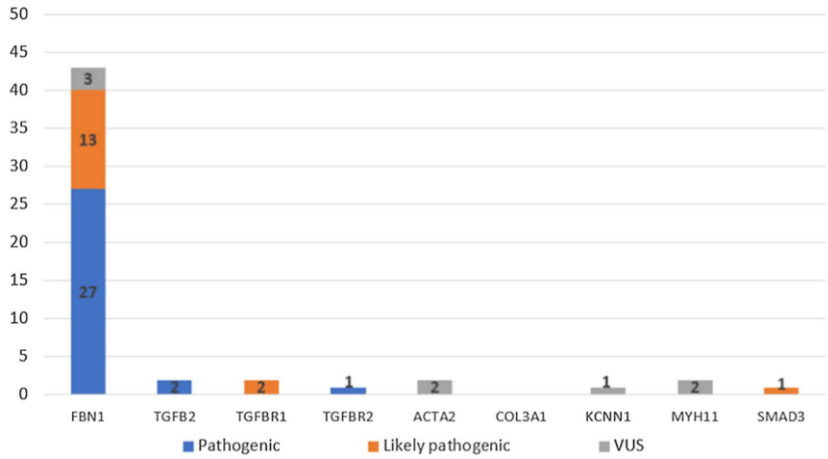

Figure 1 Distribution of the detected variants with a multi-gene panel in the second phase of our genetic testing study.

4.1.4 Overall results

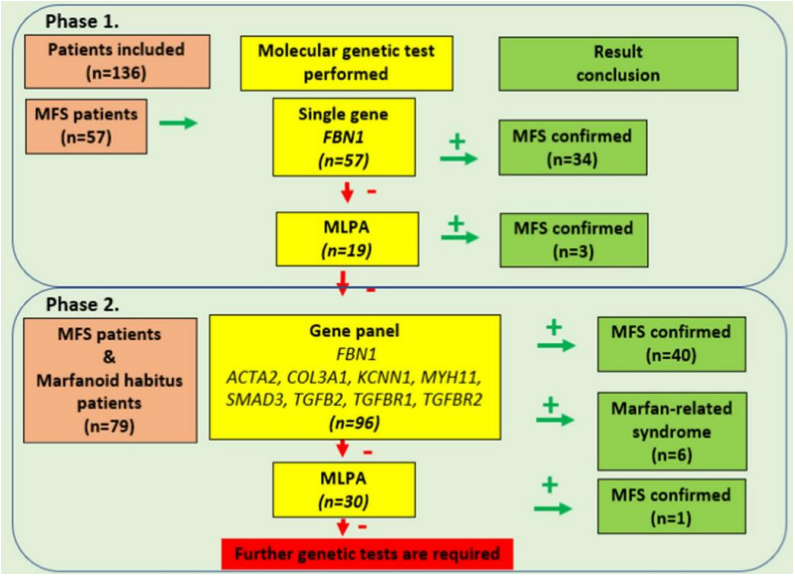

Figure 2 The two phases of genetic testing steps with the outcomes from our genotype-phenotype correlations study.

Altogether, the detection rate of *FBN1* mutations appeared to be 57% (78/136), while the overall variant detection was 62% (84/136).
The diagnosis of Marfan syndrome was confirmed in 78 individuals, while the diagnosis of LDS was established in 6 patients (*Figure 2*).

4.1.5 Genotype-phenotype correlations
DN Cys (n=18) mutations of *FBN1* led to aortic involvement significantly more frequently, than DN non-Cys (n=12) variants (89% and 50%, respectively, p=0.018).

DN non-Cys variants were less frequently associated with aortic dissection and/or dilation than the combined group of HI and DN Cys ($p<0.001$) (*Figure 3a*).
Patients with DN Cys variants required aortic surgery significantly more frequently than patients with HI (78% vs 50%, $p=0.042$) and DN non-Cys variants (78% vs 33%, $p=0.015$) (*Figure 3b*).
Of Marfan syndrome patients, 83% (65/78) presented with aortic dissection and/or dilation, while this was 100% (6/6) in case of LDS ($p=0.584$) (*Figure 3c*). However, LDS patients tended to be younger ($p=0.057$) and had significantly lower systemic score ($p=0.013$) than Marfan syndrome patients. Patients without a detected disease-causing variant presented aortic manifestations significantly less frequently than patients with a detected variant (*Figure 3c*).

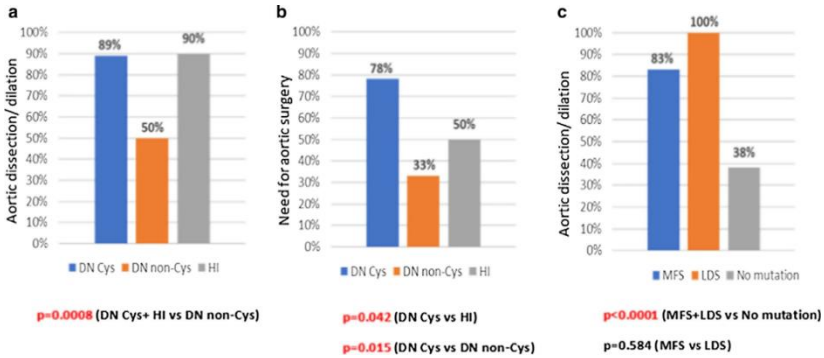

Figure 3 Aortic involvement comparisons between different *FBN1* mutation types, between Marfan syndrome and Loeys-Dietz syndrome, as well as between patients with and without identified disease-causing variant in our genotype-phenotype correlations study.

4.2 Arterial tortuosity

4.2.1 Study population

Apart from hypertension being more frequent in Group B compared to the other groups, the severity groups of Marfan syndrome patients did not differ from each other. No difference could be observed in terms of atherosclerosis risk factors between Marfan syndrome and control individuals: hypertension (p=0.832), hyperlipidemia (p=0.478), smoking (p=0.413), diabetes (p=0.663) and history of coronary artery disease (p=1.000).

4.2.2 Tortuosity of the visceral arteries in Marfan syndrome compared to controls

Distance metric (DM) of the splenic and both renal arteries was greater in Marfan syndrome patients compared to controls (2.44 [1.92-2.80] vs. 1.75 [1.57-2.18] p < 0.001; 1.16 [1.10-1.28] vs. 1.11 [1.07-1.15] p= 0.011; 1.40 [1.29-1.70] vs. 1.13 [1.09-1.23] p< 0.001 respectively) (*Figure 4A*).

Inflection count metric (ICM) of the splenic artery was significantly increased in comparison to the control individuals (31.43 [22.75-42.39] vs. 26.02 [17.86- 34.30] p= 0.026) and the right renal artery only tended to have a larger ICM 14.95 [10.65-18.53] vs. 12.03 [9.26- 15.17] p= 0.056) in patients with Marfan syndrome (*Figure 4B*).

The right and left renal arteries of Marfan syndrome patients had a significantly lower sum of angles metrics (SOAM) than the control group (0.55 [0.45-0.65] vs. 0.62 [0.53-0.71] p= 0.024; 0.43 [0.38-0.53] vs. 0.55 [0.49-0.64] p< 0.001, respectively) (*Figure 4C*).

The splenic, the right and the left renal arteries had significantly increased ICM/SOAM in Marfan syndrome patients compared to controls (73.35 [62.26-93.63] vs. 50.91 [43.19-65.62] p<0.001; 26.52 [20.69-30.24] vs. 19.95 [16.47-22.95] p< 0.001;

22.81 [18.64-30.96] vs. 18.38 [15.29-21.46] p<0.001) (*Figure 4D*).

4.2.3 Tortuosity of the visceral arteries in the severity groups of Marfan syndrome

Marfan syndrome patients without severe aortic involvement (Group A) compared to Marfan syndrome patients who underwent aortic surgery due to various indications (Group B and C) had significantly lower DM in case of the right (p=0.039 and p=0.039) and the left (p=0.041 and p=0.041) renal arteries *(Figure 5A).* In terms of the other tortuosity parameters, the difference was significant only when comparing the left renal ICM/SOAM of Group A and Group B (Kruskal-Wallis p=0.040; p=0.023) (*Figure 5B*).

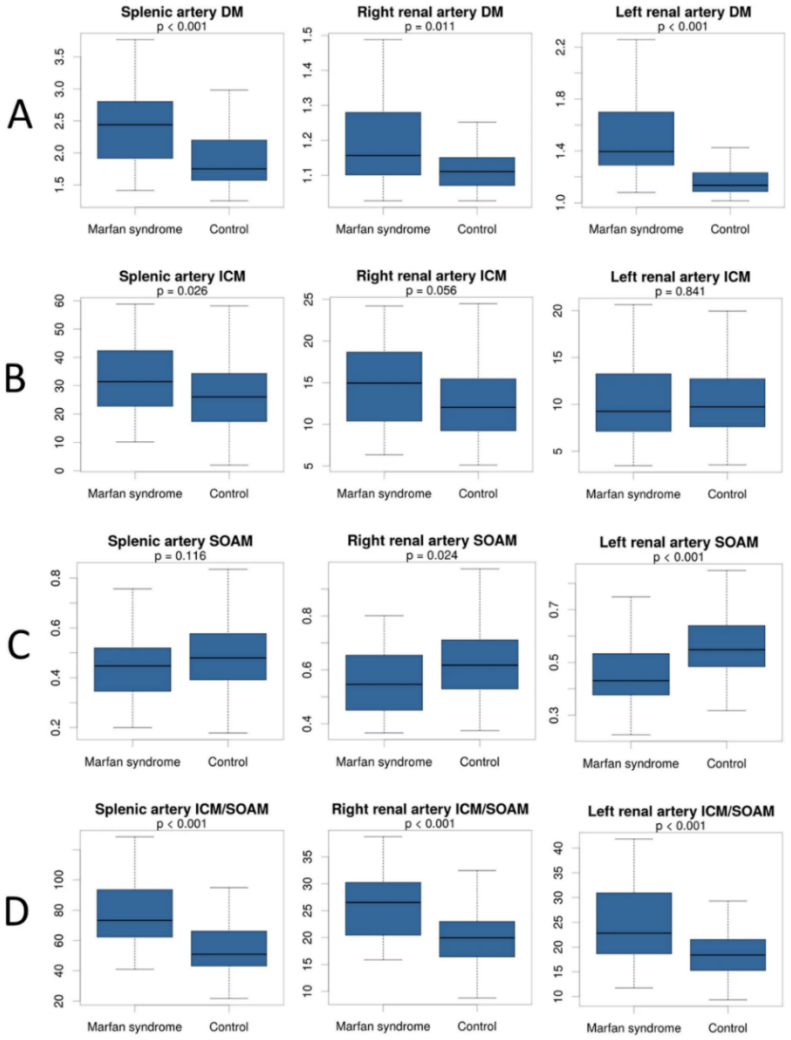

Figure 4 Comparison of the tortuosity metrics of the splenic artery and the right and left renal arteries between Marfan syndrome patients and control subjects in our visceral arterial tortuosity study.

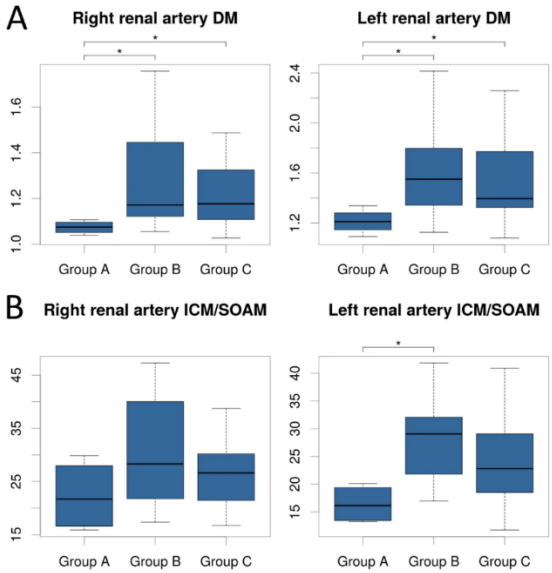

Figure 5 Comparison of the tortuosity of the right and left renal arteries between the severity groups of Marfan syndrome patients in our visceral arterial tortuosity study.

4.2.4 Following the progression of arterial tortuosity through a case of a Marfan syndrome patient

Throughout the years of follow-up of a Marfan syndrome patient with bilateral IMA aneursyms, as the RIMA and LIMA aneurysms progressed, DM demonstrated an increase in case of the RIMA and the LIMA, showing a progression of vessel tortuosity. The thoracic aorta showed an overall increase in the ICM, SOAM and ICM/SOAM parameters, indicating a progression of tortuosity with increasing amplitude and frequency of the curves, dominated by the rise in amplitude as suggested by ICM/SOAM.

5. Conclusions

The type of genetic variant and the degree of visceral arterial tortuosity could serve as potential predictors of more severe aortic involvement in Marfan syndrome patients.

Dominant negative mutations with cysteine elimination and haploinsufficient variants of the *FBN1* gene may lead to more severe aortic manifestations than dominant negative mutations without cysteine amino acid substitution. Furthermore, DN Cys variants may even be more deleterious than HI ones.

Visceral arterial tortuosity was demonstrated to be increased in Marfan syndrome patients compared to control individuals. Increased tortuosity of both renal arteries was associated with more severe aortic involvement in patients with Marfan syndrome.

Our findings indicate that Marfan syndrome patients with DN Cys and HI variants and Marfan syndrome patients with increased renal arterial tortuosity may need to undergo more frequent follow-up and a prophylactic surgery at smaller aortic diameters than patients who belong to the lower risk groups.

We emphasize the relevance of a multi-gene panel in the genetic testing of patients with Marfan syndrome, with subsequent CNV screening in negative cases. Furthermore, patients with Marfanoid habitus not meeting the clinical diagnosis of Marfan syndrome should also undergo genetic testing in order to identify LDS patients, who are likely to present with severe aortic involvement.

6. Bibliography of the candidate's publications

Publications related to the thesis:

IF=20.423

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IF=25.802

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[#] shared first authorship

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