“Apple does not fall far from the tree” – subclinical atherosclerosis in children with familial hypercholesterolemia.

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Abstract

Background

Familial hypercholesterolemia (FH) increases the risk of atherosclerosis in children and adults. Atherosclerotic cardiovascular disease in young patients FH is usually subclinical but recognition of children with more pronounced changes is crucial for adjusting effective management. Thus, we aimed to use ultrasonography with two-dimensional speckle tracking (2DST) and tonometry to evaluate atherosclerotic changes in parents and their children with FH.

Methods

Applanation tonometry and carotid arteries sonography with evaluation of the intima-media complex thickness (IMCT) and application of the 2DST were performed in 20 families with FH (20 parents and 29 children). The same size control group (age and sex matched) was included. Results were compared between peers and between generations together with the correlation analysis.

Results

Adults with FH in comparison with healthy peers presented significantly more atherosclerotic plaques (9 vs. 2, p=0.0230), had significantly thicker IMC (0.84±0.19 vs. 0.56±0.06 mm, p<0.0001) and had stiffer arterial wall (for stain: 6.25±2.3 vs. 8.15±2.46, p=0.0103). In children from both groups there were no atherosclerotic plaques and IMCT did not differ significantly (0.42±0.07 vs. 0.39±0.04, p=0.1722). However, children with FH had significantly stiffer arterial wall according to sonography with 2DST (for stain: 9.22±3.4 vs. 11.93±3.11, p=0.0057) and tonometry (for the pulse wave velocity: 4.5±0.64 vs.3.96±0.62, p=0.0047). These parameters correlated with atherosclerosis surrogates in their parents (p<0.001) but were not significantly affected by presence of presumed pathogenic gene variant.

Conclusions

Children with FH presented subclinical atherosclerosis manifesting as decreased arterial wall elasticity. Degree of stiffening was associated with advancement of atherosclerosis in their parents but did not present significant association with gene variants. Sonography with application of 2DST seems to be the best candidate for comprehensive evaluation of atherosclerosis in families with FH.
Introduction
Familial hypercholesterolemia (FH) is an autosomal dominant hereditary disease, causing life-long elevated plasma LDL cholesterol (LDL-C) levels. Homozygous form is rare but recent study suggested that heterozygous one might affect 1 in 200-250 individuals from the general population [1]. Both, pre-mortem [2] and post-mortem studies [3] confirmed FH association with development of premature atherosclerosis in children. Moreover, atherosclerotic cardiovascular disease (ASCVD) that usually starts in middle age or letter was also reported to progresses rapidly at an age of around 10 years in patients with FH. Hence, guidelines for Europe and America highlights that early diagnosis of FH and statin treatment from childhood are necessary for preventing the early-onset ASCVD [4,5].
Since atherosclerosis in children with FH typically presents in subclinical stage, standard diagnostic imaging methods might be not sufficient. Even discreet morphological changes (thickening of intima-media complex [IMC] evaluated with ultrasound [6,7]), might not be present while the function of the arterial wall is impaired already. This stiffening process can be assessed with applanation tonometry, with the pulse wave velocity (PWV) and augmentation index (AI) being its two common surrogates. Although tonometry is stated as the gold standard technique it is not routinely used in clinical practice [8].
On the other hand, a novel sonographic technique - the 2-dimensional speckle tracking (2DST) - can be an alternative that proved its usefulness in evaluation of risk groups of adults [9,10] and children as well [11]. This method allows evaluating pattern of arterial wall deformation due to the flowing pulse wave. Degree of deformation (expressed as strain in %) and its acceleration in time (expressed as strain rate in 1/s) reflect local arterial wall elasticity [9]. Thus, sonography becomes a comprehensive technique that allows tailoring the diagnostic method for each family member. We assume that in children discreet disturbances in arterial wall function could be recognized with 2DST, while in their parents atherosclerotic plaques, including their hemodynamic significance, can be assessed with 2D and Doppler ultrasound.
This study aims to compare morphological and functional surrogates of atherosclerosis between family members with FH and healthy peers. Firstly, we hypothesize that in families with FH both
parents and their offspring will present significantly more advanced atherosclerosis than their healthy counterparts. Secondly, that both diagnostic techniques (ultrasound and applanation tonometry) will allow to recognize functional abnormalities. And finally, that the degree of atherosclerotic changes in children with FH will be associated with their aggravation in parents and with type of mutation leading to FH. Confirmation of these hypotheses will enforce the usage of ultrasound with the 2DST technique as a method of choice in evaluation of atherosclerosis in children and adults with FH.

**Methods**

To this cross-sectional study we recruited 20 FH families, who are under care of the Regional Rare Disease Center in the Polish Mother’s Memorial Hospital Research Institute (PMMHRI). They comprised 20 parents (10 males, and 10 females) and their 29 children (13 males, and 16 females). The FH was confirmed based on genetic array and/or the Dutch Lipid Clinic Network (DLCN) and Simone Broome Register. In all children the next generation sequencing was performed (MiniSeq, Illumina, Inc., San Diego, US) using custom panel of 21 genes. Obtained data were analyzed with available databases and predictive programs (sorting intolerant from tolerant [SIFT] and polymorphism phenotyping [PolyPhen]). Detected variants were confirmed with the Sanger sequencing. All further examinations were performed within 6 months from the diagnosis of FH in children. Family members not affected with FH were not included. We matched the same number of healthy families (no significant difference in sex and age) to the control group. Lipid profile of children from the control group was within normal limits while adult individuals did not present significant abnormalities (levels of total cholesterol (TC), low-density lipoprotein (LDL) and triglycerides (TG) were up to borderline/borderline high levels). Eight adults from the control group reported regular treatment with statins. In both groups exclusion criteria were chronic diseases (except for hypertension) that might increase the risk of atherosclerosis (i.e.: diabetes mellitus, chronic inflammatory diseases like rheumatoid arthritis or non-specific inflammatory bowel diseases). Hypertension is a common disease and we decided to include this risk factor into analysis instead of excluding patients from the study. If the exclusion criterion was present in one family member the whole family was excluded from the study. No participant had former cardiovascular events (i.e.: acute coronary syndrome or stroke) that were
also assumed as criteria excluding the whole family from the study. Additionally, in both groups we obtained data regarding hypertension (diagnosed according to the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) guidelines as Systolic pressure >140 mmHg and/or diastolic pressure >90 mmHg [https://doi.org/10.1093/eurheartj/ehy339]) and lifetime tobacco exposure (pack years) as one of the main atherosclerosis risk factors.

**Study protocol**

In all patients blood samples were obtained up to 7 days prior to the further examinations. Lipid profile (TC, LDL, TG, high-density lipoprotein [HDL] and non-HDL cholesterol) and HbA1C % were assessed.

At appointed date family members arrived at the hospital at morning hours fasting. They were measured and weighted in light clothing and without shoes to calculate body mass index (BMI). Then the sonographic and tonometric tests were performed by two independent researches blinded to the group affiliation. Examinations techniques were standard, and the detailed description together with accuracy and reproducibility for adults and children has been reported before [9-11]. Briefly, the sonographic examination was performed with the Samsung RS80 apparatus equipped with a high-frequency linear probe (L3-12A) and Arterial Analysis™ software. The ECG trace was obtained, and the blood pressure was measured in patients lying supine. Major atherosclerotic changes were assessed first (atherosclerotic plaques – soft or calcified, focal IMC thickenings of more than 50% of the adjacent parts of the IMC layer [9]).

Then, the B-mode cine loops of the long and short axis through the common carotid artery (CCA) (just below the bulb) were stored during three consecutive heartbeats for each cross-section. If there was no plaque IMCT was assessed (semi-automatically, during end diastole, at the distance of 150-250 points, with the quality index (QI) > 0.9). To evaluate strain and strain rate in short axis cine loop the circular region of interest (ROI) was pointed along the border between the wall and arterial lumen. Parameters were assessed automatically and mean results from 6 heartbeats (three for each side) were included into the analysis.

The PWV and AI were evaluated by another researcher (9 years of experience in tonometric studies)
using a SphygmoCor applanation tonometer (SphygmoCor, AtCor Medical, New South Wales, Australia). The AI was assessed based on two measurements of the brachial artery pressure. Then, in patients laying supine, the PWV was measured by recording the arterial pressure waveform at the carotid and femoral artery sampling sites. If the operator index was lower that 75% measurements were repeated.

**Statistical analysis:**
Categorical variables were presented as number and percentages, while continuous variables as mean and standard deviation (SD). In comparison of nominal data between groups the Chi² test was applied. Normality of continues variables distribution was evaluated with the Shapiro-Wilk test. Due to distribution other than normal comparisons of means between two independent subgroups (adults with FH vs. healthy adults and children with FH vs. healthy children) were performed with the Mann-Whitney test. When subgroups were related (adults with FH vs. children with FH and healthy adults vs. healthy children) the Wilcoxon signed rank test was applied. Associations between continuous variables were evaluated with the Spearman’s rank correlation test. The analysis was performed with Statistica 12 software (StatSoft Poland, Cracow, Poland). In general a p-value lower than 0.05 was considered significant, however for multiple comparisons the Bonferroni correction was applied.

**Results**
Comparison of demographic data and laboratory tests are presented in Table 1. Individuals with FH did not differ significantly according to determined additional risk factors of atherosclerosis (BMI, hypertension, tobacco exposure, increased concentration of HbA1C). However, both parents and children with FH presented significantly higher values of TC, LDL-C, non-HDL-C and TG in comparison to their healthy counterparts, despite the fact that all adults were treated with statins.

**Morphological features of atherosclerosis.**
Adults with FH had significantly more arterial plaques (total number of 7, mean thickness 4 mm±1,2 mm) than parents from the control group (total number of 2, thickness of 1,8 mm and 2,3 mm). In analysis of IMCT adults with FH presented significantly thicker layer than observed in healthy adults (Table 2). Moreover, in both groups parents had significantly thicker IMC than their children (Table
In children there were no atherosclerotic plaque and the IMCT did not differ significantly between individuals with FH and healthy one.

**Functional features of atherosclerosis.**

Both, parents and children with FH, presented significantly increased arterial stiffness in comparison with their healthy counterparts *(Table 2)*. It was confirmed with tonometry as well as with the 2DST *(Fig. 1)*. Stiffness parameters differed also between parents and children from the same group (adults with FH vs. children with FH and healthy adults vs. healthy children, *Table 2*).

**Association of atherosclerosis markers with gens.**

In 5 children we found only single, likely pathogenic, gene variant, 14 children had 2 gene variants, 8 children had 3 variant gens, 3 children had 4 gene variants and in two children we did not find any variant from the analyzed panel. Sequencing identified *LDLR* mutations as the most common cause of FH in the tested group. In 17 patients 15 substitutions, one intragenic deletion and one intragenic duplication of *LDLR* were found. Variants were also revealed in *APOE* (6 children), *PCSK9* (4 children), *LCAT* (4 children), *ABCG5* (4 children), *ABCA1* (3 children), *GPIHBP1* (2 children), *SCAP* (2 children) and *APOA* (1 child).

We compared atherosclerosis markers between individuals in whom variants were presumed pathogenic according to prognostic programs or literature. There was only a trend towards more advanced atherosclerotic changes in children with these variants but the difference was not significant *(Table 1 Suppl.)*.

**Parameters correlations**

In both groups and for both generations there was a significant correlation between IMCT and parameters derived from tonometry and those from 2DST. Only in adults stiffness parameters correlated with IMCT. On the contrary, only in adults with FHs there was a significant correlation between IMCT and stiffness parameters with stiffness parameters in their children *(Table 3)*. In all subgroups, there was no significant association between concentrations of lipid profile components and analyzed surrogates of atherosclerosis.

**Discussion**
We showed that family members with FH, parents as well as their offspring, present more advanced atherosclerosis than their healthy peers. Adults with FH had both morphological and functional changes while in children with FH only arterial function was impaired (decreased elasticity). Moreover, in patients with FH there was a correlation of atherosclerosis surrogates between adults and their children. On the other hand, presence of presumed pathogenic gene variant in children did not result in significantly aggravated markers of atherosclerosis. Finally, disturbances of arterial wall elasticity were recognized in ultrasound and applanation tonometry and they correlated with each other. However, sonography allows for more comprehensive evaluation of atherosclerosis aggravation.

FH increases the risk of atherosclerosis and predispose to cardiovascular events in younger age than in healthy population [12,13]. Although monozygotic form is rare, as many as 1 per 250 people from general population is a heterozygote [14] what gives approximately 4.5 million individuals in Europe, of whom 20–25% are children and adolescents [15]. Despite analyses proofing cost effectiveness of FH screening [16] and treatment standards, which are developed (diet, controlling risk factors, statins [5]) or developing (gene- and cell-based therapies [17]), under-diagnosing of the disease is a major problem [18]. Our work provides another argument for introducing screening programs because, even in children with no significant morphological changes, arterial wall function can be already impaired. Diagnosis of subclinical arterial wall stiffening might improve risk stratification and clinical management in FH patients [13,19].

Many researchers have already confirmed increased risk of atherosclerosis (also subclinical [20]) and subsequent cardiovascular events in adults with FH [21]. Studies concerning children with FH are not as numerous [15,22,23,24] but it is well documented that FH children have significantly increased IMCT in carotid arteries, femoral arteries and aorta when compared with healthy children as well as healthy siblings [24,25]. Although, there are discrepancies in age from which these differences become significant (between 10-12 for siblings [24,26] and 9-11 years for nonrelated controls [22,25]) and they can also be affected by treatment with statins. Luirink et al., in their study with 20-years long follow up, proofed that although children with FH had initially significantly thicker IMC than healthy siblings, differences become insignificant during years of treatment with statins. It is worth to
notice that in aforementioned studies morphological changes were slight (fractions of millimeter). Thus, to reach the level of statistical significance study populations had to be larger than in our research. It may explain why we observed only a slight numerical trend towards thicker IMC. On the other hand, in our group we were able to detect significant deterioration of arterial wall function. It might suggest superiority of arterial wall elasticity surrogates as markers of atherosclerosis in children with FH.

Arterial wall function was reported to be impaired in children with FH, however in former researches it was proofed mainly by flow-mediated dilation (FMD) test [27,28] and markers calculated from 2D images (e.g. young elastic modulus, beta-stiffness index) [28,29]. Riggio et al. [29] investigating the group of 44 children with increased cholesterol levels (18 with FH) showed that arterial wall function was impaired in comparison with healthy control, while there was no significant difference in IMCT. Although, they also used echo-tracking software, it was applied to calculate arterial wall elasticity surrogates (beta-stiffness index, arterial compliance, AIx, local PWV, Young elastic modulus) and results were not confirmed by a gold standard method (tonometry). Aggoun et al. [28] using sonography and FMD test also showed that in children with FH (30 males, mean age of 11±2 years old) the arterial wall function was impaired when no significant changes in IMCT were observed.

Despite applying surrogates depending on blood pressure in this last study, both studies are in line with our observations, and confirm that there is a continuum of atherosclerotic changes that impairs the arterial wall function first and then affects its morphology.

To conclude the above, in our opinion application of sonography with 2DST technique is more convenient and efficient than employing other methods for evaluation of atherosclerosis. On the contrary to the FMD procedure it does not require several minutes of painful forearm compression. Currently, purchasing of 2DST software is cheaper than buying an applanation tonometer, which handling requires experience. Moreover, repeatability of the stain evaluation with 2DST is higher than reported for tonometry, while their results correlate with each other [11,30]. Finally, sonography is the most comprehensive technique because after storing a few loops of arterial wall motion not only function surrogates can be calculated in a semiautomatic manner, but also IMCT and atherosclerotic
plagues can be assessed, what would be especially important in older patients. To confirm our opinion, we were able to examine two generations of patients and showed not only that individuals with FH had significantly advanced atherosclerosis than their healthy peers, but also that there is a relation between degree of atherosclerotic changes between parents and their offspring. This last observation is unique for such a young group of patients. Till now, it was only reported that maternal or paternal origin of FH does not affect carotid IMCT or phenotype of plasma lipid levels, while type of transferred mutation does [31,32]. As far as a direct relation is concerned, in study of 154 families with other cardiovascular risk factors than FH, there was a correlation between IMCT between parents and children, however median of age for parents was 61 years and for children 36 years [33].

Recognition of genetic background of the FH becomes important nowadays because particular mutations were reported to affect phenotype of patients and due to new possibilities of treatment [33-35]. Unfortunately, we were not able to show significant association between presence of presumed pathogenic gene variants and aggravation of atherosclerosis. We think that it is due to small analyzed group of children and young age, that did not allow to develop differences in phenotype. On the other hand, we suspect that even in children genetic variability plays a significant role. It was indicated by a significant correlations of atherosclerosis markers between parents and their offspring who share the same gene variants.

**Limitations**

We excluded from the study healthy family members. In comparison of FH children with their healthy siblings significant differences in IMCT were observed from age 7 years. It would be interesting to compare strain and strain rate between them. However, our study addressed relation between adults and children affected with FH. Secondly, evaluated group did not allow for such an analysis due to not enough of siblings appropriate to enter the study.

Secondly, small study population did not allow to draw conclusions on association between genetic variance and aggravation of atherosclerosis. Nevertheless, our main aim was to indicate convenient and reliable technique for examining whole families with FH what gives foundation for future studies of larger population.
Conclusions
Children with FH presented subclinical atherosclerosis that manifested with arterial wall stiffening. Aggravation of changes was associated with advancement of atherosclerosis in their parents but was not significantly affected by a type of recognized gene variant. Sonography with application of 2DST seems to be the best candidate for comprehensive evaluation of atherosclerosis in families with FH.

Declarations

Ethics approval and consent to participate
The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Bioethical committee (approval number 28/2016). All the participants/participants’ guardians signed informed consent.

Consent for publication
All authors of the manuscript have read and agreed to its content and are accountable for all aspects of the accuracy and integrity of the manuscript in accordance with ICMJE criteria. The article is original, has not already been published in a journal, and is not currently under consideration by another journal. We agree to the terms of the BioMed Central Copyright and License Agreement, where applicable, Open Data policy.

Availability of data and material
On request.

Competing interests
MB - speakers bureau: Abbott/Mylan, Abbott Vascular, Actavis, Akcea, Amgen, Biofarm, KRKA, MSD, Sanofi-Aventis, Servier and Valeant; consultant to Abbott Vascular, Akcea, Amgen, Daichii Sankyo, Esperion, Lilly, MSD, Resverlogix, Sanofi-Aventis; Grants from Sanofi and Valeant; all other authors have nothing to declare.

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Authors' contributions
MP: research concept and design, collection and assembly of data, data analysis and interpretation, writing the article, final approval of article.

KS: collection and assembly of data, writing the article, final approval of article.

MS: recruitment of patients, collection of data, data analysis and interpretation, writing the article, final approval of article.

MP-B: recruitment of patients, assembly of data, data analysis and interpretation, writing the article, final approval of article.

AK: recruitment of patients, writing the article, final approval of article.

ES: research concept and design, data analysis and interpretation, critical revision of the article, final approval of article.

MT: research concept and design, data analysis and interpretation, critical revision of the article, final approval of article.

SG: research concept and design, data analysis and interpretation, critical revision of the article, final approval of article.

LR: performing and analyzing genetic tests, writing the article, critical revision of the article, final approval of article.

AG: performing and analyzing genetic tests, critical revision of the article, final approval of article.

ML: research concept and design, assembly of data, data analysis and interpretation, writing the article, final approval of article.

PG: research concept and design, critical revision of the article, final approval of article.

MB: research concept and design, data analysis and interpretation, critical revision of the article, final approval of article.

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Tables

Table 1. Clinical characteristics of the patients.

| Feature          | Adults with FH | Healthy adults | P | Children with FH | Healthy children | P |
|------------------|----------------|----------------|---|------------------|------------------|---|

| Age [years (SD)] | 37.5 (7.4) | 38.6 (6.4) | 0.5772 | 11.1 (4.5) | 9.8 (4.0) | 0.3148 |
| Sex | Females [n (%)] | 10 | 10 | 1.000 | 16 | 15 | 0.7924 |
| | Males [n (%)] | 10 | 10 | 13 | 14 |
| BMI [kg/m2 (SD)] | 24.2 (1.9) | 23.9 (1.8) | 0.5778 | 18.5 (3.2) | 17.2 (2.1) | 0.1087 |
| Smoking (pack years) | 6.26 (2.8) | 6.28 (4.0) | 0.9929 | 0 | 0 | 1.000 |
| Hypertension [n (%)] | 7 (35) | 6 (30) | 0.7924 | 0 | 0 | 1.000 |
| HbA1C | 4.89 (0.38) | 5.01 (0.15) | 0.1523 | 3.99 (1.29) | 4.23 (1.18) | 0.8473 |
| TC | 316.55 (65.57) | 158.52 (16.81) | <0.0001* | 365.38 (99.23) | 107.30 (18.09) | <0.0001* |
| LDL | 217.50 (53.47) | 80.70 (16.97) | <0.0001* | 242.45 (58.33) | 57.00 (12.15) | <0.0001* |
| HDL | 68.91 (32.32) | 58.17 (18.67) | 0.1772 | 48.73 (14.82) | 52.30 (8.35) | 0.3128 |
| Non-HDL | 247.63 (79.82) | 100.35 (21.07) | <0.0001 | 316.65 (98.05) | 55.00 (18.70) | <0.0001 |
| TG | 111.63 (25.21) | 93.04 (16.88) | 0.0056* | 99.35 (32.22) | 59.78 (14.33) | <0.0001* |

*Significant differences according to Mann-Whitney test; HbA1C - Glycosylated Hemoglobin, Type
A1C; TC - total cholesterol; LDL - Low-density lipoprotein; HDL - High-density lipoprotein; TG - Triglycerides; P - p-value.

Table. 2. Comparison of atherosclerosis surrogates between patients with FH and healthy control according to generations.

| Feature | Adult FH | Healthy Adult | P | Child FH | Healthy Child | P | Adult FH | Healthy Adult | P |
|---------|----------|---------------|---|---------|---------------|---|---------|---------------|---|
| Atherosclerotic plaques [n (%)] | 9 | 2 | 0.023 | 0 | 0 | 1.000 |
| Mean IMCT [mm (SD)] | 0.84 (0.19) | 0.56 (0.06) | 0.001 | 0.42 (0.07) | 0.39 (0.04) | 2 | 0.84 (0.19) | 0.42 (0.07) | 0.001 |
| PWV [m/s (SD)] | 6.12 (1.08) | 5 (1.38) | 0.005 | 4.50 (0.64) | 3.96 (0.62) | 7 | 6.12 (1.08) | 4.50 (0.64) | 0.001 |

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### Table 3. Correlations between atherosclerosis surrogates (cumulated data for all subgroups and between parents and children with FH.)

IMCT – intima media complex thickness; PVW – pulse wave velocity; AI – augmentation index; P – p-value.
### Parameters

| Parameters | Strain | Strain Rate |
|------------|--------|-------------|
|            | R      | P           | R          | P          |
| IMCT       | -0.7043| 0.0001      | -0.6659    | 0.0001     |
| PWV        | -0.8080| 0.0001      | -0.7788    | 0.0001     |
| AI         | 0.8793 | 0.0001      | 0.8229     | 0.0001     |

### Children with FH

| Parameters | PWV   | Al      | Strain | Strain Rate |
|------------|-------|---------|--------|-------------|
|            | R     | P       | R      | P           | R   | P   | R   | P   |
| A          | 0.5427| 0.009   | -0.6520| 0.001       | -0.6055| 0.002| -0.67| 0.000 |
| d          | 1     | 0       | 8      | 02          | 6    |
| ul         | 0.6497| 0.001   | -0.7891| <0.0        | -0.6970| 0.000| -0.71| 0.000 |
| ts         | 1     | 001     | 3      | 13          | 2    |
| wi         | -0.5629| 0.006 | 0.6650 | 0.000       | 0.5739| 0.005| 0.623| 0.002 |
| th         | 4     | 7       | 2      | 1           | 0    |
| F          | -0.5964| 0.003 | 0.7482 | 0.000       | 0.6374| 0.001| 0.718| 0.000 |
| H          | 4     | 1       | 4      | 2           | 2    |
| Strain     | -0.6551| 0.000 | 0.8113 | <0.0        | 0.7248| 0.000| 0.776| <0.0 |
| n          | 9     | 001     | 1      | 7           | 001  |

**IMCT** – intima media complex thickness; **PWV** – pulse wave velocity; **AI** – augmentation index; **P** – p-value

**Figures**
Examples of graphs presenting strain and strain rate in each group of patients. Colored lines represent segments of vessel while dotted white line is a mean value.

Supplementary Files
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Table 1 Suppl.docx