Full title: Human Pulmonary Vein Myocardial Sleeve Autonomic Neural Density and Cardiovascular Mortality

Short title: Pulmonary Vein Autonomic Innervation

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Abstract

Myocardial sleeves around pulmonary veins (PVs) are highly innervated structures with heterogeneous morphological and electrophysiological characteristics. Autonomic nerve dysfunction in the myocardium may be associated with an increased risk of cardiovascular morbidity and mortality. This paper studied autonomic neural remodeling in myocardial sleeves around PVs and atrial-PV ostia with immunohistochemical and morphometrical methods with clinicopathological correlations. PVs were collected from 37 and atrial-PV ostia from 17 human autopsy hearts. Immunohistochemical analysis was performed using antibodies against tyrosine hydroxylase, choline acetyltransferase, and growth-associated protein 43. In the PV cohort, subjects with immediate cardiovascular cause of death had significantly decreased sympathetic nerve density in fibro-fatty tissue vs. those with non-cardiovascular cause of death (1624.53 vs. 2522.05 $\mu m^2/mm^2$, P=0.038). In the atrial-PV ostia cohort, parasympathetic nerve density in myocardial sleeves was significantly increased in subjects with underlying cardiovascular cause of death (19.48 $\mu m^2/mm^2$) than subjects with underlying non-cardiovascular cause of death with no parasympathetic nerves detected (P=0.034). Neural growth regionally varied in sympathetic nerves and was present in most of the parasympathetic nerves. Heterogeneous autonomic nerve distribution and growth around PVs and atrial-PV ostia might play role in cardiovascular morbidity and mortality. No association in nerve density was found with atrial fibrillation.

Keywords

myocardial sleeves, pulmonary veins, autonomic nervous system, nerve density, cardiovascular mortality, morphometry

1. Introduction

Pulmonary veins (PVs) and adjacent atrial-PV ostia participate in the regulation of atrial fibrillation (1–3). Myocardial sleeves are extensions of the atrial myocardium that reach proximal portions of PVs (4) and they are morphologically heterogeneous (3,5). In addition, PVs are associated with variable responses to autonomic activity and unique electrophysiological characteristics (6).

The autonomic nervous system (ANS) regulates heart activity by stimulatory and inhibitory neurotransmitters. In pathologic conditions, such as congestive heart failure and myocardial ischemia, myocardial remodeling and dysregulation of ANS increase the risk for lethal arrhythmias and sudden cardiac death (7–13). The process is usually associated with structural, electrophysiological, and neural remodeling of the myocardium (7,14,15).

Uneven regional distribution of the autonomic nerves and various patterns of innervation were studied in caval veins (16), PVs, and atrial-PV ostia (17–21). However, the role of myocardial sleeves around PVs and in atrial-PV ostia in the pathogenesis of cardiovascular mortality and various heart diseases awaits more studies.

In our recent study, we assessed nerve density in caval veins and provided evidence of sympathetic denervation to be associated with cardiovascular mortality (16). We hypothesized that similar mechanisms may apply also in the PVs and atrial-PV ostia area. Thus, in the current study, we scoped on autonomic neural remodeling in and around myocardial sleeves in PVs and atrial-PV ostia using immunohistochemistry and computerized morphometry to measure the distribution of ANS nerves and ganglia in myocardial sleeves and surrounding fibro-fatty tissue in PVs and atrial-PV ostia of autopsy hearts.

2. Materials and Methods

2.1 Characterization of Pulmonary Vein Cohort

PVs were collected from 37 autopsies (mean age ± standard deviation (SD) 68±9 years; M:F ratio 22:15; mean heart weight ± SD 484±140 g). The most common immediate cause of death was cardiovascular (CVS) (n=12), followed by respiratory (n=9), infectious (n=6), brain death (n=4), and miscellaneous in the rest of the cases (n = 6). Out of 12 subjects with documented immediate CVS cause of death, 8 subjects (66.6%) were diagnosed with heart failure. Underlying CVS cause of death was diagnosed in 17 cases and malignancies in 9 cases. Other underlying causes of death included central nervous system diseases (n=3), chronic obstructive pulmonary disease (n=1), congenital connective tissue disorder (n=1), chronic tubulointerstitial nephritis (n=1), diabetes mellitus (n=1), and anemia (n=1). According to the autopsy referral, 14 subjects were diagnosed with atrial fibrillation (AF) and 23 subjects had documented sinus rhythm (SR). Detailed clinical information of the PV cohort is shown in Table 1.

2.2 Characterization of Atrial-Pulmonary Vein Ostia Cohort

Atrial-PV ostia were collected from 17 autopsies (mean age ± SD 63±15 years; M:F ratio 10:7; mean heart weight ± SD 484±139 g). The most common immediate cause of death was cardiovascular in 8 cases followed by infectious in 3 cases, respiratory in 2 cases, gastrointestinal and brain death each in 1 case, and miscellaneous in 2 cases. Underlying cardiovascular cause of death was diagnosed in 11, malignant in 1, gastrointestinal in 1, hematologic in 1, and miscellaneous in 3 subjects. Out of 11 subjects with documented underlying CVS cause of death, 8 subjects (72%) were diagnosed with systemic atherosclerosis. According to the autopsy referral, 7 subjects had documented AF and 10

subjects had documented SR. More details on the atrial-PV ostia cohort are shown in Table 2.

2.3 Sample Collection

PVs and atrial-PV ostia were collected from adult autopsy hearts at the Fingerland Department of Pathology, Charles University Hospital, Hradec Králové, Czech Republic (3).

After the excision of the heart, PVs were separated from the left atrium at the level of the atrial-PV ostium, which was macroscopically determined. All PVs were longitudinally cut into pieces, spread, fixed in 10% buffered formalin, and processed into paraffin blocks. The collection technique was described in detail previously (3). Atrial-PV ostia were collected and processed correspondingly. The samples consisted of 94 PVs (24 right upper, 25 right lower, 24 left upper, 19 left lower, one right common and one left common PVs) in the PV cohort. The atrial-PV ostia cohort included ostia samples from 51 PVs (11 right upper, 10 right lower, 9 left upper, 11 left lower, 4 right common and 6 left common PVs). In total, 125 samples of PVs tissue and 90 samples of atrial-PV ostia tissue were included in the present study. The presence of myocardial sleeves was microscopically verified in all included samples.

2.4 Immunohistochemical Analysis

The collected tissue samples were cut into 5 µm thick sections and stained as described previously (16). In brief, the Ventana Automatic System (Ventana Medical Systems, Tucson, AZ, USA) was used for the immunohistochemical staining. Anti-tyrosine hydroxylase antibody (TH; dilution 1:100, AB152, Chemicon, Merck KGaA, Darmstadt, Germany) was used to detect sympathetic nerves and ganglia, anti-choline acetyltransferase antibody (CHAT; dilution 1:300, AB143, Chemicon, Merck KGaA, Darmstadt, Germany) was used to label parasympathetic nerves and ganglia, and growth-associated protein 43 antibody

(GAP43; dilution 1:100, AB5220, Chemicon, Merck KGaA, Darmstadt, Germany) was used to detect neural growth.

2.5 Morphometrical Analysis

The samples were scanned (NanoZoomer-XR, Hamamatsu Photonics, Hamamatsu, Japan) at 40x magnification. Bioimage analysis software was used for histomorphometry (QuPath, Queens University, Belfast, Northern Ireland (22)). Nerve density ($\mu m^2/mm^2$) was quantified as in the previous study (16). Briefly, the nerves were detected in the whole slide images, which were divided into two regions: myocardial sleeves and fibro-fatty tissue outside the sleeves. Total areas (mm^2) of the whole section, myocardial sleeves, and fibro-fatty tissue were measured in all samples. The nerve area (μm^2) was measured by manually delimiting positive nerves and ganglia at 20x magnification separately for TH-stained, CHAT-stained, and GAP43-stained sections.

To evaluate the subset of nerves showing neural growth (regenerative activity) in TH- and CHAT-positive nerves, the percentages of nerves stained positively with GAP43 antibodies were calculated in TH- and CHAT-stained samples. The nerves stained for both TH and GAP43, and CHAT and GAP43, respectively, were manually selected in all 3 analyzed areas (whole section, myocardial sleeves, and fibro-fatty tissue) within each pulmonary vein and atrial-PV ostium.

2.6 Statistical Analysis

Data are presented as mean ± standard deviation (SD). Categoric variables were evaluated as the count and percentage. Because of the small cohort size, the Mann–Whitney non-parametric test was used for continuous variables, and the chi-square test was used for categoric analysis. An average percentage value used to express the representation of neural

growth (GAP43-positive nerves) in TH- and CHAT-positive nerves, was calculated from all the TH- and CHAT-stained pulmonary vein samples. Statistical analysis was performed by the Statistical Package for the Social Sciences version 24.0 (SPSS Inc., Chicago, IL, USA). A *P*-value of < 0.05 was considered significant for these comparisons.

2.7 Ethical Statement

The study was approved by the Pirkanmaa Health Care District Ethical Committee (permission application number R15013). The collection and research use of the samples were approved by the Ethical Committee of the University Hospital, Hradec Králové, Czech Republic. The study was done in accordance with the Declaration of Helsinki.

3 Results

3.1 Autonomic Nervous System Characteristics and Localization

Myocardial sleeves were found in 62.8% of studied PVs (59/94), and 90.2% of atrial-PV ostia (46/51). TH-, CHAT-, and GAP43-positive nerves were identified both in muscle sleeves and surrounding fibro-fatty tissue, with a predominant location in the latter (Fig. 1A, B). TH- and GAP43-positivity dominated over CHAT-positive nerves in all PVs and atrial-PV ostia samples. Variably sized and shaped nerve bundles consisted of nerve fibers. Ganglia consisted of large ganglion cells and nerve fibers (Fig. 1, 2). Nerve bundles with colocalizing TH- and CHAT- positive fibers (i.e. intermediate nerves) were evaluated according to the predominant positivity.

3.2 Quantitative Evaluation

3.2.1 Study Cohorts' Characteristics

No differences were found in age, sex, and heart weight among subjects with cardiovascular mortality and the presence of atrial fibrillation in both studied cohorts (Table 1B, 2B). Additionally, no association in nerve density was found among subjects in age, sex, heart weight, heart rhythm, and cardiovascular mortality. Topographically, our study did not show any association in nerve density gradient among individual PV and atrial-PV ostia locations. Results of nerve densities of all pooled PVs and ostia are given below.

3.2.2 Pulmonary Veins

The total PV nerve density of TH-positive nerves was 2090.28±2607.59 $\mu m^2/mm^2$ in the whole section, 1248.67±2406.27 $\mu m^2/mm^2$ in myocardial sleeves, and 2187.86±2994.48 $\mu m^2/mm^2$ in the fibro-fatty tissue. The total CHAT-positive nerve density was 204.56±409.83

$\mu m^2/mm^2$ in the whole slide, 65.75±217.21 $\mu m^2/mm^2$ in the myocardial sleeves, and 241.30±598.71 $\mu m^2/mm^2$ in the fibro-fatty tissue. The GAP43-positive nerve density was 1754.71±2130.90 $\mu m^2/mm^2$ in the whole section, 1514.62±2973.23 $\mu m^2/mm^2$ in the myocardial sleeves, and 1622.63±2197.77 $\mu m^2/mm^2$ in the fibro-fatty tissue.

Although mean TH-positive nerve density of PV area was decreased in all measured regions (whole slide, myocardial sleeves, fibro-fatty tissue) in subjects with documented immediate CVS death (Table 3, Fig. 1C-F), only the fibro-fatty tissue region showed a statistically significant difference in comparison to subjects with documented immediate non-CVS cause of death (1378.5±2159.68 $\mu m^2/mm^2$ vs. 2512.53±2771.53 $\mu m^2/mm^2$, *P*=0.064; 633.22±1550.85 $\mu m^2/mm^2$ vs. 1613.77±2740.45 $\mu m^2/mm^2$, *P*=0.105; 1624.53±3148.36 $\mu m^2/mm^2$ vs. 2522.05±2874.43 $\mu m^2/mm^2$, *P*=0.038, respectively). The results of nerve distribution are illustrated in Figure 3.

The mean density of CHAT-positive nerves did not differ in the PV area between subjects with documented immediate CVS and non-CVS cause of death (whole section: 217.72±397.39 $\mu m^2/mm^2$ vs. 196.76±420.2 $\mu m^2/mm^2$, *P*=0.948; myocardial sleeves: 20.98±79.72 $\mu m^2/mm^2$ vs. 92.31±264.57 $\mu m^2/mm^2$, *P*=0.053; fibro-fatty tissue: 305.56±664.62 $\mu m^2/mm^2$ vs. 203.18±558.43 $\mu m^2/mm^2$, *P*=0.696, respectively) (Table 3, Fig. 1G-J).

Table 3 and Fig. 1K-N show a slight decrease in GAP43-positive nerve densities of the whole section, myocardial sleeves and fibro-fatty tissue regions between subjects with documented immediate CVS vs. non-CVS cause of death, yet with no statistically significant difference (1330.31±1548.63 $\mu m^2/mm^2$ vs. 2006.47±2387.84 $\mu m^2/mm^2$, *P*=0.270;

962.65±2202.73 $\mu m^2/mm^2$ vs. 1842.06±3322.26 $\mu m^2/mm^2$, *P*=0.330; 1374.62±1769.91 $\mu m^2/mm^2$ vs. 1769.76±2418.56 $\mu m^2/mm^2$, *P*=0.559, respectively).

On average, 51% of TH-positive (sympathetic) nerves in the PV whole section were also stained with anti-GAP43 antibodies, a marker of neural growth. These TH-positive nerves represented 24.26% of nerves in the myocardial sleeves and 52.67% of nerves in the fibro-fatty tissue. Similarly, CHAT-positive nerves with GAP43 activity constituted 72.19% of nerves in the whole section, 63.64% of nerves in the myocardial sleeves, and 75.45% of nerves in the fibro-fatty tissue (Fig.4).

3.2.3 Atrial-Pulmonary Vein Ostia

The total mean TH-positive nerve density in the atrial-PV ostia was 1972.77±2660.69 $\mu m^2/mm^2$ in the whole section, 1302.49±2107.76 $\mu m^2/mm^2$ in the myocardial sleeves, and 1940.02±2475.84 $\mu m^2/mm^2$ in the fibro-fatty tissue. The CHAT-positive nerve density was 35.13±68.14 $\mu m^2/mm^2$ in the whole section, 11.84±36.62 $\mu m^2/mm^2$ in the myocardial sleeves, and 51.15±108.03 $\mu m^2/mm^2$ in the fibro-fatty tissue. The total GAP43-positive nerve density was 2015.67±3086.62 $\mu m^2/mm^2$ in the whole section, 1404.77±2018.74 $\mu m^2/mm^2$ in the myocardial sleeves, and 1881.56±2979.04 $\mu m^2/mm^2$ in the fibro-fatty tissue.

In the atrial-PV ostia area, CHAT-positive nerve density in the myocardial sleeves was significantly higher in subjects with documented underlying CVS cause of death (19.48±45.62 $\mu m^2/mm^2$) vs. non-CVS cause of death group with no parasympathetic nerves detected (0±0 $\mu m^2/mm^2$) (*P*=0.034). CHAT-positive nerve density in the whole slide and fibro-fatty tissue regions did not show a difference between subjects with documented underlying CVS and non-CVS cause of death as shown in Table 4 and Fig. 2C-F (33.87±57.95 $\mu m^2/mm^2$ vs.

37.08±83.12 $\mu m^2/mm^2$, *P*=0.613; 41.39±80.95 $\mu m^2/mm^2$ vs. 66.27±141.3 $\mu m^2/mm^2$, *P*=0.839, respectively) (Fig.5).

Table 4 and Fig. 2G-J compare the densities of TH-positive nerves between subjects with documented underlying CVS and non-CVS cause of death showing an increase within all three regions, however without statistical significance (2331.47±3305.04 $\mu m^2/mm^2$ vs. 1416.78±920.3 $\mu m^2/mm^2$, *P*=0.421; 1714.17±2581.65 $\mu m^2/mm^2$ vs. 664.38±680.05 $\mu m^2/mm^2$, *P*=0.421; and 2136.82±3010.26 $\mu m^2/mm^2$ vs. 1634.98±1289.21 $\mu m^2/mm^2$, *P*=0.546, respectively).

Similarly, the densities of GAP43-positive nerves within atrial-PV ostia were higher in the whole section, myocardial sleeves, and fibro-fatty tissue in subjects with documented underlying CVS cause of death, but the differences did not reach a statistical significance (2392.16±3863.55 $\mu m^2/mm^2$ vs. 1432.1±955.47 $\mu m^2/mm^2$, *P*=0.546; 1718.89±2469.42 $\mu m^2/mm^2$ vs. 917.89±827.95 $\mu m^2/mm^2$, *P*=0.763; 2150.66±3672.5 $\mu m^2/mm^2$ vs. 1464.45±1325.67 $\mu m^2/mm^2$, *P*=0.763, respectively) (Table 4, Fig. 2K-N).

In the atrial-PV ostia area, GAP43-positive neural growth in the TH-positive nerves was detected in 45.56% in the whole section, 27.93% in the myocardial sleeves, and 55.03% in the fibro-fatty tissue. Lastly, 70.85% of CHAT-positive nerves in the whole section, 78.79% of CHAT-positive nerves in the myocardial sleeves, and 77.43% of CHAT-positive nerves in the fibro-fatty tissue were concomitantly stained with anti-GAP43 antibodies.

4 Discussion

4.1 Main Findings

The most significant finding of this study is that myocardial sleeves around PVs and atrial-PV ostia contain heterogeneous autonomic innervation. Furthermore, autonomic nerves were associated with cardiovascular mortality in our autopsy cohort. Sympathetic nerve density was significantly decreased in the fibro-fatty tissue surrounding myocardial sleeves of the PVs in subjects with documented immediate CVS cause of death compared to subjects with documented immediate non-CVS cause of death (P=0.038). Parasympathetic nerve density was significantly increased in the myocardial sleeves of the atrial-PV ostia in subjects with documented underlying CVS cause of death compared to subjects with documented underlying non-CVS cause of death (P=0.034). No association was found between TH-, CHAT-, and GAP43-positive nerve densities and a history of atrial fibrillation. Additionally, no differences in nerve density topography among individual PVs were found when related to age, sex, heart weight, heart rhythm, and cardiovascular mortality. The proportion of GAP43-positive neural growth in the autonomic nervous system showed a similar pattern in both PV and atrial-PV ostia cohorts. GAP43-positive neural growth was observed in nearly one-fourth of the TH-positive nerves in the myocardial sleeves, and in roughly half of the TH-positive nerves in the surrounding fibro-fatty tissue. Surprisingly, the majority of CHAT-positive nerves was immunoreactive to GAP43 in all the studied regions.

4.2 Autonomic Nervous System and Heart Failure

Dysregulation of ANS in heart failure has been investigated due to the impact on morbidity and mortality. As shown previously, heart failure is associated with decreased neuronal density in both atrial (23) and ventricular myocardium (24–28). Conversely to the

decreased sympathetic nerve density, sympathetic activity is increased to preserve the cardiac output in systolic heart failure as a part of the neurohumoral compensatory mechanism (24,29). These sympathetic nerve alterations in heart failure may be associated with increased oxidative stress (28,30) and can further impair cardiac function and promote arrhythmogenesis (31). In addition, the plasticity of ANS may play a role in heart failure. It has been reported that sympathetic nerve fibers may become cholinergic through a transdifferentiation process, which could explain sympathetic innervation decrease in heart failure (31,32).

In our recent study, we showed decreased TH- and GAP43-positive nerve densities in the superior vena cava myocardial sleeves in subjects with cardiovascular mortality (16). Furthermore, in agreement with these results, the current study shows that sympathetic innervation was decreased in the myocardial sleeves and the fibro-fatty tissue around PV in patients with documented immediate CVS cause of death, of which 66.6% of subjects were diagnosed with heart failure. However, the difference reached the statistical difference only in fibro-fatty tissue. Interestingly, Parisi et al. (33) demonstrated a significant correlation between the thickness of epicardial adipose tissue and the extent of myocardial sympathetic denervation in subjects with systolic heart failure. The epicardial adipose tissue thickness can be used as a clinical predictor for myocardial sympathetic dysfunction. However, parasympathetic nerve density around PVs and atrial-PV ostia did not differ in subjects with documented immediate CVS cause of death. This finding is in agreement with a previous canine study observing atrial neural remodeling in congestive heart failure (34).

4.3 Autonomic Nervous System and Myocardial Ischemia

Altered autonomic innervation has been associated with increased atherosclerosis (35), which is the most common underlying cause of CVS death in our cohort of PVs. Previous studies provided strong evidence of myocardial sympathetic denervation in ischemic heart disease (36,37) causing hypersensitivity to adrenergic stimulation (38). This can lead to lethal arrhythmias and sudden cardiac death (12). In contrast to earlier findings, in our study, there was a trend of increased sympathetic innervation in atrial-PV ostia in subjects with documented underlying CVS cause of death. However, these results did not reach statistical significance.

Interestingly, we found that parasympathetic nerve density in atrial-PV ostia myocardial sleeves was significantly increased in subjects with documented underlying cardiovascular cause of death compared to subjects with documented underlying non-cardiovascular cause of death (P=0.034). In the atrial-PV ostia cohort, 72% of subjects with documented underlying CVS cause of death were diagnosed with systemic atherosclerosis. Increased parasympathetic innervation may be explained by the cardioprotective mechanism of the parasympathetic nervous system against myocardial ischemia to reduce myocardial injury in subjects with ischemic heart disease (39).

4.4 Pulmonary Veins, Atria and Atrial Fibrillation

Myocardial sleeves around PVs were identified as the most common morphological substrate of atrial fibrillation (40), especially in the atrial-PV ostia region (17,41). Previous studies suggested a relationship between the myocardial sleeves' length and the presence of atrial fibrillation, reporting that subjects with diagnosed atrial fibrillation had longer myocardial sleeves than subjects with sinus rhythm (3,42). Also, the association of atrial fibrillation with scaring and amyloid deposits in the myocardial sleeves of PVs was shown

previously (2,43). Sympathetic innervation was found to be heterogeneously increased in the right atrial appendage compared to the left atrial appendage in persistent atrial fibrillation (44). Moreover, Chang et al. (45) reported a significant increase in sympathetic innervation and nerve sprouting in canine models with sustained atrial fibrillation, with higher immunohistochemical positivity in the right atrial tissue than in the left atrial tissue (45). Similarly, animal studies found a significant increase in the parasympathetic density and atrial parasympathetic innervation heterogeneity in chronically rapidly-paced atria (46,47). Interestingly, Nguyen et al. (20) described a substrate of chronic atrial fibrillation in PV myocardial sleeves characterized by the presence of periodic acid-Schiff (PAS)-positive cells, interstitial Cajal-like cells, fibrotic tissue, inflammatory cells, and sympathetic nerves. They found increased sympathetic nerve density in the atria and the PV myocardial sleeves in subjects with atrial fibrillation compared to subjects with sinus rhythm, but without statistical significance.

Although abnormal autonomic innervation has been shown as a contributing factor to the initiation and maintenance of atrial fibrillation, neither autonomic nerve density nor neural growth in PV myocardial sleeves and atrial-PV ostia were associated with the presence of atrial fibrillation in our study. This also accords with our earlier observations on caval veins myocardial sleeves, which showed no association of atrial fibrillation with autonomic nerve densities and neural growth (16). In addition, the present study material was previously tested for Leu-7 neural marker with no association with a history of atrial fibrillation (48). The immunohistochemistry was underlined by electron microscopy with the detection of neuroendocrine granules corresponding to Leu-7 intensity (48).

4.5 Neural growth detected by GAP43 in TH- and CHAT-positive nerves

GAP43 is a polypeptide expressed in growing axons (49) and measuring the density of GAP43-positive nerves can be used to approach the growing activity of nerves rather than stable ANS innervation (50). This study found that the positive GAP34 staining patterns in the TH-positive nerves differed between the myocardial sleeves and surrounding fibro-fatty tissue in the study cohorts. The GAP43 neural growth activity was about two times decreased in the myocardial sleeves compared to the fibro-fatty tissue in the PV cohort (24% vs. 53%) and the atrial-PV ostia cohort (28% vs. 55%). Thus, the increased sympathetic growth in the surrounding fibro-fatty tissue might be involved in pathologic processes causing the formation of more mature synapses than in the myocardial sleeves (50). Another interesting finding is that between 64-79% of CHAT-positive nerves show GAP43-positive neural growth activity within the studied regions in both study cohorts. Such colocalization implies that parasympathetic nerve growth was enhanced despite its sparse nerve distribution. These notable differences in the TH, CHAT, and GAP43 immunoreactivity suggest an interindividual variance in nerve density and neural growth genetic regulation. The combination of our findings provides possible support for future therapeutic interventions in targeting not only the distribution, but also the neural growth of autonomic nerves, and their topography.

4.6 Study Limitations

This is a morphometric study with samples obtained post-mortem, therefore, we did not perform functional electrophysiological measurements. Clinical data from the autopsy referrals were only available in this study. Our study showed substantial variability in nerve densities among subjects in both study cohorts, which possibly resulted in wide data distribution and high standard deviations. Such variation may be also attributable to the

underlying health conditions influencing autonomic innervation, small series size, and tissue sample sizes with an uneven innervation pattern.

4.7 Conclusions

Variations in autonomic nerve density may be associated with cardiovascular mortality as we observed sympathetic denervation in PVs in patients with documented immediate CVS death, and increased parasympathetic innervation in atrial-PV ostia in patients with documented underlying CVS death. Sympathetic neural growth was found to be enhanced in the fibro-fatty tissue rather than in the myocardial sleeves, and neural growth activity was present in the majority of detected parasympathetic nerves. Knowledge of cardiac ANS remodeling in various disorders may provide further light on pathophysiology and treatment development in cardiovascular diseases.

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Competing Interests

The authors declare they have no competing interests.

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Author Contributions

DD designed the study, performed the morphometric analysis and drafted the manuscript. AM performed the statistical analysis of data and interpreted the results. RV performed part of the morphometric analysis. TP designed the study and interpreted the results. IK designed the study, interpreted the results and drafted the manuscript. All authors have read and approved the final manuscript.

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6 Figure Legends and Figures

Figure 1: Autonomic nervous system in pulmonary veins as detected by immunohistochemistry. Low-power sections of two PVs and representative sections of positively stained autonomic nerves and ganglia. A: Low-power longitudinal section of a left lower PV (marked by blue line) in a subject with documented immediate CVS death. The whole section contains TH-positive nerves and ganglia (marked by red line) in MS (demarked by green line) and surrounding FFT (TH immunochemistry). B: Low-power longitudinal section of a left lower PV (marked by blue line) in a subject with documented immediate non-CVS death. The whole section contains TH-positive nerves and ganglia (marked by red line) in MS (demarked by green line) and surrounding FFT (TH immunochemistry). Note increased innervation in the surrounding FFT in comparison to A. C: a transverse section of TH-positive nerve (arrow) surrounded by adipose tissue. D: a transverse section of TH-positive nerve (arrow) embedded in FFT. E: a transverse section of a nerve stained for TH (arrow) in the middle of MS. F: a transverse section of TH-positive nerve (arrow) in the MS with profound fatty replacement. G: a transverse section of CHAT-positive nerve (arrow) in an adipose tissue. H: a transverse section of CHAT-positive ganglion (arrow) in the FFT compartment. I: a transverse section of CHAT-positive nerve (arrow) on the edge of MS partially surrounded by fatty tissue. J: a transverse section of two CHAT-positive nerves (arrows) in MS with fibro-fatty replacement. K: a transverse section of GAP43-positive ganglion (arrow) close to vessels structures surrounded by FFT. L: a transverse section of GAP43-positive nerve (arrow) embedded in FFT. M: a transverse section of GAP43-positive nerve (arrow) in the middle of MS surrounded by a small amount of fatty replacement. N: a transverse section of GAP43-positive nerve (arrow) in MS with mild fibrosis and fatty replacement. Scale bars: A and B=800 µm, C-F: bar=50 µm. (CVS – cardiovascular, FFT –

fibro-fatty tissue, MS – myocardial sleeve, TH – tyrosine hydroxylase detecting sympathetic nerve tissue, CHAT – choline acetyltransferase detecting parasympathetic nerve tissue, GAP43 – growth-associated protein 43 detecting neural growth)

Figure 2: Autonomic nervous system in atrial-PV ostia as detected by immunohistochemistry. Low-power sections of two atrial-PV ostia compartments and representative sections of positively stained autonomic nerves. A: Low-power longitudinal section of left common atrial-PV ostium (marked by blue line) in a subject with documented underlying CVS death. The whole section contains CHAT-positive nerves and ganglia (marked by red line) in MS (demarked by green line) and surrounding FFT (CHAT immunochemistry). B: Low-power longitudinal section of left upper atrial-PV ostium (marked by blue line) in a subject with documented underlying non-CVS death. The whole section contains TH-positive nerves and ganglia (marked by red line) only in surrounding FFT. Note that there was no positive nerve tissue detected in the MS (demarked by green line) (CHAT immunochemistry). C: a transverse section of TH-positive nerve (arrow) embedded in an adipose tissue. D: a transverse section of TH-positive nerve (arrow) surrounded by adipose predominant FFT. E: a transverse section of two TH-positive nerves (arrows) in the MS. F: a transverse section of TH-positive nerve (arrow) in the hypertrophic MS. G: a transverse section of CHAT-positive nerve (arrow) in an adipose tissue. H: a transverse section of a nerve stained for CHAT stain (arrow) surrounded by an abundant adipose tissue. I: a transverse section of CHAT-positive nerve (arrow) in MS with myocardial hypertrophy and fatty replacement. J: a section of MS without detected positive nerve tissue (CHAT immunochemistry). K: a transverse section of GAP43-positive nerve (arrow) surrounded by FFT. L: a transverse section of two GAP43-positive nerves (arrows) with vessels and adipocytes in FFT. M: a transverse and a

longitudinal section of GAP43-positive nerves (arrow) in the MS surrounded by a small amount of fibrous tissue. N: a longitudinal section of GAP43-positive nerve (arrow) in hypertrophic MS. Scale bars: A and B=800 µm, C-F: bar=50 µm. (CVS – cardiovascular, FFT – fibro-fatty tissue, MS – myocardial sleeve, TH – tyrosine hydroxylase detecting sympathetic nerve tissue, CHAT – choline acetyltransferase detecting parasympathetic nerve tissue, GAP43 – growth-associated protein 43 detecting neural growth)

Figure 3: Pulmonary veins: Comparison of autonomic nerve densities ($\mu m^2/mm^2$) between subjects with documented immediate CVS cause of death and immediate non-CVS cause of death. (WS – whole section, MS – myocardial sleeves, FFT – surrounding fibro-fatty tissue outside the sleeve, TH – tyrosine hydroxylase, CHAT – choline acetyltransferase, GAP43 – growth-associated protein 43, CVS – cardiovascular)

Figure 4: Longitudinal section of a left upper pulmonary vein showing GAP43-positivity in TH- and CHAT-positive nerves. Both A and B figures represent the same topographic area of the PV. Continuous myocardial bundles containing vessels and nerves form MS surrounded by abundant FFT. A: TH-stained section. Nerves stained with both GAP43 and TH antibodies are marked in yellow, and GAP43-negative and TH-positive nerves are marked in red. B: CHAT-stained section. Nerves positively stained with both GAP43 and CHAT antibodies are marked in yellow. Only CHAT-positive nerves that are GAP43-negative, are marked in red. Scale bar=800 µm. (PV – pulmonary vein, MS – myocardial sleeves, FFT – fibro-fatty tissue, TH – tyrosine hydroxylase, CHAT – choline acetyltransferase, GAP43 – growth-associated protein 43)

Figure 5: Atrial-pulmonary vein ostia: Comparison of autonomic nerve densities ($\mu m^2/mm^2$) between subjects with documented underlying CVS cause of death and underlying non-CVS cause of death. (WS – whole section, MS – myocardial sleeves, FFT – surrounding fibro-fatty tissue outside the sleeve, TH – tyrosine hydroxylase, CHAT – choline acetyltransferase, GAP43 – growth-associated protein 43, CVS – cardiovascular)

7 Tables and Table Legends

Table 1A Clinical data in PV cohort

ID no.	Age	Sex	Heart weight (g)	Heart rhythm	Underlying cause of death		Immediate cause of death	
1	86	female	510	SR	ASVD	A	acute on chronic HF	A
2	70	female	470	SR	multiple myeloma	B	HF	A
3	57	male	540	SR	liver cholangiocarcinoma	B	respiratory failure	B
4	62	female	620	SR	congenital connective tissue disorder	B	HF	A
5	65	female	530	SR	ASVD	A	HF	A
6	56	female	180	SR	cervical carcinoma	B	bronchopneumonia	B
7	73	female	380	SR	choledocholithiasis	B	respiratory failure	B
8	70	female	390	SR	chronic tubular nephritis	B	sepsis	B
9	63	male	620	AF	mitral stenosis	A	HF	A
10	70	male	630	AF	ASVD	A	acute on chronic HF	A
11	57	female	490	AF	multiple myeloma	B	respiratory failure	B
12	42	male	870	AF	mixed aortic disease	A	extreme obesity	B
13	60	male	600	AF	abdominal aortic aneurysm	A	pulmonary artery embolism	B
14	81	male	560	AF	ASVD	A	brain death	B
15	74	female	600	SR	acute pancreatitis	B	cardiorespiratory insufficiency	A
16	77	male	370	AF	renal cell carcinoma	B	bronchopneumonia	B
17	61	male	550	SR	renal papillary carcinoma	B	right respiratory failure	B
18	73	male	460	SR	diabetes mellitus	B	cardiorespiratory insufficiency	A
19	70	male	510	SR	myelodysplastic syndrome	B	respiratory failure	B
20	79	male	400	SR	ASVD	A	posthemorrhagic shock	B
21	55	male	430	SR	anemia	B	lung edema	B
22	75	female	380	AF	ASVD	A	brain death	B
23	66	male	600	SR	ASVD	A	myocardial infarction	A
24	72	female	320	SR	bronchioalveolar carcinoma	B	respiratory failure	B
25	77	male	500	SR	ASVD	A	acute on chronic HF	A
26	71	male	770	AF	ASVD	A	multiorgan failure	B
27	61	female	620	SR	infective endocarditis	A	HF	A
28	57	male	340	SR	meningomyelitis	B	brain death	B
29	69	female	290	SR	brain hemorrhage	B	brain death	B
30	69	male	400	SR	postchemotherapy status	B	sepsis	B
31	78	male	460	AF	ASVD	A	pulmonary fat embolism	B
32	67	male	410	AF	gastric ulcer disease	B	posthemorrhagic shock	B
33	75	male	470	SR	ASVD	A	bronchopneumonia	B
34	73	male	280	AF	COPD	B	respiratory failure	B
35	56	male	550	SR	arterial hypertension	A	food aspiration	B
36	76	female	540	AF	ASVD	A	sepsis	B
37	71	female	280	AF	epilepsy	B	cardiorespiratory insufficiency	A

A – cardiovascular cause of death, B – non-cardiovascular cause of death, AF – atrial fibrillation, SR – sinus rhythm, ASVD – atherosclerotic vascular disease, COPD – chronic obstructive pulmonary disease, HF – heart failure

Table 1B Subjects characteristics in PV cohort

PV cohort	Immediate CVS death	Immediate non-CVS death	Underlying CVS death	Underlying non-CVS death
Subjects (n)	12	25	17	20
Age (years±SD)	70±7	67±10	70±11	67±7
Female (%)	58	32	29	50
Heart weight (g±SD)	537±103	459±150	565±122	416±116
AF n (%)	3 (25)	11 (44)	9 (53)	5 (25)

CVS – cardiovascular, PV – pulmonary veins, AF – atrial fibrillation, SD – standard deviation

Table 2A Clinical data in atrial-PV ostia cohort

ID no.	Age	Sex	Heart weight (g)	Heart rhythm	Underlying cause of death		Immediate cause of death	
1	63	female	480	AF	mixed mitral valve disease	A	HF	A
2	57	male	600	AF	ASVD	A	respiratory failure	B
3	71	male	450	SR	ASVD	A	acute myocardial infarction	A
4	79	male	520	SR	aortic stenosis	A	HF	A
5	70	male	770	SR	ASVD	A	myocardial infarction	A
6	68	male	670	AF	schizophrenia	B	pulmonary embolism	B
7	79	male	390	AF	ASVD	A	HF	A
8	64	male	450	SR	leukemia	B	bronchopneumonia	B
9	69	female	430	AF	ASVD	A	cardiogenic shock	A
10	76	female	360	SR	peptic ulcer	B	hemorrhagic shock	B
11	54	male	610	SR	ASVD	A	HF	A
12	39	female	290	SR	chronic alcoholism	B	hemorrhagic shock	B
13	33	female	290	SR	chronic alcoholism	B	respiratory failure	B
14	77	female	620	AF	ASVD	A	HF	A
15	46	female	320	SR	vascular malformation	A	brain death	B
16	80	male	410	AF	ASVD	A	urosepsis	B
17	52	male	570	SR	laryngeal carcinoma	B	bronchopneumonia	B

A – cardiovascular cause of death, B – non-cardiovascular cause of death, AF – atrial fibrillation, SR – sinus rhythm, ASVD – atherosclerotic vascular disease

Table 2B Subjects characteristics in atrial-PV ostia cohort

Atrial-PV ostia cohort	Immediate CVS death	Immediate non-CVS death	Underlying CVS death	Underlying non-CVS death
Subjects (n)	8	9	11	6
Age (years±SD)	70±9	57±16	68±11	55±17
Female (%)	38	44	36	50
Heart weight (g±SD)	534±126	440±142	509±130	438±156
AF n (%)	4 (50)	3 (33)	6 (55)	1 (17)

CVS – cardiovascular, PV – pulmonary veins, AF – atrial fibrillation, SD – standard deviation

Table 3 Pulmonary veins cohort autonomic nerve densities

	Area	Immediate CVS death	Immediate non-CVS death	*P*-value	Underlying CVS death	Underlying non-CVS death	*P*-value
TH	Whole section	1378.5±2159.68	2512.53±2771.53	0.064	2398.22±3178.98	1819.3±1968.93	0.428
	Myocardial sleeves	633.22±1550.85	1613.77±2740.45	0.105	1444.65±3183.23	1076.2±1425.62	0.976
	Fibro-fatty tissue	1624.53±3148.36	2522.05±2874.43	0.038*	2428.47±3638.68	1976.13±2302.7	0.761
CHAT	Whole section	217.72±397.39	196.76±420.2	0.948	292.18±533.96	127.46±235.83	0.112
	Myocardial sleeves	20.98±79.72	92.31±264.57	0.053	67.29±174.64	64.4±250.55	0.637
	Fibro-fatty tissue	305.56±664.62	203.18±558.43	0.696	386.19±812.81	113.81±255.19	0.081
GAP43	Whole section	1330.31±1548.63	2006.47±2387.84	0.270	2145.99±2726.03	1410.38±1355.85	0.100
	Myocardial sleeves	962.65±2202.73	1842.06±3322.26	0.330	1847.84±3724.25	1221.38±2102.14	0.807
	Fibro-fatty tissue	1374.62±1769.91	1769.76±2418.56	0.559	1897.13±2780.29	1381.08±1502.27	0.361

* – statistical significance, CVS – cardiovascular, TH – tyrosine hydroxylase, CHAT – choline acetyltransferase,

GAP43 – growth-associated protein 43

Table 4 Atrial-pulmonary vein ostia cohort nerve densities

	Area	Immediate CVS death	Immediate non-CVS death	P-value	Underlying CVS death	Underlying non-CVS death	P-value
TH	Whole section	2656.02±3924.35	1531.96±1238.17	0.501	2331.47±3305.04	1416.78±920.3	0.421
	Myocardial sleeves	1679.73±2814.97	1059.11±1493.24	0.630	1714.17±2581.65	664.38±680.05	0.421
	Fibro-fatty tissue	2425.29±3584.95	1626.94±1348.53	0.700	2136.82±3010.26	1634.98±1289.21	0.546
CHAT	Whole section	34.74±50.28	35.38±78.34	0.287	33.87±57.95	37.08±83.12	0.613

	Myocardial sleeves	16.43±42.59	8.87±32.6	0.072	19.48±45.62	0	0.034*
	Fibro-fatty tissue	39.34±59.5	58.77±130.59	0.770	41.39±80.95	66.27±141.3	0.839
GAP43	Whole section	2521.52±4665.85	1689.31±1345.47	0.923	2392.16±3863.55	1432.1±955.47	0.546
	Myocardial sleeves	1325.42±2540.51	1455.96±1642.44	0.386	1718.89±2469.42	917.89±827.95	0.763
	Fibro-fatty tissue	2375.27±4440.04	1563.03±1427.68	0.847	2150.66±3672.5	1464.45±1325.67	0.763

* – statistical significance, CVS – cardiovascular, TH – tyrosine hydroxylase, CHAT – choline acetyltransferase,

GAP43 – growth-associated protein 43