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Safety and efficacy of catheter-based renal denervation for the management of resistant hypertension: clinical, imaging and immunological assessment

Tese no âmbito do Programa Doutoral em Ciências da Saúde, ramo de Medicina, orientada pelo Professor Doutor Lino Gonçalves e pelo Professor Doutor Manuel Santos Rosa e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Tese de Doutoramento do Programa Doutoral em Ciências da Saúde, apresentada à Faculdade de Medicina da Universidade de Coimbra, para candidatura ao grau de Doutor em Ciências da Saúde – ramo de Medicina, especialidade de Cardiologia. A Tese foi realizada sob a orientação científica do Professor Doutor Lino Gonçalves e do Professor Doutor Manuel Santos Rosa.

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Abstract

Hypertension (HT) is the most common modifiable cardiovascular risk factor and is responsible for significant mortality and morbidity. It is estimated that 30-45% of the adult population has HT, with its prevalence increasing with advancing age. The employment of lifestyle changes and drug therapy reduces the risk of major cardiovascular events such as acute myocardial infarction, stroke and heart failure and increases life-expectancy. However, despite the availability of safe and efficacious anti-hypertensive drugs, the prevalence of uncontrolled HT continues to rise, with non-adherence to therapy being the most important contributor of poor blood pressure control. HT is considered true resistant when a documented failure to achieve blood pressure guideline-recommended targets is observed and, secondary HT, non-adherence and pseudo-hypertension have been excluded. The pathophysiology of resistant HT is complex and not clear. The vasoconstrictor effects of the sympathetic nervous system and its implication on the pathogenesis of refractory hypertension have long been known, turning it into an attractive therapeutic target.

Renal denervation (RDN) is a minimally invasive percutaneous technique which consists on the delivery of radiofrequency energy on the renal artery wall in order to achieve a selective disruption of sympathetic nerve endings. First generation randomized trials were rather controversial, specifically HTN-3, given the results were not the expected and renal denervation did not achieve a higher blood pressure reduction than the control arm. Several questions have been raised by this 'negative' study and prompted investigation around the technique. Up to the current date, RDN has been employed to treat patients with resistant HT and second generation trials have included a wider cohort of patients, in order to assess safety and efficacy of the procedure, based on the development of more advanced devices and the recent knowledge regarding renal nerve distribution.

In the current study, we used RDN to treat patients with true resistant HT, in order to assess i) the safety of the technique, through the performance of intra-arterial optical coherence tomography (OCT) in a porcine model and, its efficacy through the histologic evaluation of the treated renal artery; ii) the impact of the technique on the sympathetic cardiac innervation, in a subgroup of patients; iii) the impact of the technique in the immune system and, possibly,

identify a biomarker to predict response; iv) the efficacy of the technique through a clinical evaluation and ambulatory blood pressure monitoring v) the impact of renal denervation on several parameters such as left ventricular ejection fraction, diastolic function, arrhythmic profile, renal function, glycemic and lipid profiles, NT pro-BNP and vitamin D.

In the first part of our research, we performed unilateral pre-bifurcation RDN, with a multi-electrode (second generation) catheter, in twelve domestic pigs. The contralateral artery was untreated (control). OCT was performed pre and immediately post procedure and follow-up with renal angiogram and OCT was performed after one month. We demonstrated that RDN caused acute vessel wall changes (intimal disruption (edema/spasm) and intraluminal thrombus formation) visible by OCT but, after one month, the vessel was completely healed, with no evidence of renal stenosis in any of the treated subjects. The histological analysis revealed nearly absent tyrosine hydroxylase immunostaining and a statistically significant increase in the amount of collagen fibers in the denervated artery, compatible with a decrease in nerve terminals and an increase in fibrosis, compared to the control, suggesting an efficacious delivery of the radiofrequency energy to the vessel wall. No differences were found in the norepinephrine or epinephrine renal tissue levels between the treated and contralateral kidney.

Subsequently, we performed ^{123}I-labelled meta-iodobenzylguanidine (MIBG) cardiac scintigraphy, in a subgroup of patients, before and six months after RDN, to evaluate the impact of the procedure on cardiac sympathetic activity. Early heart to mediastinum ratio (HMR) was significantly lower in *responders* at baseline but similar after six months follow-up. Late HMR was statistically similar in both groups pre- and post RDN but reduced in comparison to values reported in healthy subjects. Accordingly, there were no statistically significant differences regarding the washout rate but above normal baseline values were detected, a difference more evident in the *non-responders* (sympathetic overdrive? increased risk of cardiovascular events?), with both group values converging after the RDN. None of the evaluated rates altered significantly at follow-up, translating an absence of deleterious sympathetic nerve disruption. In conclusion, RDN is a safe procedure, in terms of cardiac nerve integrity, but none of the evaluated MIBG parameters were useful to predict response.

In the third part our research, we investigated the cellular immune profile in our cohort of 23 patients with resistant HT and treated with RDN. A preliminary assay, which included an extended analysis of T, B and natural killer cells, monocytes and dendritic cells, guided the

forthcoming study in order to select T cells (CD4 and CD8, memory and activated subsets) as possible biomarkers of response to RDN. Blood samples were obtained in six timings, pre and post procedure. Response to RDN was evaluated at six-months and one year and was observed in 69.6 and 82.6% of patients, respectively. Absolute values of HLA-DR+ double negative T cells were significantly lower in the group of *responders* at one year. Additionally, interaction between the timings were found in three T cell subsets (T CD4, T CD8 and naïve T CD8 cells), with the *responders* tending to present with lower absolute values and little inter-timing variation.

Afterwards, our research focused on investigating the behavior of cytokines in 21 RDN patients. We analyzed 45 protein targets which included cytokines, chemokines and growth factors. Response to RDN was evaluated as described previously. Venous blood samples were obtained at four timings, pre and post procedure. 66.7% of the patients were *responders* at six months and 85.7% were *late-responders*. Levels of regulated on activation, normal T cell expressed and secreted (RANTES) were significantly lower in *responders*, both at baseline and at 30 days (p=0.037). As there is evidence that Angiotensin II inhibits RANTES expression and, the renin-angiotensin-aldosterone system is directly linked to the sympathetic nervous system, we could hypothesize that low levels of RANTES are associated with higher levels of angiotensin II and therefore to an over activated sympathetic nervous system, turning these patients more prone to a RDN response.

Finally, we assessed serum vitamin D concentration as a predictor of blood pressure response to RDN. Additionally, we evaluated overall long-term safety and efficacy, as well as echocardiographic parameters, in the cohort of 24 patients submitted to RDN. Response at six-months (*early-responders*) and one year was evaluated. We observed that *responders* at six-months had significantly higher baseline and six-month follow-up values of vitamin D than *non-responders*. *Responders* at one year (including late-responders) continued to present with higher vitamin D levels than *non-responders*, although it didn't reach statistical significance, probably due to the low number of the later at this stage. In the long-term follow-up (mean 52 months), 70.8% of the patients maintained a clinical response. Regarding echocardiographic parameters, there was an improvement in diastolic function in non-responders, finding that could reflect benefit from the RDN, even though no effect on blood pressure was observed. No other relevant echocardiographic differences were found.

In conclusion, RDN effectively lowered the blood pressure in the majority of the studied patients, with an optimal safety pattern. Our study contributed to better comprehend the clinical, immunological and hemodynamic profiles of patients submitted to this procedure and, to identify potential biomarkers of denervation success. Our findings may allow for a better identification of patients who could really derive benefit from RDN and prompt further investigation around this area.

Keywords: Hypertension; Resistant; Renal Denervation; Sympathetic Nervous System; Renin-Angiotensin-Aldosterone; Optical Coherence Tomography; Radiofrequency; Fibrosis; Catecholamine; Immune System; Double-Negative T cells; Inflammation; Cytokines; RANTES; Vitamin D.

Resumo

A hipertensão (HT) é o factor de risco cardiovascular modificável mais comum e é responsável por morbilidade e mortalidade significativas. É estimado que 30-45% da população adulta tenha HT sendo que, a sua prevalência aumenta com o avanço da idade. A implementação de alterações do estido de vida e a terapêutica com fármacos reduz o risco de eventos cardiovasculares major com o enfarte agudo do miocárdio, acidente vascular cerebral e insuficiência cardíaca e, aumenta a expectativa de vida. No entanto, apesar da disponibilidade de medicamentos seguros e eficazes para controlar a HT, a prevalência de HT não controlada continua a aumentar sendo que, o mais importante contribuidor para um mau controle tensional é a não aderência à terapêutica. A HT é considerada verdadeiramente resistente quando não se conseguem atingir os valores de pressão arterial recomendados pelas *guidelines* e, quando são excluídos HT secundária, não-aderência e pseuso-hipertensão. A patofisiologia da HT resistente é complexa e pouco clara. Os efeitos vasoconstrictores do sistema nervoso simpático e a sua implicação na patogénese da HT resistente é conhecida, tornando-o num alvo terapêutico atrativo.

A desnervação renal (RDN) é uma técnica percutânea minimamente invasiva que consiste na aplicação de energia de radiofrequência na parede da artéria renal, com o objectivo de provocar lesão nos terminais nervosos simpáticos. Os ensaios randomizados de primeira geração foram controversos, especificamente o HTN-3, uma vez que os resultados não foram os esperados e a RDN não se associou a uma maior queda na pressão arterial, em relação ao grupo de controlo. Várias questões foram colocadas por este estudo 'negativo' e motivou uma maior investigação da técnica. Até ao presente, a RDN tem sido usada para tratar doentes com HT resistente e os ensaios de segunda geração já incluiram uma população de doentes mais ampla, para avaliação da segurança e eficácia do procedimento, baseando-se no desenvolvimento de um catéter mais avançado, multi-eléctrodo, e no conhecimento recente relativo à distribuição dos nervos renais.

Na presente tese, utilizámos a RDN para tratar doentes com HT resistente verdadeira, com o objectivo de avaliar i) a segurança da técnica, através da realização de tomografia de coerência óptica (OCT) intra-arterial num modelo porcino e, a sua eficácia através da avaliação

histológica da artéria renal tratada; ii) o impacto da técnica na inervação simpática cardíaca, num subgrupo de doentes; iii) o impacto da técnica no sistema imunitário e, potencialmente, identificar um biomarcador predictor de resposta; iv) a eficácia da técnica através de uma avaliação clínica e de monitorização ambulatória da pressão arterial; v) o impacto da RDN em vários parâmetros como a fração de ejeção do ventrículo esquerdo, função diastólica, perfil disrítmico, função renal, perfis glucídico e lipídico, NT pro-BNP e vitamina D.

Na primeira parte da nossa investigação, realizámos, em doze porcos domésticos, RDN unilateral, pré-bifurcação, com um catéter multi-eléctrodo (segunda geração). A artéria contra-lateral não foi tratada (controlo). Foi realizado OCT pré e imediatamente após o procedimento e, após um mês, *follow-up* com angiografia renal e OCT. Demonstrámos que a RDN causou alterações agudas na parede do vaso (alterações da intíma (edema/espasmo) e formação de trombo intra-luminal) visíveis por OCT mas, após um mês, o vaso estava totalmente recuperado, não se observando estenose da artéria renal em nenhum caso. A análise histológica revelou uma quase ausência de coloração para tirosina hidroxilase e um aumento estatisticamente significativo na quantidade de fibras de colagénio na artéria tratada, alterações compatíveis com uma redução dos terminais nervosos e um aumento da fibrose, comparativamente com o controlo, sugerindo uma entrega eficaz da energia de radiofrequência na parede arterial. Não se encontraram diferenças estatisticamente significativas em relação aos níveis tecidulares renais de norepinefrina e adrenalina, entre o rim tratado e o contra-lateral.

Subsequentemente, realizámos cintigrafia cardíaca com ^{123}I meta-iodobenzylguanidina (MIBG), num subgrupo de doentes, antes e seis meses depois da RDN, para avaliação do impacto do procedimento na actividade simpática cardíaca. O *ratio* coração/mediastino (HMR) precoce foi significativamente mais baixo nos *respondedores* pré-procedimento, mas semelhante entre os grupos após seis meses de *follow-up*. O HRM tardio foi estatisticamente semelhante entre os grupos, pré- e pós-RDN, mas apresentando valores mais baixos do que os reportados em indivíduos saudáveis. Por conseguinte, não se verificaram diferenças estatisticamente significativas na taxa de *washout,* mas valores basais acima do normal foram detectados, sendo esta diferença mais evidente nos *não-respondedores* (hiperactivação simpática? risco aumentado de eventos cardiovasculares?), verificando-se uma aproximação entre os 2 grupos após a RDN. Nenhum dos parâmetros avaliados se alterou significativamente no *follow-up*, o que poderá traduzir uma ausência de lesão dos nervos simpáticos. Em conclusão, a RDN é um

procedimento seguro, em termos de integridade nervosa cardíaca, mas nenhum dos parâmetros de MIBG avaliados foram úteis para predizer resposta.

Na terceira parte da nossa investigação, avaliámos o perfil imunológico celular na nossa população de 23 doentes com HT resistente e tratada com RDN. Um estudo preliminar, que incluiu uma análise exaustiva de células T, B e *natural killer*, monócitos e células dendríticas, orientou o o estudo posterior e permitiu identificar as células T (CD4 e CD8, memória e activadas) como possíveis biomarcadores de resposta na RDN. Foram obtidas amostras de sangue em seis instantes de tempo, pré e pós-procedimento. A resposta à RDN foi avaliada aos seis meses e um ano e observou-se em 69.6 e 82.6% dos doentes, respectivamente. Os valores absolutos das células T duplas-negativas activadas foram significativamente inferiores no grupo de *respondedores* ao ano. Verificou-se adicionalmente uma interação entre os diferentes tempos em 3 subgrupos de células T (T CD4, T CD8 e T CD8 *naive*), com tendência para os *respondedores* apresentarem valores absolutos inferiores e pouca variação entre os tempos.

Seguidamente, a nossa investigação focou-se em avaliar o comportamento das citocinas em 21 doentes tratados com RDN. Foram analizados 45 alvos proteicos que incluiram citocinas, quimiocinas e factores de crescimento. A resposta à RDN foi avaliada de acordo com o descrito previamente. Amostras de sangue venoso foram obtidas em quatro instantes de tempo, pré e pós-procedimento. 66.7% dos doentes foram *respondedores* aos 6 meses e 85.7% foram *respondedores-tardios*. Os níveis de RANTES (regulada sob activação, expressada e secretada por células T normais) foram significativamente mais baixos nos *respondedores*, tanto basalmente como aos 30 dias (p=0.037). Como há evidência que a Angiotensina II inibe a expressão da RANTES e, o sistema renina-angiotensina-aldosterona está directamente conectado ao sistema nervoso simpático, podemos colocar a hipótese de que baixos níveis de RANTES estão associados a valores elevados de angiotensina II e consequentemente, a um sistema nervoso simpático sobreactivado, tornando estes doentes mais susceptíveis de responderem à RDN.

Por último, avaliámos a concentração da vitamina D sérica, colocando a hipótese de potencial preditora de resposta à RDN. Adicionalmente, avaliámos a segurança e eficácia a longo-prazo, assim como os parâmetros ecocardiográficos, na população de 24 doentes submetida a RDN. Foi avaliada a resposta aos 6 meses (*respondedores precoces*) e ao ano. Observámos que os *respondedores* apresentaram valores basais e aos seis meses

significativamente mais elevados de vitamina D que os *não-respondedores*. Os *respondedores* ao ano (que inclui os *respondedores* tardios) continuaram a apresentar valores mais elevados de vitamina D que os *não-respondedores*, apesar de ausência de significado estatístico, provavelmente pelo baixo número de *não-respondedores* nesta altura. No *follow-up* a longo-prazo (média de 52 meses), 70.8% dos doentes mantiveram resposta clínica. Em relação aos parâmetros ecocardiográficos, verificou-se uma melhoria da função diastólica nos *não-respondedores*, achado que poderá reflectir benefício da RDN, apesar de não se ter observado efeito na pressão arterial. Não foram encontradas outras diferenças de relevo nos parâmetros ecocardiográficos.

Em conclusão, a RDN efectivamente reduziu a pressão arterial na maioria dos doentes estudados, apresentando um óptimo padrão de segurança. O nosso estudo contribuiu para uma melhor compreensão do perfil clínico, imagiológico, imunológico e hemodinâmico dos doentes submetidos a este procedimento e, para a identificação de potenciais biomarcadores de sucesso. Os nossos resultados poderão permitir uma melhor seleção de doentes, para que possam realmente beneficiar da RDN, e potenciar investigação nesta área.

Palavras Chave: Hipertensão; Resistente; Desnervação renal; Sistema Nervoso Simpático; Renina-Angiotensina-Aldosterona; Tomografia de Coerência Óptica; Radiofrequência; Fibrose; Catecolaminas; Sistema Imunitário; Células T Duplas-Negativas; Inflamação; Citocinas; RANTES; Vitamina D.

Thesis Outline

This thesis is organized in three parts, whose content is summarized below.

Part I is a general introduction to the thesis, giving an overview of the state of the art in the field of renal denervation and, is composed by one review article and one editorial comment. In the **review paper**, entitled *Renal denervation in the era of HTN-3. Comprehensive review and glimpse into the future*, we presented an overview of the sympathetic nervous system and its implication in the pathogenesis of hypertension, examined the evidence known to date regarding renal denervation and shared some insights and future expectations regarding this technique. The **editorial comment** entitled *Renal denervation in heart failure: Modulating the sympathetic nervous system*, examined a paper which evaluated the effects of renal denervation in patients with reduced left ventricle ejection fraction and reviewed the role of the sympathetic nervous system in the pathophysiology of heart failure. These two papers were very important to delineate the ideas and the research presented in the current thesis.

Part II of this thesis contains five original articles, published or submitted for publication in international and national peer-reviewed journals:

- **Original Article 1**: *Intravascular imaging, histopathological analysis, and catecholamine quantification following catheter-based renal denervation in a swine model: the impact of prebifurcation energy delivery*.
- **Original Article 2**: *Activated double-negative T cells (CD3+CD4−CD8−HLA-DR+) define response to renal denervation for resistant hypertension*.
- **Original Article 3**: *Low plasmatic levels of RANTES/CCL5 are associated with a positive response to renal denervation in patients with resistant hypertension*.
- **Original Article 4**: *Risk stratification and cardiac sympathetic activity assessment using myocardial [123I] MIBG imaging in renal denervation.* **Original Article 5**: *Renal Sympathetic Denervation in Resistant Hypertension: the association between Vitamin D and Positive Early Response in Systolic Blood Pressure*.

Part III of the thesis provides a section which comprises the clinical and scientific advances regarding renal denervation, to date, since the publication of the Review Article, an integrated discussion summarizing the main results of our investigation and addressing future research in the area.

The **supplements** include a list of presentations given in the field of resistant hypertension and renal denervation, and one case report, addressing the clinical cases of two patients in whom it was necessary to perform a second renal denervation, stating the possibility of re-innervation. This section also comprises important documents necessary to for the completion of this thesis.

Purposes

The overall goal of this thesis was to evaluate the safety and efficacy of catheter-based renal denervation in a cohort of patients with true resistant hypertension, based on a clinical, imagiological, immunological and histological evaluation. Blood pressure was measured in the office and along 24 hours (ambulatory blood pressure monitoring - ABPM). The employed imaging methods were optical coherence tomography (in a porcine model), ^{123}I-labelled meta-iodobenzylguanidine (MIBG) cardiac scintigraphy (in a subgroup of patients), transthoracic echocardiography and renal angiography, pre- (renal anatomy evaluation, technique feasibility) and post-procedure (patency of the renal arteries). Additionally, a cellular and humoral immunological study was performed.

In order to thoroughly organize and determine main and secondary purposes, several research questions have been defined, according to identified gaps in science regarding renal denervation:

1. Is renal denervation efficacious? Does radiofrequency energy effectively destroy sympathetic nerves fibers around renal vasculature? Does renal denervation effectively lower blood pressure in patients with resistant hypertension?
2. Is renal denervation safe? Can it be safely performed in human patients without being associated to severe disruptions in the renal vasculature?
3. What is the behaviour of the immune system in the context of renal denervation? Can a biomarker to clinical response be identified?
4. Does renal denervation have any impact on cardiac sympathetic innervation?
5. What is the impact of renal denervation on echocardiographic parameters (cardiac wall thickness, diastolic function or systolic function) and on vitamin D concentration?

The **main purposes** of the thesis were:

i) To assess the safety of pre-bifurcation renal denervation, with a multi-electrode catheter, through the performance of optical coherence tomography in a

porcine model (pre-procedure, post-procedure and at one month follow-up) and, its efficacy, through the histologic analysis of the treated artery and surrounding tissues and renal tissue catecholamine quantification (with the contralateral side as the control) – *addresses research questions number 1 and 2 and answered in Original Article 1.*

ii) To evaluate the impact of renal denervation on the immune system, by exploring the cellular immune response and the cellular mediated immunity and, potentially identify a biomarker to predict a beneficial blood pressure response – *addresses research question number 3 and answered in Original Articles Number 2 and 3.*

iii) To evaluate the efficacy of renal denervation in a cohort of patients with resistant hypertension, through a regular clinical evaluation and ABPM – *addresses research question number 1 and answered in Original Articles 2, 3, 4 and 5*.

The **secondary purposes** of this thesis were:

i) To evaluate the impact of renal denervation on the sympathetic cardiac innervation, in a subgroup of patients, through the performance of ^{123}I-labelled MIBG cardiac scintigraphy pre and six months after the procedure – *addresses research question number 4 and answered in Original Article 4.*

ii) To assess the impact of renal denervation on several echocardiographic parameters (left ventricular ejection fraction, diastolic function, left ventricle hypertrophy) and on serum vitamin D concentration – *addresses research question number 5 and answered in Original Article 5.*

In order to achieve these goals, the inclusion criteria were the following:

- Age over 18 years old
- Presence of idiopathic resistant hypertension – ambulatory mean systolic blood pressure > 130mmHg or diurnal mean systolic blood pressure > 135mmHg and, office systolic blood pressure > 150mmHg, plus anti-

hypertensive treatment with 3 or more drugs, in maximum tolerated dosages, including a diuretic.

- Preserved renal function (glomerular filtration rate ≥ 45 ml/min/1.73m^2).
- Compatible renal anatomy: absence of stenosis > 50%, previous renal angioplasty with stent implantation or fibromuscular dysplasia.

Patients were excluded if untreated secondary hypertension was detected, if hemodynamically significant valvular disease was present, if they had had a stroke or acute coronary syndrome in the previous six months, if life expectancy was bellow one year or if they refused to sign the informed consent.

Publication List

I – Review article

Renal denervation in the era of HTN-3. Comprehensive review and glimpse into the future.

Delgado Silva J, Costa M, Gersh BJ, Gonçalves L.

J Am Soc Hypertens 2016 Aug;10(8):656-70. doi: 10.1016/j.jash.2016.05.009.

Q1. IF 3,206. Citations 17.

II – Editorial Comment

Renal denervation in heart failure: Modulating the sympathetic nervous system.

Delgado Silva J.

Rev Port Cardiol 2017 Jan;36(1):53-54. doi: 10.1016/j.repc.2016.11.003.

Q3. IF 0,827. Citations 0.

III – Original Article Number 1

Intravascular imaging, histopathological analysis, and catecholamine quantification following catheter-based renal denervation in a swine model: the impact of prebifurcation energy delivery.

Delgado Silva J, Fernandes R, Pita IR, Pereira FC, Jaguszewski M, Gutiérrez-Chico JL, Ribeiro-Rodrigues T, Girão H, Ioannou A, Gonçalves L.

Hypertens Res 2018 Sep;41(9):708-717. doi: 10.1038/s41440-018-0072-y.

Q1. IF 3,217. Citations 3.

IV – Original Article Number 2

Activated double-negative T cells (CD3 + CD4 - CD8 - HLA-DR +) define response to renal denervation for resistant hypertension.

Delgado Silva J, Almeida JS, Rodrigues-Santos P, Santos Rosa M, Gonçalves L.

Clin Immunol 2020 Sep; 218:108521. doi: 10.1016/j.clim.2020.108521.

Q2. IF 3,368. Citations 0.

V – Original Article Number 3

Low plasmatic levels of RANTES/CCL5 are associated with a positive response to renal denervation in patients with resistant hypertension.

Delgado Silva J and Rodrigues-Santos P, Almeida JS, Santos Rosa M, Gonçalves L.

Submitted to *International Journal of Cardiology: Hypertension*.

Q2. IF 2.21.

VI – Original Article Number 4

Risk stratification and cardiac sympathetic activity assessment using myocardial [123I] MIBG imaging in renal denervation.

Delgado Silva J, Moreira AP, Costa G, Gonçalves L.

Accepted for publication in *Arq Bras Cardiol*.

Q3. IF 1,450.

VII – Original Article Number 5

Renal Sympathetic Denervation in Resistant Hypertension: the association between Vitamin D and Positive Early Response in Systolic Blood Pressure.

Costa G and Delgado Silva J, Monteiro E, Campos D, Gonçalves L.

Accepted for publication in *Rev Port Cardiol*.

Q3. 0,960.

Supplements

Case Report:

Reinnervation After Denervation – A Myth?

Monteiro E, Costa G, Delgado Silva J, Gonçalves L.

Submitted to *Arq Bras Cardiol*.

Q3. IF 1,450.

Part I: Review Articles

Review Article

Renal denervation in the era of HTN-3. Comprehensive review and glimpse into the future.

J Am Soc Hypertens. 2016 Aug;10(8):656-70.

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Abstract

The pathophysiological role of sympathetic overactivity in conditions such as hypertension has been well documented. Catheter-based renal denervation (RDN) is a minimally invasive percutaneous procedure which aims to disrupt sympathetic nerve afferent and efferent activity through the application of radiofrequency energy directly within the renal artery wall. This technique has emerged as a very promising treatment with dramatic effects on refractory hypertension but also in other conditions in which a sympathetic influence is present. Several studies have evaluated the safety and efficacy of this procedure, presently surrounded by controversy since the recent outcome of Symplicity HTN-3, the first randomized, sham-control trial, which failed to confirm RDN previous reported benefits on BP and cardiovascular risk lowering. Consequently, although some centers halted their RDN programs, research continues and both the concept of denervation and treatment strategies are being redefined to identify patients who can drive the most benefit from this technology. In the United States, the Food and Drug Administration (FDA) has appropriately mandated that RDN remains an investigative procedure and a new generation of sham-controlled trials are ongoing and aimed to assess not only its efficacy against pharmacotherapy but also trials in drug free patients with the objective of demonstrating once and for all whether the procedure actually does lower BP in comparison to a placebo arm. In this article, we present an overview of the sympathetic nervous system and its role in hypertension, examine the current data on RDN, and share some insights and future expectations.

Keywords: Hypertension; sympathetic nervous system; renal denervation; HTN-3.

Introduction

The relationship between hypertension (HT) and cardiovascular (CV) and renal events is well known and has been the subject of several observational studies. [2-3] Office blood pressure (BP) is independently associated with the incidence of major adverse CV events, such as stroke, myocardial infarction, sudden death, heart failure and also end-stage renal disease. [4-8] Prevalence of resistant HT is largely unknown and is compounded by the documented lack of drug compliance in many series. [9] It has been reported to range between 5-30%, with a probable true prevalence below 10%. [10-11] Patients with true resistant HT are at the utmost point of an already high CV risk and are currently being referred for renal denervation (RDN), as an hyperactivated sympathetic nervous system (SNS) may be responsible for HT refractoriness and its partial inhibition is believed to be achievable resourcing to a rather simple and safe percutaneous technique, as demonstrated in several studies [12, 40-44].

RDN is a percutaneous procedure which consists of the application of radiofrequency (RF) energy in quadrant-based points in the renal arteries and has emerged as an attractive method to target the SNS, as most trials (without sham controls) show it can significantly reduce BP in resistant HT at the long term. However, the initial hype surrounding this novel approach was dramatically tempered by the results of the HTN-3 clinical trial, [12] which caused interventionalists, hypertension specialists and referring doctors to reconsider whether the procedure is indeed effective, to revise the definitions of resistant HT and to perform a more exhaustive exclusion of secondary causes in order to redefine the patient who can truly benefit from this technique.

In this review we focus our attention on RDN and its role on the treatment of resistant HT. We also perform an overview of the SNS, examine the most important clinical trials and share some insights and future expectations.

The sympathetic nervous system – why is it a target?

Surgical treatment of essential hypertension - historical perspective

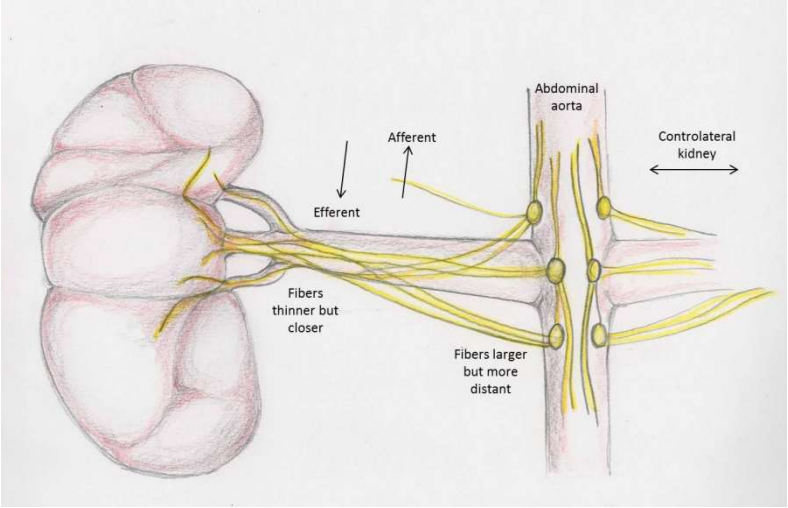
HT has been misunderstood for years and became the focus of attention of many investigators in the last century, in an attempt to elucidate its pathophysiological background.

The vasoconstrictor effects of the SNS and its implication in the pathogenesis of HT has long been known but the concept continues to evolve. As antihypertensive drug therapy was not available before World War 2 and untreated malignant HT was associated with a 100% five-year mortality, [13] surgical techniques were developed and the first sympathectomy was conducted by the surgeon Fritz Bruening in 1923. In the following years, surgeons like Peet and Smithwick attempted to treat essential severe HT with radical sympathectomy and later by thoracolumbar splanchnicectomy. The so called Smithwick procedure consisted on a lumbodorsal sympathectomy and was associated with considerable side effects (severe orthosthatic hypotension, intestinal disturbances, fecal and urinary incontinence, anydrosis and erectile dysfunction, among others) and high mortality rates. [14-18] As the drug era begun, around the 1960s, this aggressive approach was discontinued and the knowledge regarding the relation between the SNS, kydneys and HT was used, almost one century later, to comprise an utterly intrusive operation into a more selective approach that aims to target the renal nerves and, consequently, reduce the activity of the SNS without the severe adverse effects but with the same or even superior efficacy, in regard to BP control.

The sympathetic nervous system and renal anatomy – a very close relation (Figure 1)

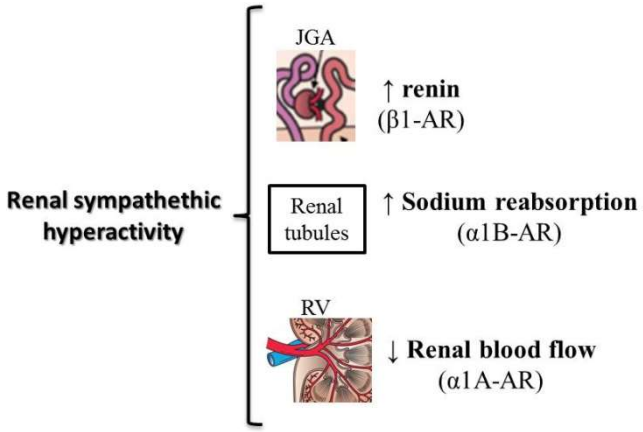
The function of several organs, like the heart, vasculature and the kidneys, is controlled by the SNS, through an efferent nerve stream. The SNS activity is not constant or predictable and is modulated by an afferent sympathetic outflow and also by the contralateral kidney. Afferent sensory nerve fibers are located mainly in the renal pelvic wall and communicate with the contralateral kidney in order to maintain diuresis and natriuresis despite unilateral disturbances. This mechanism is called the renorenal reflex and coordinates the excretory function of the two kidneys to maintain homeostatic regulation of sodium and water balance. [19]

Figure 1: The kidney and the sympathetic nervous system. Renal fibers are thought to be thinner but closer to the arterial lumen in the distal segments.



The efferent nerves are predominantly unmyelinated small fibers which enter the kidneys through the hilum and then divide, following the ramifications of the arterial circulation. [20-21] Renal sympathetic nerves play a potentially pathophysiologic role in HT, contributing to both its development and persistence. They lead to three major alterations (mediated by the primary neurotransmitter norepinephrine): (1) increased renin secretion via stimulation of β1-adrenoreceptors on the juxtaglomerular apparatus, (2) increased renal tubular sodium reabsorption via stimulation of α1B-adrenoreceptors on renal tubular epithelial cells and (3) decrease of renal blood flow via stimulation of α1A-adrenoreceptors on the renal arterial vasculature (figure 2).

Figure 2: The role of renal sympathetic nerves in hypertension through three major pathophysiologic alterations: increased renin secretion on the juxtaglomerular apparatus (JGA) via stimulation of β1-adrenoreceptors (β1-AR), increased sodium reabsorption via α1B-adrenoreceptors (α1B-AR) and decreased renal blood flow on the renal arterial vasculature (RV) via α1A-adrenoreceptors (α1A-AR).



The SNS is directly linked to the renin-angiotensin-aldosterone system as increased renin secretion leads to angiotensin I and angiotensin II (Ang II) generation. Ang II rapidly binds to its main receptor (Ang II type 1 receptor, AT1R) followed by internalization and accumulation in cells. Activation of AT1R induces several deleterious alterations like vasoconstriction, endothelial dysfunction, inflammation, growth and remodeling. It also increases the formation of reactive oxygen species which results in cell growth, expression of pro-inflammatory genes and the production of extracellular matrix proteins. Ang II type 2 receptors are thought to counterbalance these effects (controversial finding) by blocking mitogen-activated protein kinase and activating nitric oxide synthase, inducing vasorelaxation. [22-23] This knowledge has led to the development of several substances that target these pathways at different levels and have become the hallmark of HT treatment.

The advent of a technique that uses an intra-vascular approach to deliver energy in order to ablate/interrupt the sympathetic outflow and thus destroying nerve bundles, lead to the investigation of their actual distance to the vessel lumen as an attempt to predict the level of denervation. Some studies have shown that sympathetic fibers are located mainly in the adventitia and outer layers of the renal vasculature. [24] Atherton et al. [25] conducted a histological study in post-mortem human renal arteries and concluded that there are fewer,

larger nerve trunks in the proximal renal artery and more, smaller nerves distally. Nerves were evenly distributed around the circumference of the renal artery and 50% of all nerves detected resided within 0.5-1mm of the lumen (90.5% within 2.0 mm). These findings suggested that renal nerves were accessible to RF energy delivery and that applying the energy prior to the bifurcation, following a helical pattern from distal to proximal was appropriate. However, more recent animal studies showed that nerve distribution is rather homogenous throughout the artery length and renal artery nerves were more frequently found in the proximal segment of the renal artery and decreased gradually distally, where they were closer to the arterial wall. [26-27] Sakakura et al. [28] examined the arteries and peri-renal nerve anatomy from human autopsy subjects and concluded that, although there were fewer nerves in the distal segments of the arteries, they are closer to the lumen and therefore may be an attractive target for RDN. This led to an evolution of the denervation technique with investigators now proposing energy application to the branches, if appropriately sized [29], as it apparently leads to a significant reduction in both norepinephrine and axon density, as opposed to treating only the main artery. [30] Nerve injury and ablation also appears to be determined by tissue microanatomy at the electrode site, with power density peaking at discontinuities between fatty adventitia and water-rich tissues. [31]

The duration of the BP lowering effect has also been a matter of discussion, since there are reports that point to the fact that renal fibers may regenerate and regrow into functionally active nerves. [58] However, studies with long follow-ups (up to 36 months, table 1) show that BP remains significantly lower than baseline after RDN. Rousselle et al. [32] conducted an animal study that aimed to characterize nerve response at 7, 30 and 90 days after RDN and observed that a progressive regenerative response occurred as early as 7 days but resulted in neuromatous tangles with disrupted architecture and apparent low potential for functional activity, at the RF lesion sites. According to these findings, nerve fibers behavior after RDN still needs to be fully elucidated but possibly have a very low capacity for regenerating after RF ablation.

The sympathetic nervous system – assessing the sympathetic drive

The SNS is involved in the pathogenesis of multiple clinical entities associated with marked sympathetic CV drive, such as advanced heart failure, obesity and sleep apnea syndrome, and increases the risk of life-threatening cardiac arrhythmias and sudden death. [33-36] Sympathetic

activity is highly complex as it functions at several levels of the body. Therefore, there is no single method to obtain an overall activity assessment and diverse non-imaging techniques that complement each other have been used over the years to measure neuro-adrenergic activity [37]:

- Heart rate: controlled partially by the SNS and the parasympathetic system influence on the sinus node. Highly variable, may be normal even in diseases associated with a high sympathetic drive and, as such, cannot be a reliable marker of SNS activity. Heart rate variability and turbulence may be useful when combined with additional indices of autonomic function.
- Microneurography: minimally invasive technique that permits direct measurement of the sympathetic outflow to either the skin or the skeletal muscle (usually via the radial or the peroneal nerves) providing a dynamic assessment of the SNS. Unfortunately, it does not produce direct information of the sympathetic stream to internal organs like the kidneys or the heart.
- Plasma norepinephrine: low sensitivity as the levels released by the sympathetic nerve terminals are very different from the circulating neurotransmitter. However, can be a complimentary method to microneurography.
- Regional and total norepinephrine spillover: infusion of small dosages of radiolabelled norepinephrine that permits its precise quantification both at the nerve terminals and the circulation. Selective quantification is one of the main advantages of this method as it provides specific organ information regarding the sympathetic drive. The major limitation is the need to catheterize both the renal arteries and the coronary sinus for assessment of cardiac and renal spillover, respectively.

Renal denervation – the procedure

RDN is a minimally invasive percutaneous procedure which consists on the delivery of RF energy on the renal arterial wall in order to achieve a selective disruption of the sympathetic nerve endings. Catheter based RF ablations techniques have been used successfully in electrophysiology for more than two decades to treat diseases such as pre-excitation syndromes, supraventricular and ventricular arrhythmias. To date, RDN has been used to treat patients with resistant HT and RF energy has been delivered using either a single-tip electrode catheter or multi-electrode systems. The purpose is to apply the energy in a circular pattern, according to the distribution of the renal nerves, without re-crossing previously treated sites (to

avoid renal stenosis). Currently, there are six devices approved (one of which is ultrasound-based and another allows for denervation via the radial approach) but the largest amount of evidence clearly exists for the Symplicity TM RDN Systems (Medtronic, Minneapolis, MN, USA). [38] The preferred approach is via the femoral artery through a 6-8 French (F) sheath. The Symplicity Flex TM RDN Catheter is a 6F guide-compatible steerable catheter which has a self-orienting tip for atraumatic vessel contact. It is rather easy to use but cumbersome as the intended circumferential pattern is difficult to achieve. This technology has been further developed into a multi-electrode ablation catheter (Symplicity Spyral TM multi-electrode RDN catheter) which allows for the delivery of consistent four quadrant ablation pattern through a one minute ablation time and simultaneous-firing electrodes. The deliverability is over a standard 0.014" guidewire, is 6F compatible and conforms to sizes 3-8mm (renal diameter) [39] (figure 3). The multi-electrode EnligHTN TM is the St. Jude system (St. Jude Medical, St Paul, MN, USA) and has four electrodes mounted on a non-occluding nitinol basket that can be deployed by rotating a handle. It is 8F compatible, has an atraumatic tip and allows for a predictable pattern. The basket has two sizes that are suitable for RDN from 4-8mm diameters (Figure 4). [40] The Iberis ® System (Terumo Medical Corporation, Tokyo, Japan) is unipolar and allows the radial access (Figure 5). The One Shot TM RDN Device (Covidien Campbell, CA, USA) and the Vessix V2 TM System (Boston Scientific, Marlborough, MA, USA) have the electrodes mounted on a balloon. The Paradise ® System (ReCor Medical, Ronkonkoma, NY, USA) uses ultrasound technology.

Figure 3: Symplicity Spyral ™ multi-electrode RDN catheter. A: Positioning. B: Confirmation of correct location. C: Deliverability is over a standard 0.014'' guidewire.

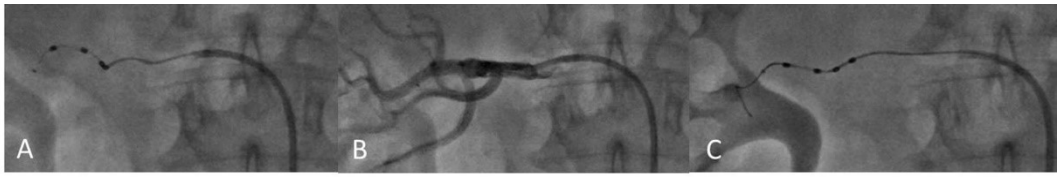


Figure 4: Multi-electrode EnligHTN ™ RDN catheter. A: Left renal artery. B: Positioning of the basket. C: Opening of the basket.

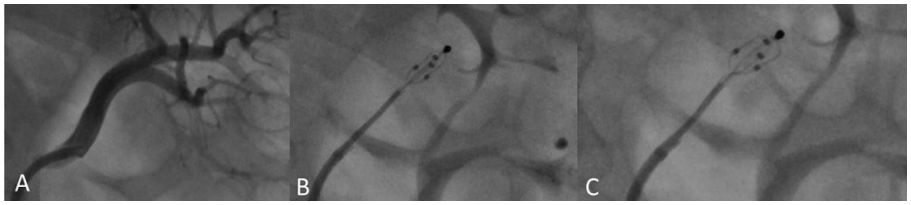
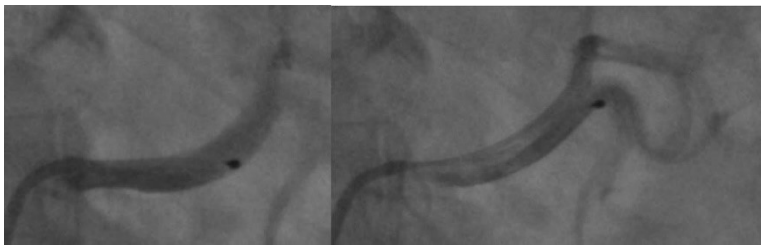


Figure 5: Iberis ® System (unipolar).



Clinical studies on renal denervation – The tide has changed

Several clinical trials have been conducted since catheter based RDN first became available but the current evidence relies mostly on the Medtronic catheter, Symplicity Flex.

The Symplicity HTN-1 was the first multicenter, proof-of-concept and safety study (nonrandomized) in patients with resistant HT. [41-42] Fifty patients were enrolled from five European and Australian Centers. Primary outcomes were assessment of office BP and safety data. Three-year results confirmed the long-term benefits of RDN, with a drop of 10mmHg or more in systolic office BP observed in 93% of patients and with safety being maintained.

Symplicity HTN-2 [43] was conducted shortly after HTN-1 and was a multicenter, prospective, randomized trial, unblinded, which included 106 patients (52 underwent RDN vs 54 in the control group). Office systolic and diastolic BP was significantly reduced at 6 months whereas the control group had only minor reductions. After the 6-month endpoint was met, cross-over of patients from the control to the RDN group was allowed and these last patients had a significant drop in office BP similar to the one observed in the initial RDN group. Sustained BP

reduction at 1-year was confirmed. [44] Three-year follow-up [45] continued to show a sustained lowering effect of RDN on office systolic and diastolic BP.

Many questions arose from the mentioned results and HTN-3 was eagerly awaited. HTN-1 and 2 had a number of inconsistencies like the discrepancies between office BP and Ambulatory Blood Pressure Monitoring (ABPM) (performed in a small subset of patients; the BP reduction was only 41% and 34% in both studies, respectively). What is particularly interesting is the complete lack of any benefit in the control arm (something very unusual in the field of HT drug trials). One potential explanation is the “reverse Hawthorn” effect because patients knew beforehand that they could cross-over to RDN after 6 months if their BP was still uncontrolled. This knowledge might have resulted in reduced compliance in order to remain eligible for RDN. [46] The success of RDN is difficult to assess and hence, in HTN-1, renal norepinephrine spillover was measured in a subset of patients. Unfortunately, it is a cumbersome method and infeasible in real world practice.

After the first reports on HTN-1 and 2 trials were disclosed, a considerable enthusiasm surrounded RDN. Several articles mentioning emerging new indications that surpassed the restricted group of patients with resistant HT were published and the SNS became the subject of many lectures, as it appeared to be possible to interrupt it with a minimally invasive procedure and target disease states whose main pathophysiological basis was sympathetic overdrive.

Symplicity HTN-3 [12] is a prospective, single-blind, randomized, sham-controlled trial which included patients with resistant HT (n=535) who were randomly assigned in a 2:1 ratio to undergo RDN with a single-tip electrode catheter (Symplicity Flex, Medtronic, MN, USA) or a sham procedure. Six-month results showed no significant differences between the RDN group and the sham group regarding both systolic office BP and mean systolic ABPM. There were no differences concerning safety. Authors provided several explanations which could possibly explain the disparities between this study and the previously published ones such the Hawthorne effect, [47] lack of blinded control groups in prior trials and non-confirmation of medication adherence. Subgroup analysis revealed that some groups responded worse to RDN than others like patients older than 65 years old, with an estimated glomerular filtration rate < 60ml/min/1.73m2 and the African-American population. Multivariate analysis identified baseline systolic BP ≥ 180mmHg, prior treatment with an aldosterone antagonist and total ablation attempts as positive predictors of BP change at 6 months. Prior prescription of a

vasodilator was a negative predictor. Twelve-month results [48] revealed that the changes in systolic office BP were significantly greater than at 6 months in the RDN group but there were still no differences regarding ABPM.

Results of Symplicity HTN-3 had great impact in the medical community and many centers stopped their RDN programs after the disclosure of these results. Supporters of RDN have tried to provide an explanation and critically review HTN-3 outcomes, bearing in mind the knowledge provided by past research. The most obvious explanation could be that RDN is, in fact, ineffective in lowering BP in the studied population. Pocock and Gersh [49] consider that the previous findings may indeed reflect a substantial placebo effect and regression to the mean, which possibly turned patients with 'refractory HT' adherent to drug therapy once enrolled into the trial. The power of the placebo has been continuously underestimated by inadequately-designed randomized clinical trials, with consequent exaggerated findings, and well-designed RCTs are urgently needed to clarify the concrete role of RDN in the management of HT. Kandzari et al. [50-51] provided an excellent analysis and investigated key factors that could have contributed to the differences between the sham group and the RDN group in HTN3. Possible accountable factors were the following:

- Medication adherence and stabilization was not altogether clear. The great response observed in the sham group is possibly the result of behavioral changes as these patients were observed in very frequent clinical visits (not usual in clinical practice). Also, between randomization and 6 month follow-up, 39% of patients had a change in medication, mainly due to side-effects.

- African-Americans comprised 26.2% of the overall population and were more frequently prescribed with vasodilators. Although there are differences regarding pathophysiological and genetic mechanisms, [52] these could not explain the marked reduction of BP in the sham group. This effect was probably due to medication adherence/change.

- No method for assessing procedural success exists which is a major setback in the evaluation not just of these patients but a limitation to the procedure as a whole.

- Regarding the procedure itself, several considerations arise. In HTN-3, more than half of the operators performed at most two RDN procedures and 31% performed only one during the trial. This insufficient experience could be correlated with the lack of ablations in all four

quadrants (6% received bilateral four quadrant ablations, 20% received unilateral four quadrant ablation and 74% received no four-quadrant ablation) and reduced number of ablations attempts (only 84% were complete ablations of 120 seconds duration), as these are thought to be predictors of a positive 6-month BP change. Patients receiving ≥ 14 ablations had the greater BP fall, with a 12mmHg increase with <8 ablations. The true meaning of this finding is not completely clarified.

More recently, some interesting studies focusing on RDN were published. The DENERHTN [53] is a French multicenter, prospective, open-label randomized controlled trial with blinded endpoint evaluation, performed in patients with resistant HT, confirmed by ABPM. This study was different from the previous ones as a standardized stepped-care antihypertensive treatment (SSAHT) was added to RDN, which was performed with the single electrode Symplicity Flex catheter. After 4 weeks of standardized triple therapy, patients with confirmed resistance were randomly assigned to receive either RDN plus SSAHT or SSAHT alone (53 in each group).The primary efficacy endpoint was the mean change in daytime ambulatory systolic BP from baseline to 6 months Treatment compliance was assessed by performing a validated questionnaire. At six months, patients in the RDN group had a modest but significant decrease in daytime ambulatory systolic BP with a mean baseline-adjusted difference between the two groups of −5.9 mm Hg, p=0•0329 (this reduction, even though it was statistically significant, is not close to the huge reductions observed in other studies). Similar results were observed for nocturnal and 24 hour mean BP (without reaching statistical significance). Multivariate analysis was performed and revealed that male gender, high adherence to medication, high baseline daytime ambulatory systolic BP, large changes in daytime ambulatory heart rate from baseline to 6 months, but not race, were independently and significantly associated with greater changes in daytime ambulatory systolic BP. Interestingly, the number of ablations was not a predictor of response. The incidence of adverse events was not significant in both groups. Even though there was no sham procedure, sequential blocking of the SNS (bisoprolol, prazosin, and rilmenidine) was implemented in both groups. Authors comment on the fact that these results diverge from the Simplicity HTN-3 study, probably due to variability between the patients who were included in both trials and the study design itself. The authors also refer the absence of a sham procedure as a limitation, having selected an opel-label study design both for ethical and operational reasons. Main strengths of this study include the use of ABPM as the primary endpoint, thus reducing variability of baseline measurements and potential clinician related bias, and the

implementation of a standardized pharmacological treatment, which reduced inter-individual variability and unexpected medication changes.

The Global Symplicity Registry (GSR) is a prospective, open-label, multicenter registry that aims to assess safety and efficacy of RDN in an uncontrolled global clinical setting. It includes patients not only with HT but also with other conditions associated with SNS activation, like heart failure. The only inclusion criteria are age ≥ 18 years old and eligibility for RDN, according to the local system. The first report [54] acquaints on 998 patients, 323 of which had severe HT. In this cohort, baseline systolic BP was 179.3 ± 16.5 mmHg and decreased to 159 ± 21.5 mmHg at six months ($p<0.0001$). Baseline 24h systolic BP (available in 221 patients) was 159 ± 15.6 mmHg and dropped to 150 ± 18mmHg at six months ($p<0.0001$). Regarding safety, RDN was associated with low rates of CV, renal and periprocedural complications (<1%). In the discussion the authors comment on the clear difference between the response in this registry and Symplicity HTN-3, calling to attention: (1) the modifications in medication after RDN (34% in GSR vs 39% in HTN-3), (2) the differences in the interventionalists experience (59% of the GSR operators had performed ≥ 15 RDN before the registry started), (3) the average number of completed 120 second ablations was 9.2 in HTN-3 vs 13.7 in severe HT cohort of GSR and (4) the possibility of regression to the mean. [55] Mentioned limitations were directly related to the fact that it is a registry-based study and included: non-assessment of the specific indications for RDN, non-standardization of follow-up procedures (which may compromise the report of safety events) and the possibility of placebo/Hawthorne effect. Nevertheless, GSR provided further evidence that RDN appears to safely reduce BP in patients with resistant HT.

The SPYRAL HTN Global Clinical Trial Program was recently announced by Medtronic and consists of two global, prospective, randomized, sham-controlled trials conducted simultaneously by experienced interventionalists, aiming to investigate the impact of RDN both in the absence of, and in the presence of antihypertensive medications. [56] They will include approximately 100 patients each with moderate to high-risk HT and will be conducted at approximately 20 centers in the United States and other countries. The multi-electrode Symplicity-Spyral catheter will be used, the number of ablations will increase and distal branches will be targeted, raising safety issues. This program was designed to address the results of HTN-3 and maybe will shed some light on the meaning of published results.

Patient selection – according to the new evidence, who can benefit?

At the present time, RDN is, at the very least, in an impasse. The most recent guidelines and position paper [4, 58] state that patients are eligible to RDN if they have severe refractory essential HT, are adherent to medication and have eligible renal anatomy (no renal stenosis, no prior stent intervention and an adequate artery diameter). Although patients with renal dysfunction, cardiovascular instability and multiple renal arteries have been excluded from most trials, RDN can be performed after careful consideration by a vascular team. Advice is given as to handle patient's expectations, as the intent is to control HTper se and reduce the cardiovascular burden, and not to lessen the medication. Many authors believe that patients with refractory HT may not be the ideal targets for RDN (should HT be targeted in an earlier phase?). Recent studies comparing RDN effect in isolated systolic HT (ISH) vs combined HT [59] showed a less pronounced reduction of HT in ISH, as ISH appears to be associated with stiffer arteries. [60] RDN also appears to lower total peripheral resistance, a change not associated with significant changes in cardiac output. [61] An elevated central pulse pressure (CPP), as an indicative of this arterial stiffening and enhanced vascular ageing, may be helpful in predicting BP reduction after RDN. Ott C et al. [62] stratified RDN patients according to the median CPP and found that BP reduction following RDN is greater in patients with lower CPP, which is indicative of a lower degree of arterial damage. As the SNS greatly influences the vessel vasoconstrictive tone, a decrease in sympathetic activity has the potential to lower BP if the vasculature still retains the ability to react and vasodilate, a capacity apparently lost in patients with high CPP.

However, with all the controversy and a myriad of trials, registries, reviews and editorials, the main focus remains on several questions that are still unanswered (table 2):

No 1 – <u>Does it work? And if so in whom</u>

- Is it possible to identify, prospectively, patients who can truly benefit from an invasive approach to treat their untreatable HT and therefore lower their CV morbidity? An interesting meta-analysis by Fadl Elmula et al. [63], aiming to sum up the randomized evidence on the efficacy and safety of RDN with the Symplicity catheter in the treatment of resistant HT, analyzed seven randomized controlled trials and a total of 985 patients (397 in control and 588 in RDN).

Women were underrepresented in all trials (proportions ranging from 10 to 42.5%). The two major findings of this review were, first, that the BP lowering effect of RDN, when compared to optimized medical therapy, was only modest, both in the office and ABPM and second, the procedure is safe, with similar risk of major adverse CV events both in RDN and control groups. It seems that a minority of patients really experiences the BP lowering effect of RDN and the future will be determined by the correct identification of this responder population.

- If these patients are in fact identified, how will the interventionalist know how much RF energy to deliver and how will the ablation success be measured? The search for a reliable biomarker is ongoing but until the moment, no such predictor of BP response exists. Dörr et al. [64] executed a very interesting study based on the fact that HT is associated with endothelial dysfunction. The authors evaluated whether the angiogenic factors soluble fms-like tyrosine kinase-1 (sFLT-1), intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are predictive markers for BP lowering after RDN. They observed that responders showed significantly higher serum levels of the studied molecules at baseline compared with non-responders, identifying them as potential biomarkers. Since this was a small study, with a limited number of patients, further investigation is required. Another interesting study by Dörr et al. [65] evaluated whether brain-derived neurotrophic factor (BDNF) would provide an immediate assessment of successful denervation, as BDNF is an important modulator of the SNS. The study included 100 consecutive patients with resistant HT who underwent RDN. The authors observed a significant decrease in BDNF levels immediately after RDN and a significant correlation between the extent of an early decrease and systolic BP reduction at six-month follow-up. Although BDNF analysis was restricted to 2 hours post procedure, it appears promising as a biomarker to evaluate the immediate success of RDN. Renal nerve stimulation is also being investigated as a potential marker of RDN response, with small studies showing an increase in BP prior to RDN, a response that is significantly blunted after the procedure. [66] This method may allow for the identification of the exact location of nervous endings and therefore overcome the difficulties associated with anatomical variability. Further investigation is ongoing and awaited.

- How can adherence to medication be assured? Should RDN be offered to patients who cannot/will not comply to multiple drug treatment? Does adherence have an impact on the response to RDN? Ewen et al [67] investigated adherence to prescribed antihypertensive

treatment by liquid chromatography-high resolution tandem mass spectrometry in plasma and urine at baseline and 6 months in 100 RDN patients and observed that mean adherence was significantly reduced from 85.0 % at baseline to 80.7 % at 6 months (p = 0.005). The authors concluded that RDN can reduce office and ambulatory BP in patients with resistant HT despite a significant reduction in adherence to antihypertensive treatment after 6 months.

No 2 - Should we expand the indication of RDN to less severe stages of HT?

- Desch et al. conducted a randomized, blinded, sham-controlled trial [68] whose main objective was to evaluate the effect of RDN on patients with resistant HT and day-time systolic BP of 135-149mmHg and diastolic of 90-94mmHg, assessed by ABPM. Patients were randomized to either RDN (with the Symplicity Flex catheter, n=29) or an invasive sham procedure (n=34). In this trial, RDN failed to show a significant reduction in the primary endpoint of ABPM systolic BP lowering at six months in the intention to treat analysis, but in the per-protocol population RDN was associated to a significant drop in BP, compared with the sham group. The REDUCE HTN: REINFORCE study [69] is currently recruiting participants and aims to determine whether the Vessix Reduce TM Denervation System (Boston Scientific, Marlborough, MA, USA) shows acceptable performance for treating uncontrolled HT (off-treatment office systolic blood pressure ≥150 mmHg and ≤180 mmHg) when compared to a sham procedure.

No 3 - Should RDN (only) be offered to patients who experience side effects to medication?

- Spironolactone has shown to markedly improve BP control when added to a three anti-hypertensive drug regimen [70] and was a marker of response in HTN-3. It is well known for its adverse effects such as hyperkalemia, painful gynecomastia or erectile dysfunction.

- The Prague-15 is a prospective, randomized, open-label multicenter study aiming to evaluate the efficacy of RDN (with the single electrode Symplicity Flex catheter) vs intensified pharmacological treatment including spironolactone (if tolerated). Confirmation of true resistance was accomplished through ABPM, exclusion of secondary HT and evaluation of treatment compliance. Six-month results [71] showed significant BP reductions in both groups (although not between groups) but spironolactone side effects, leading to treatment

discontinuation (anti-androgen effect was the most common), were present in 35% of the patients, with 4% even refusing to start the drug. Eplerenone was offered as an alternative but was rejected by all patients due to financial costs. Twelve-month results were recently published [72] and corroborated that RDN was not superior to intensified drug-treatment during this period. Per-protocol analysis showed a significant reduction in 24-hour systolic BP, more accentuated in the pharmacological cohort, with evident between-group difference (p=0.04). However, only 19 of the initial 54 patients randomized to intensified treatment were able to maintain spironolactone up to one year. Major caveats of this study include the use of a single electrode catheter (which prevented the application of an adequate number of four-quadrant ablations), the reduced recommended number of ablations (minimum four per side) and the absence of a sham procedure. Although these findings suggest RDN may be as effective as an intensified drug treatment, they also show a high rate of non-adherence/drug discontinuation due to adverse effects and, as such, further investigation with improved procedural technique and patient selection are needed to clarify these data.

No 4 - Is RDN safe?

- Studies have demonstrated acceptable safety profiles following RDN, including HTN-3. Procedure related events are rare, even during long-term follow-up, and include renal artery dissection, stenosis and vascular access complications, such as femoral pseudoaneurysm. Pucci G et al. [73] described a case of renal artery stenosis accelerated progression after RDN, associated with a sustained increase in 24 hour-BP, suggesting that RF application may worsen the progression of pre-existing renal artery atherosclerotic lesions and therefore affect RDN efficacy. Clinical and imaging patient monitoring after RDN is of the utmost importance to assure safety.

Until many of these doubts are clarified, RDN should only be offered to patients in the setting of clinical research and in highly skilled referral centres. The European clinical consensus conference for RDN [74] for the design of future randomized controlled trials recommended a rigorous approach, focusing on three main topics: (1) Selection of a suitable patient population (including patients with moderate instead of just resistant HT and exclude patients with

increased vascular stiffness), (2) Attain a proper study design by avoiding wash out periods (unless considered necessary and only in tertiary centres), avoiding sham procedures in mild to moderate HT (as the associated risk is not negligible) and considering drug adherence monitoring and pharmacological therapy standardization and (3) Proper selection of study outcomes like ABPM and accessible predictors of efficacy. White et al. [75] authored a scientific statement which summarized the conclusions of a reunion co–sponsored by the American Society of Hypertension, the United States Food and Drug Administration and the National Heart, Lung, and Blood Institute and aimed to delineate a strategy to undertake clinical studies, as they are pivotal to evaluate the efficacy and safety of new devices. Participants of this forum did not consider that Symplicity HTN-3 represented a complete failure of RDN but rather highlighted the need to define pathways for the future development of device therapies for HT. They recommended the use of preclinical models to evaluate RDN, the identification of reliable biological markers of RDN, the assessment of anatomical completion of RDN, the selection of cohorts with a high response probability, and hence most likely to drive some benefit from RDN, and the design of randomized clinical trials to assess treatment effect.

Radiofrequency alternatives for the treatment of resistant hypertension: a valuable option?

- Cryoablation: Cryoenergy is the application of intense cold within the target tissue which causes cells to die and be replaced with fibrosis. Its main use has been in the setting of cardiac arrhythmias ablation but more recently, is being investigated as an alternative to RF for sympathetic denervation of the renal artery. Animal studies [76] suggest cryoenergy may be more effective in decreasing the density of nervous fibers than RF delivery. Prochnau et al. led a small observational study [77] in which ten RF non-responders were treated with cryoenergy for RDN and observed a significant drop both in office systolic BP and systolic and diastolic BP in ABPM, with reduced periprocedural pain and discomfort. Although these are interesting results, the authors state it is unknown if a second RF delivery, instead of cryoenergy, would have yielded the same outcome. Cryonenergy is an interesting technique but its efficacy still has to be confirmed in large and adequately designed multicenter trials.

- Carotid baroreflex activation therapy: Arterial baroreceptores are stretch sensors located in the blood vessels, which are pressure-sensitive, being stimulated by arterial wall distortion induced by pressure oscillations. They are activated when BP rises, sending impulses to the brain that increase parasympathetic outflow and therefore inducing bradycardia and peripheral vasodilation. As baroreceptores become partially blunted in the setting of resistant HT, mainly due to increased vascular stiffness, electrical stimulation of these receptors may lead to reductions in sympathetic activity and hence, the BP. Surgical implantable carotid baroreceptors devices, which electrically stimulate the carotid sinus, are currently under investigation and appear to be a promising alternative for patients with resistant HT. [78]

Summarizing, HT is a multifactorial disease, difficult to assess and treat and, in most of the cases, associated with multiple life-long antihypertensive medication. Patients with resistant HT are at the end of the line, exposed to a huge cardiovascular risk and facing few options to choose from. A diagnostic workup to exclude secondary forms of hypertension and confirm resistance (observational studies have shown that white coat HT can be detected in one third of patients diagnosed with resistant HT) [79] should be thoroughly conducted and afterwards, a therapeutic strategy should be implemented, always including lifestyle counseling, the necessary and recommended adjustments to the antihypertensive regimen and device-based therapies.

Several studies have revealed that RDN significantly reduces noradrenaline spillover, muscle sympathetic nerve activity and the augmentation index, which appears to be independent of BP. [80] This disruption of the SNS has the potential to influence many other clinical entities highly dependent on SNS hyperactivity, such as the sleep apnea syndrome, [81] chronic heart failure, [82] atrial fibrillation (AF) and ventricular arrhythmias, [83] metabolic syndrome, [84] chronic kidney disease [85] and polycystic ovary syndrome. [86] Most of these studies are small and purely observational. Regarding cardiac arrhythmias, SYMPLICITY AF is a prospective, randomized, multi-center, feasibility clinical study investigating pulmonary vein isolation and renal denervation compared to pulmonary vein isolation alone, for the treatment of paroxysmal or persistent AF in patients with both AF and HT. Patients in both arms will receive an insertable cardiac monitoring device in order to detect and record the net recurrence of abnormal heart rhythms after therapy randomization. Enrollment has recently started. [87]

Presently, an integration of the available therapies and an individualized assessment may be the optimum path, while awaiting further insight on the matter.

Conclusions

Although the knowledge surrounding renal denervation is conflicting, current evidence suggests that this technique modulates the hyperactivity of the sympathetic nervous system and appears to be associated with some benefit regarding the reduction of cardiovascular risk in several disease states associated with sympathetic overdrive. More research aiming to detect which patients attain the most beneficial effects of this invasive procedure, to rearrange the procedural technique (as it is rather complex) and to discover a reliable biomarker on procedural success is of the utmost importance for the future of hyper-adrenergic states targeting.

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Tables

Table 1: Synopsis of Pivotal Trials assessing safety and efficacy of RDN in resistant HT

Study	Type	Location	N	RDN catheter	Longer FU	Primary outcome	Main results
Symplicity HTN 1 [42-43]	Proof-of-concept	Europe and Australia	153	Single-tip electrode Symplicity Flex™(Medtronic) (6F)	36 M (88 pts)	* OBP * Safety	ABPM not mandatory. Drops of 10 mm Hg or more in systolic BP: 69% (1M), 81% (6M), 85% (12M), 83% (24M) and 93% (36M).
Symplicity HTN 2 [44-46]	Randomized	Europe, Australia and New Zealand	106 (52 in RDN vs 54 in control gp)	Single-tip electrode Symplicity Flex™ (Medtronic) (6F)	Initial RDN gp: 36M (40 pts) Crossover gp: 30M (30 pts)	*Systolic OBP *Procedural safety * CV events	ABPM not mandatory. Crossover to RDN gp allowed at 6M. A sustained lowering of BP at 3 years was observed, without serious safety concerns.
EnligHTN I [58]	First in human, non-randomized	Australia and Greece	46	Multi-electrode catheter EnligHTN™ (St. Jude Medical) (8F)	24 M	* OBP * Safety	ABPM at baseline and FU. Sustained reduction of office, ambulatory and home BP at 24M. Safe method.
Symplicity HTN 3 [12,48]	Randomized, sham-controlled	United States	535 (364 in RDN vs 171 in sham gp)	Single-tip electrode Symplicity Flex™ (Medtronic) (6F)	Initial RDN gp: 12M (319 pts) Crossover gp: 6M (93 pts)	* OBP *Mean systolic ABPM * Safety	ABPM at baseline and FU. Crossover to RDN gp allowed at 6M. Lack of significant reduction of systolic BP at 6 and 12M. Safe method. In-depth analysis performed (see text).
DENERHTN [53]	Randomized	France	106 (53 in RDN vs 53 in SSAHT gp)	Single-tip electrode Symplicity Flex™ (Medtronic) (6F)	6 M	* Daytime systolic ABPM * Safety	ABPM at baseline and FU. RDN plus an SSAHT decreases ABPM more than the same SSAHT alone at 6 M. Safe method.
Global Symplicity Registry [54]	Open-label	Europe	323 in severe HT cohort	Symplicity RDN system (Flex™ or Spyral™) (Medtronic) (6F)	6 M	* OBP and ABPM * Safety	ABPM not mandatory (real world setting). RDN resulted in significant reductions in OBP and ABPM with a favorable safety profile.

RDN: renal denervation; gp: group; M: months; pts: patients; OBP: office blood pressure; ABPM: ambulatory blood pressure monitoring; BP: blood pressure; CV: cardiovascular; FU: follow-up; SSAHT: standardized stepped-care antihypertensive treatment; HT: hypertension; PHAR: Intensified anti-hypertensive therapy group.

Table 2: *Brief summary*

What is known	
Renal denervation has good safety but results are conflicting regarding the lowering of blood pressure.	More well designed randomized clinical trials needed.
Symplicity HTN-3 failed	In-depth analysis showed several confounding factors.
Symplicity HTN-1, HTN-2, EnliHTN-1, DENERHTN, Global simplicity Registry with good efficacy and safety results	Several limitations
Single-tip catheters not compatible with four quadrant ablation	Multi-electrode catheters are now at disposal and probably more efficacious
What is not known	
How to measure ablation success.	The search for a molecular/imaging/clinical biomarker continues.
What are the ideal spots of ablation?	Should RF energy be delivered more distally, including the branches? It appears so.
Should accessory or polar arteries be treated if appropriately sized?	Unknown
Number of ablations needed?	The more the better?
Selection criteria for the ideal candidate	Only resistant HT or also milder degrees of HT?
Should RDN be offered to patients who refuse medication? Or experience severe side effects?	Unknown.

RDN: renal denervation; RF: radiofrequency; HT: hypertension.

Editorial Comment

Renal denervation in heart failure: Modulating the sympathetic nervous system

Rev Port Cardiol. 2017;36(1):53---54

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Conflict of Interest: none.

Heart failure (HF) is frequently the end-stage of many cardiovascular diseases and remains a major cause of morbidity and mortality, with 26 million patients now affected worldwide.[1] Its prevalence is around 1-2% in developed countries, rising to ≥10% among people over 70 years of age. The terminology of HF has recently been redefined and patients with an ejection fraction (EF) of 40-49% (the so-called 'gray area') now being classified as having 'heart failure with mid-range ejection fraction', as opposed to 'HF with reduced EF' (<40%) and 'HF with preserved EF' (EF ≥50%).[2]

The pathophysiology of HF is highly complex and involves the activation of compensatory mechanisms including the renin-angiotensin-aldosterone system, the sympathetic nervous system (SNS) and arginine vasopressin release, probably as a consequence of hemodynamic changes induced by a dysfunctional myocardium.[3] Activation of the SNS leads to excessive release and decreased uptake of norepinephrine. This autonomic hyperactivity has long been known to be directly related to the worsening of HF, by inducing myocyte enlargement and interstitial growth and remodeling, leading to increased myocardial mass and chamber dilatation.[4] The SNS thus became a therapeutic target, and there is evidence that continuing pharmacological beta-blockade has favorable prognostic implications in both ischemic and nonischemic cardiomyopathies.[5]

Renal denervation (RDN) is a percutaneous procedure which aims to achieve selective disruption of the sympathetic nerve endings in the renal arterial wall. It is currently under investigation as a promising technique for the treatment not only of hypertension (with varying treatment effects) but also of other clinical entities associated with an increased sympathetic drive such as advanced HF, sleep apnea and life-threatening cardiac arrhythmias.[6] Two pilot studies have been published that aimed to prove the effectiveness of RDN in patients with HF. The REACH-Pilot was a first-in-man study which assessed the safety of RDN in seven patients with symptomatic chronic systolic HF (New York Heart Association [NYHA] class III or IV), EF of 45±15% and without hypertension. At six months all patients were symptomatically improved, there was an increase in the six-minute walk distance (Δ=27.1±9.7m, p=0.03) and diuretics were reduced or stopped in four patients (p=0.046). No significant differences were observed in EF. This study had clear limitations (population and design, among others) and was underpowered for several parameters, but established the need for additional evidence.[7] Chen et al. conducted a randomized prospective pilot study which included 60 patients (30 in the RDN group and 30 in

the optimal medical therapy group) in NYHA II-IV with EF ≤40%. Renal denervation was performed with a saline irrigated catheter. At six-month follow-up EF (from 31.1±5.7% to 41.9±7.9%, p<0.001), the six-minute walk distance (from 285.5±84.3 m to 374.9±91.9 m, p=0.043), NYHA classification (p<0.001) and NT-pro-BNP levels (p<0.001) were significantly improved in the RDN group. There were no safety concerns and there was no difference in blood pressure between the groups.[8] Again, this study had several limitations, such as sample size, and data from two randomized trials which are currently recruiting are eagerly awaited.[9, 10]

As the relationship between HF and the SNS is robust and the available clinical data is promising, evaluation of the effects of RDN in patients with reduced EF is appropriate. Is this issue of the Journal, Gao et al.[11] present the results of a prospective, open, single-arm study which included fourteen patients with an EF below 45% (eight with ischemic cardiomyopathy), in class NYHA III or IV and on optimized medical therapy. Patients with severe renal failure (glomerular filtration rate below 30 ml/min/1.73 m2), type 1 diabetes or hypotension or in the acute phase of myocardial infarction or cerebrovascular accident were excluded. RDN was performed using a radiofrequency catheter with temperature control and 4-6 ablation points were delivered to each artery. At six-month follow-up improvements were observed in the six-minute walk distance (from 152.9±38.0 m to 334.3±94.4 m, p<0.001), EF (from 36±4.1% to 43.8±7.9%, p<0.003), NYHA functional class (p<0.001) and BNP levels (p<0.008). The recovery in EF was more significant in the group of hypertensive patients (34.5±4.3% to 52.3±6.1%, p<0.005) than in the non-hypertensive group (36.6±3.8% to 41±6.2%, p=0.07), a fact that the authors attribute to the blood-pressure lowering effect of RDN and suggesting a more beneficial effect of RDN in HF due to hypertensive disease. There were no safety issues. These results are different from those of the REACH-Pilot study in terms of change in EF, but are comparable to the results published by Chen et al., indicating a favorable effect of RDN in HF patients. However, several limitations must be taken into consideration such as the small sample size, the absence of a control group and non-randomization.

To summarize, this article highlights the role of RDN in modulating autonomic tone in specific conditions, including HF, and emphasizes the importance of further investigation in this area. The authors should be encouraged to continue their research on RDN in various clinical settings, and perhaps in the future some light will be shed on RDN's 'autonomic' significance.

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Part II: Original Articles

Original Article Number 1

Intravascular imaging, histopathological analysis, and catecholamine quantification following catheter-based renal denervation in a swine model: the impact of prebifurcation energy delivery

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Conflict of Interest: none.

Abstract

The purpose of this study was to evaluate the impact of prebifurcation renal denervation in a swine model and assess its safety through optical coherence tomography (OCT). Prebifurcation renal denervation with a multi-electrode catheter was performed in one renal artery of 12 healthy pigs, with the contralateral artery and kidney being used as controls. Angiograms and OCT pullbacks were obtained peri-procedurally and 1 month post procedure. Renal tissue catecholamines were quantified, and the arterial wall and peri-adventitial tissue were analyzed histologically. Intraluminal changes (endothelial swelling, spasm, and thrombus formation) were observed acutely by OCT in most of the treated arteries and were no longer visible at follow-up. Histology revealed a statistically significant accumulation of collagen (fibrosis) and a near absence of tyrosine hydroxylase labeling in the denervated artery, suggesting a clear reduction in nervous terminals. Renal tissue catecholamine levels were similar between both sides, probably due to the low number of ablation points and the renorenal reflex. The present study demonstrates that renal denervation is associated with acute intimal disruptions, areas of fibrosis, and a reduction in nervous terminals. The lack of difference in renal tissue catecholamine levels is indicative of the need to perform the highest and safest number of ablation points in both renal arteries. These findings are important because they demonstrate the histological consequences of radiofrequency energy application and its medium-term safety.

Keywords: Hypertension; Renal sympathetic denervation; Pre-clinical; Optical coherence tomography; Catecholamine.

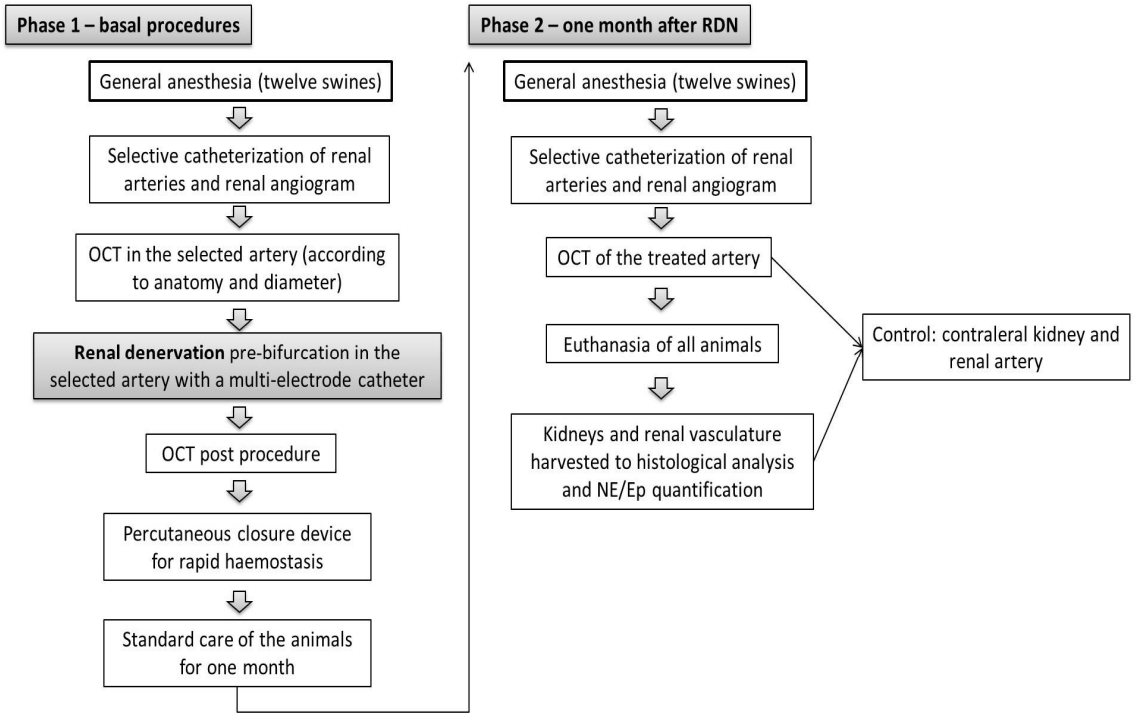
Abbreviations List: RF: radiofrequency; RDN: renal denervation; Ep: epinephrine; NE: norepinephrine; OCT: optimal coherence tomography; TH: For tyrosine hydroxylase

Introduction

Sympathetic nervous system hyperactivation is associated with several pathological conditions, such as arterial hypertension [1]. Radiofrequency (RF) catheter-based renal denervation (RDN) has emerged as a minimally invasive percutaneous procedure, designed to potentially disrupt the sympathetic fibers of the renal vasculature and hence decrease the sympathetic drive. Several proof-of concept randomized controlled trials have been conducted in resistant hypertensive patients [2–5], but the results have been inconsistent. Disbelief in the true efficacy of this technique has arisen, especially after the disclosure of the HTN-3 trial [6]. So far, the outcomes of RDN have been unpredictable, and several hypotheses, such as the Hawthorne effect, operator inexperience, an inability to achieve circumferential energy delivery, location inadequacy, and catheter design, have been extensively discussed as reasons for response variability [7]. Early investigations revealed that sympathetic fibers are mainly located in the adventitia of renal vessels with larger bundles in the proximal segment, compared to the thinner fibers found distally [8, 9]. However, more recent research on renal nerve distribution showed that even though sympathetic nerve fibers are concentrated in the proximal and middle arterial segments, the distance from the lumen is significantly higher than that of distal fibers, which are fewer but probably more vulnerable to RF injury [10]. Safety is a major concern after RDN, and the occurrence of renal stenosis has been described in a few patients. However, the actual incidence in the medium term and long term is not known. Currently, there is insufficient data regarding the correlation between prebifurcation RF induced vessel injury, assessed by intra-arterial imaging, and the levels of fibrosis, density of nervous terminals, and effects on renal tissue catecholamine levels in the medium term.

The main purposes of this study are: (1) to evaluate the effect of RF energy application (using a multielectrode RDN catheter) on the proximal/middle segment of the renal artery (prebifurcation) of a swine model, through histological analysis of the arterial wall and peri adventitial tissue and to compare the levels of fibrosis with the untreated contralateral side (control) and (2) to assess the disruption of the arterial wall acutely (pre and post procedure) and at 1 month of follow-up with intrarenal optical coherence tomography (OCT). A secondary endpoint is the assessment of renal tissue epinephrine (Ep) and norepinephrine (NE) levels in both sides.

Figure 1: Study design chart. *Ep: epinephrine; NE: norepinephrine; OCT: optical coherence tomography.*



Methods

Study design and animal procedures

All animal procedures were performed in the Veterinary Medical School of the Lisbon University, Portugal and were approved by the Ethical and Animal Welfare Commission (CEBEA) of the institution and by the Portuguese National Authority for Animal Health (DGAV).

This study was executed in two phases (Fig. 1).

- Phase 1: RF energy was delivered to one renal artery of twelve domestic swine (nine males Duroc X and three females F1 Large White X Landrace, 31 ± 1.2 kg) using the contralateral artery as the histological control and the contralateral kidney as the Ep/NE control. The animals were fasted 12 h prior to the procedure. Two certified veterinarians were present in every procedure and were responsible for the administration of anesthetic drugs, which included 2 mg/kg of azaperone, 0.04 mg/kg of atropine, and 0.2 mL/kg of ketamine, via an intramuscular route. In the catheterization laboratory, venous access was obtained from the marginal ear vein, followed by the administration of 6 mg/kg of 5% sodium thiopental with subsequent orotracheal intubation and mechanical ventilation. Anesthetic maintenance was obtained through the administration of volatile 2% isoflurane in a continuous oxygen flow. A 0.9% saline infusion was kept flowing until the end of the procedure, and a thermal blanket was used for temperature control. Anticoagulation with heparin was used to achieve an activation clotting time > 250 s. An 8-F sheath was placed in the right femoral artery, and selective catheterization of the renal arteries was performed under fluoroscopic guidance. A basal angiogram was obtained with prior administration of 0.5–1 mg isosorbide dinitrate to determine suitability of the artery. Suitable arteries were required to be ≥4 mm in diameter and ≥20 mm in length. OCT was performed pre- and post RDN. The multi-electrode catheter was placed prior to the bifurcation, and 6–8 ablation points were delivered. A final angiogram was obtained, and a percutaneous closure device was used in the femoral puncture for rapid hemostasis. Continuous hemodynamic monitoring was maintained during the procedure. Rimadyl ® was used in the following 3 days for analgesia. Acetylsalicylic acid (100 mg) and antibiotics were administered empirically in the following 7 days. No complications or adverse effects were observed in the animals in either of the two study phases.

- Phase 2: Medium-term efficacy and safety were assessed by renal angiography and OCT evaluation 1 month after RDN. Subsequently, the animals were euthanized according to the standard protocols, with intravenous sodium thiopental. Both kidneys, including the renal vasculature with peri-adventitial tissue, and abdominal aorta were harvested for histological purposes. The samples were embedded in optimal cutting temperature (Sakura® Finetek Inc, Torrance, CA, USA) compound and transported frozen in dry ice. The animals underwent a brief necropsy to exclude any pathological conditions (none found).

Technique description

Renal denervation system

The EnligHTNTM Multi-Electrode RDN system (St. Jude Medical, MN, USA) was used in this study. The ablation catheter consists of an expandable basket with four electrodes, providing a 60-s ablation time per electrode, set with the purpose of obtaining a circumferential ablation pattern. The system allows the independent selection of electrodes. It is compatible with an 8-F sheath and is powered by the EnligHTN TM generator. Final OCT evaluation and a renal angiogram were obtained.

Optical coherence tomography

OCT was performed pre- and post RDN and at 1-month follow-up, using the non-occlusive acquisition technique, to access the presence of intraluminal disruptions such as spasm, thrombus, or dissection. Images were acquired using the IlumienTM OptisTM System and the DragonflyTM Imaging Catheter (St. Jude, MN, USA). The catheter was advanced over a standard 0.014'' guidewire, positioned distally to the major bifurcation, and pulled back automatically with simultaneous manual injection of a nonionic contrast medium. OCT analysis was done by two independent and experienced analysts. Minimum, mean and maximum vessel areas and diameters were calculated through the analysis of cross-sectional images frame by frame, using the main bifurcation as the distal reference point. Lumen volume was calculated based on the minimum lumen area.

Histological analysis

The samples were removed from the dry ice, sectioned at 5 µm, and stored at −80 °C. Three artery regions, prebifurcation, were evaluated and compared with the contralateral side (proximal, medium, and distal). Fibrosis area was assessed in frozen sections with Masson's trichrome staining, according to the instructions of the Trichrome Stain kit (Abcam, Cambridge, MA, USA). Briefly, frozen slides were fixed with Bouin's solution. After incubation in Weigert's

iron hematoxylin solution, the slides were stained with Biebrich Scarlet-Acid Fuchsin and Aniline Blue and dehydrated in ethanol and xylene. Extensive washes were done between each staining. The collagen fibers were stained blue, the nuclei were stained black and blue, and the cytoplasm was stained red. Artery sections were immunostained as follows: frozen sections were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline, permeabilized

with 0.2% (vol/vol) Triton X-100, and incubated in 2.5% bovine serum albumin to block unspecific staining. For TH immunodetection, sections were incubated with primary antibody (ab137869, Abcam, Cambridge, MA, USA) overnight at 4 °C. Thereafter, the samples were incubated with the secondary antibody for an additional hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The specimens were mounted with MOWIOL 4–88 Reagent (Calbiochem®). Fluorescence microscopy images were collected using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany).

Norepinephrine and epinephrine quantification in the renal cortex and medulla

NE and Ep were quantified in the renal cortex and medulla by an experienced researcher who was blinded to the treated artery. From each kidney, 100 mg of renal cortex and medulla were collected, distributed in Eppendorf tubesTM, sonicated in ice-cold 0.2M perchloric acid and centrifuged. Supernatants were filtered using 0.2 µm Nylon microfilters (Costar® Spin-X® Centrifuge Tube Filter) and stored at −80 °C until further analyses. The pellet was resuspended in 1M NaOH and stored at −80 °C for total protein quantification by the bicinchoninic acid protein assay (Thermo Fisher Scientific, MA, USA). A reversed-phase high-performance liquid chromatography method using isocratic elution and electrochemical detection was applied to quantify kidney NE and Ep levels, as described previously [11–13]. NE was quantified by an

analytical cell (model 5011, ESA Analytical, Dorton Aylesbury, Buckinghamshire, UK) set at a potential of 0.25 V and attached to a Coulochem-II electrochemical detector. Ep was quantified by a glassy carbon electrode vs. Ag/AgCl reference electrode set at a potential of 0.75 V and attached to an amperometric electrochemical detector. A flow rate of 0.5 mL/min and a sensitivity of 100 nA were used to detect renal cortical and medulla NE (coulometric detector). A flow rate of 0.3 mL/min and a sensitivity of 0.5 nA were used to detect renal cortical and medulla Ep (amperometric detector). Catecholamine concentration in each sample was determined by comparison with peak areas of standards and is expressed in nanograms per milligram of protein.

Statistical analysis

This study was planned to evaluate independent cases, with one control per case, with a previously determined sample size. The statistical tests used in the different sets of data were Student's t-test and the non-parametric Friedman test. All reported p values < 0.05 were considered statistically significant. Analyses were performed using SPSS statistics version 22 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 5 (GraphPad Software, Inc.).

Results

All animals survived the procedure and were in good health after 1 month. A weight change from a baseline of 31 ± 1.2 to 43.8 ± 4.5 kg at follow-up was documented. No vascular complications on the renal angiogram or at the femoral level were observed. RDN was performed in 12 renal arteries (six in the left and six in the right); a total of 6–8 ablations were delivered to each artery (mean 6.3 ± 0.8).

OCT analysis

A total of 36 pullbacks were evaluated by two independent operators. All peri-procedural pullbacks had excellent quality, with good lumen and vessel layer visualization. At follow-up, 75% of the pullbacks had excellent quality, but 25% (three animals) were not analyzed due to the greater artery dimensions, which prevented full lumen/artery visualization. The lumen area, diameter and volume quantification are displayed in Table 1. Statistically significant differences were found between the minimum area, diameter, and volume in the three timings, mainly due to differences between post-procedural and follow-up values, probably indicating a higher degree of spasm at sites where the vessel is narrower. A normal and physiological vessel growth and healing after 1 month was observed. Postprocedurally, angiographically clear vessel notches were distinguished and correlated with the sites where RF energy was delivered. In OCT cross-sections, endothelial edema and vessel spasm were present in 100% of the arteries, without associated flow compromise. Intraluminal thrombus formation was present in 11 of the 12 treated arteries. One minor dissection was visualized (2 frames). At 1-month follow-up, all arteries appeared to be completely healed, as none of the previously described lesions or renal artery stenosis was present (Figs. 2 and 3).

Table 1: Optical Coherence Tomography assessment at three different timings. Statistically significant differences were found between the minimum area, diameter and volume in the three timings, mainly due to differences between post-procedural and follow-up values, probably indicating a higher degree of spasm at sites where the vessel is narrower, and a normal and physiological vessel growth and healing after one month.

	Before RDN	Immediately after RDN	Follow-up (one month)	p value
Lumen area (mm2)				
- Mimimum	9.4±4.4	6.3±3.3	12.3±3.7	**0.003**
- Mean	12.5±2.6	9.2±3.2	13.5±4.9	0.097
- Maximum	14.7±2.5	12.7±3.6	17.7±3.9	0.097
Lumen diameter				
- Minimum	3.3±0.98	2.7±0.79	3.9±0.62	**0.003**
- Mean	3.9±0.45	3.2±0.68	4.3±0.5	0.062
- Maximum	4.3±0.37	3.99±0.59	4.7±0.5	0.097
Lumen Volume (mm3)	136±58	123.4±75.1	204.6±83.1	**0.032**

Values are presented as mean ± SD

Figure 2: Optical coherence tomography assessment. *Bif: bifurcation; T: thrombus; yellow arrow: spasm and endothelial edema.*

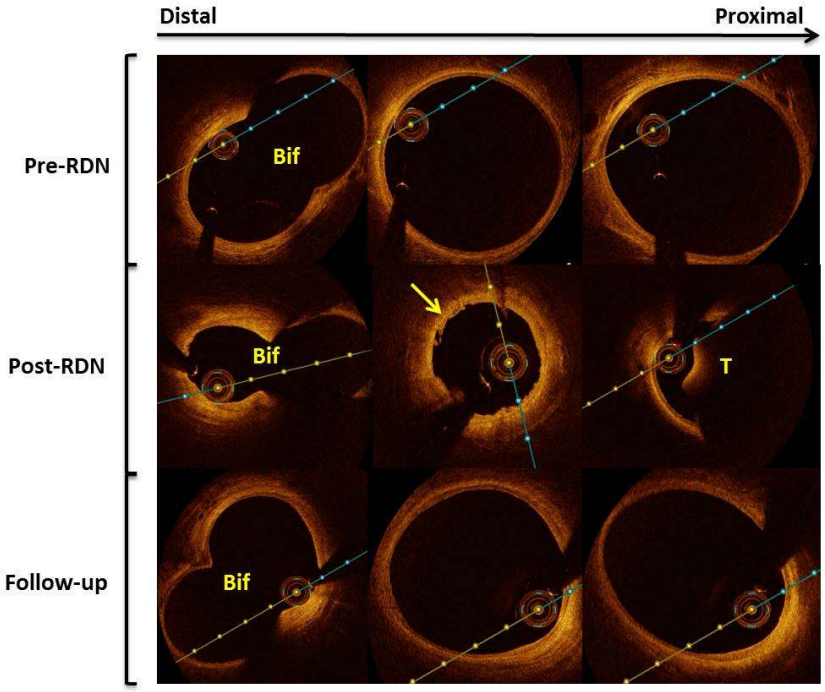
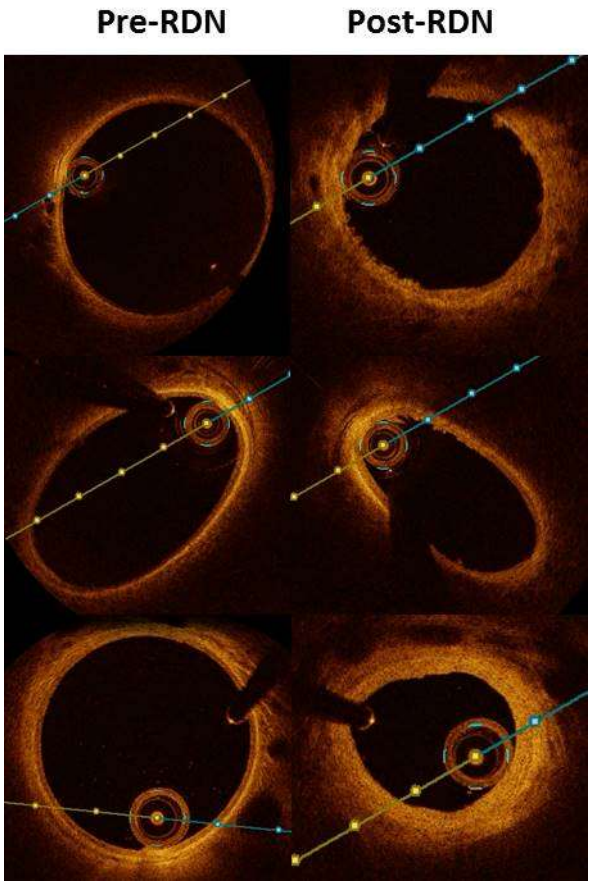


Figure 3: Several degrees of vasoconstriction (spasm) and endothelial edema as assessed by optical oherence tomography. Left column: prerenal denervation (RDN); right column: immediately after RDN. *RDN: renal denervation*



Histological analysis

The presence of neuronal enervation was assessed by TH immunostaining in three zones of the renal artery, according to the distance from the bifurcation and the RF delivery. The images clearly showed that TH immunostaining in denervated arteries was significantly lower than in the nondenervated controls, in all three zones analyzed, suggesting a near absence of nerve terminals in the treated artery. The presence of fibrosis was evaluated using Masson's trichrome staining (in which blue color stains for collagen and red stains for muscle fibers). The images show that in denervated arteries, more collagen was embedded in the media, suggesting an

increase in fibrosis (with the red layer (media) becoming less red and more blue). Collagen accumulation was quantified and revealed a statistically significant increase in collagen in the treated artery vs the control (Figs. 4–6).

Norepinephrine and epinephrine assessment

NE and Ep levels in the renal cortex and medulla were similar when comparing the treated side to the contralateral untreated control. Overall, NE was significantly higher in the medulla than in the renal cortex, while Ep was similar in both regions (Fig. 7).

Figure 4: Tyrosine hydroxylase immunostaining (THI) in three zones of the renal artery (a–f: control; g–l: denervated artery. THI is clearly lower in denervated areas than the control, translating a reduction in nervous terminals. Masson's trichrome staining is shown in the right columns (a–c: control; d–f: denervated artery). Blue stain is collagen, whereas red stain is muscle fibers. In denervated areas, the amount of collagen embedded in the media is higher, translating into an increase in fibrosis (the red layer (media) becomes less red and bluer).

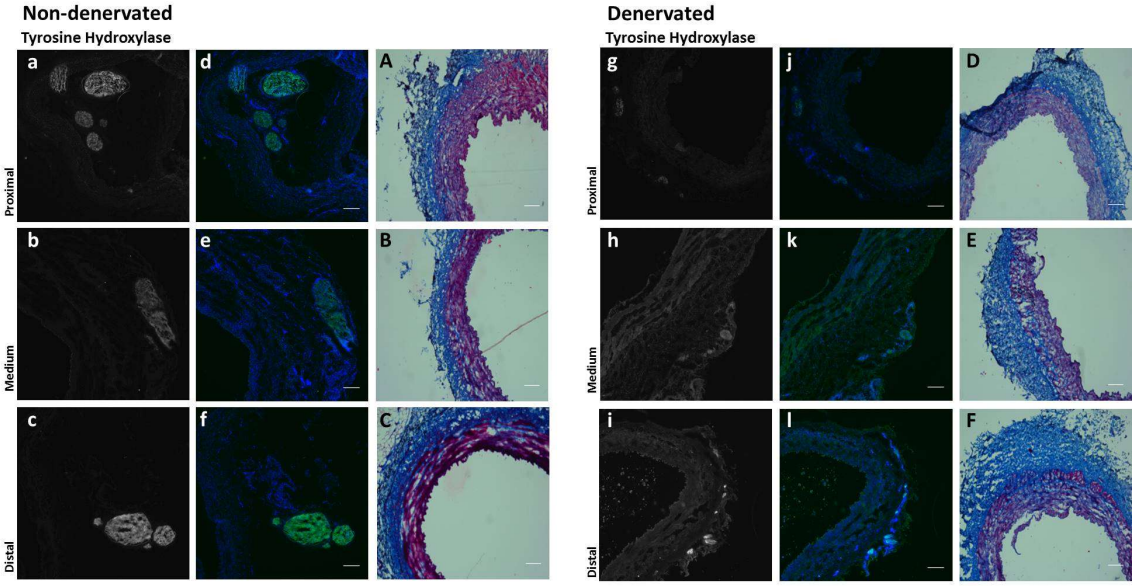


Figure 5: Tyrosine hydroxylase immunostaining showing a statistically significant difference between the denervated artery (near absence of nervous terminals) vs the control. **p < 0.01

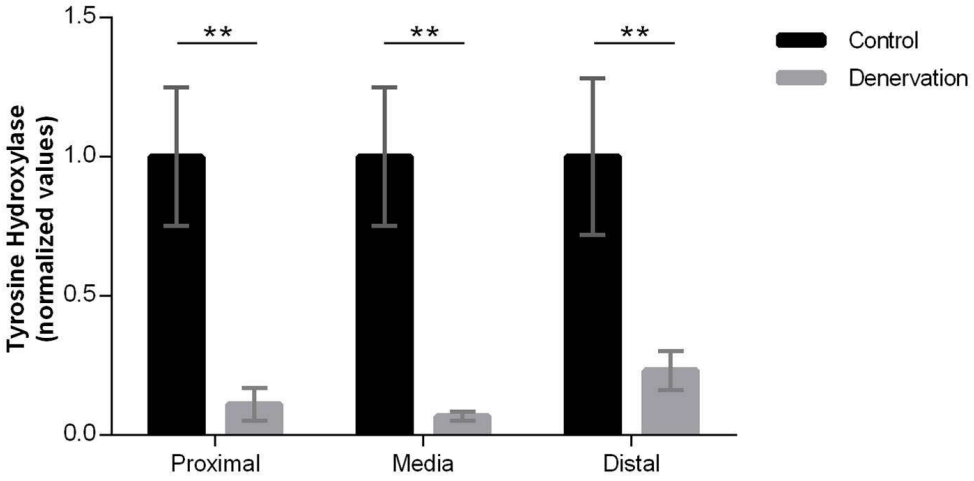


Figure 6: Collagen accumulation as assessed by Masson's trichrome staining in the renal arteries 1 month after renal denervation was performed. There was a statistically significant increase in collagen levels in the denervated arteries compared to control. *p < 0.05.

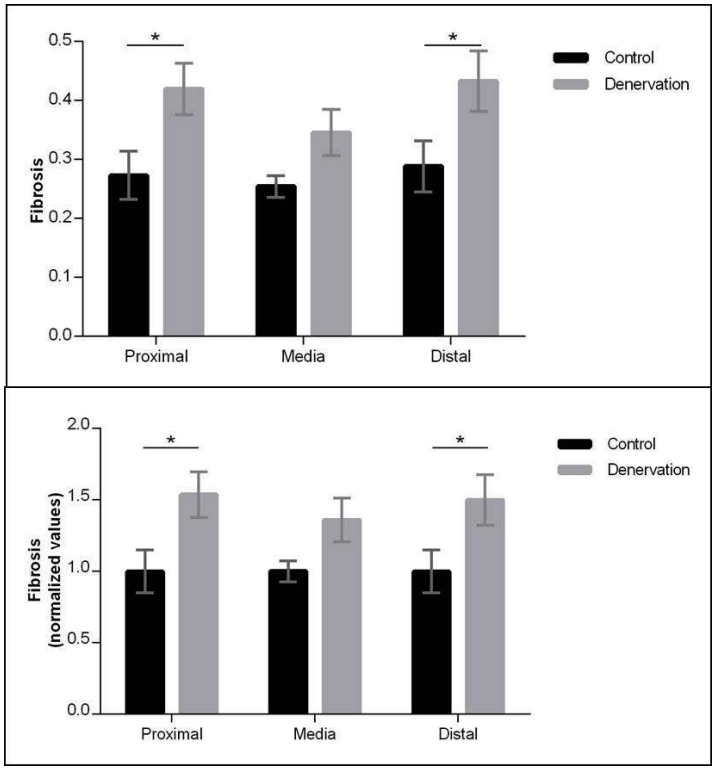
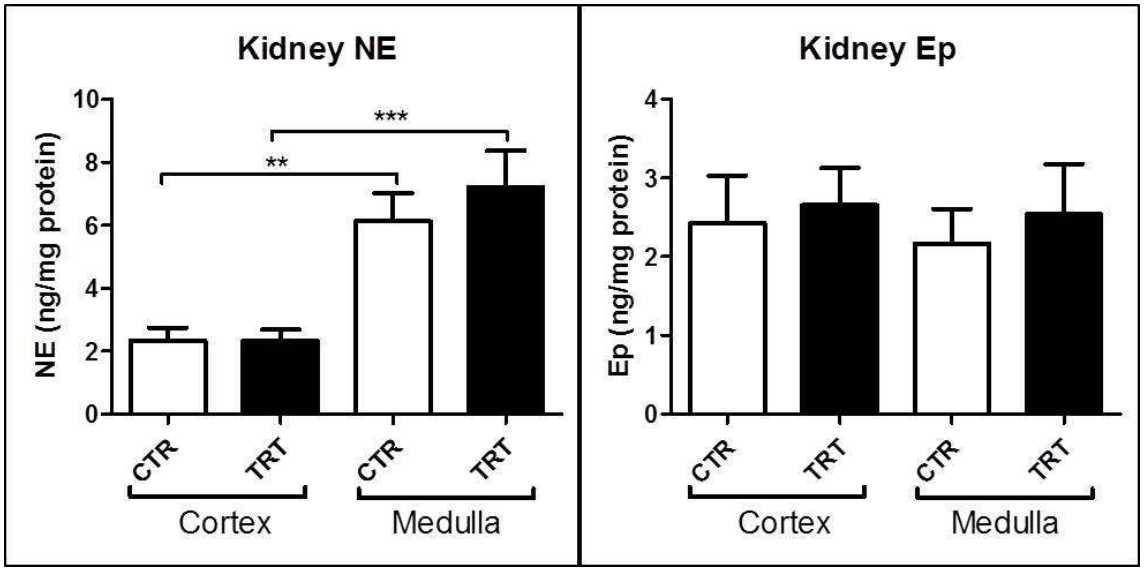


Figure 7: Data are mean ± SEM of norepinephrine (NE) and epinephrine (Ep) levels (ng/mg protein). **p < 0.01, ***p < 0.001 (medulla vs. cortical region, using an unpaired Student's t-test). *CTR: control side; TRT: treated side.*



Discussion

Our study demonstrated several main findings: (1) RDN performed in the proximal/medial segment of the renal artery (prebifurcation), with a multi-electrode device, causes acute vessel wall changes, revealed by OCT, such as intimal disruption (edema/spasm) and intraluminal thrombus formation. (2) RDN appears to be safe in the medium term. At the 1-month follow-up, OCT revealed a completely healed vessel and the absence of ‘‘de novo’’ stenosis. The only dissection detected was no longer visible at this point in time. (3) The histological analysis revealed nearly absent TH immunostaining and a statistically significant increase in the amount of collagen fibers in the denervated artery, compatible with a decrease in nerve terminals and an increase in fibrosis, compared to the control, suggesting an efficacious delivery of the RF energy to the vessel wall. No differences were found in the NE or Ep renal tissue levels between the treated and contralateral kidney.

The recent HTN-3 trial [6, 7] raised several questions regarding the efficacy of RDN in the proximal main renal artery but is virtually the only negative trial among numerous randomized trials. Several aspects of the procedure and the Hawthorne effect are thought to have contributed to the unexpected results [14], and research is currently focusing on updating the present knowledge to achieve experimental and subsequent clinical success. Recent Ambulatory blood pressure monitoring (ABPM) data from the Simplicity Spyral Global registry are very encouraging (presented at EuroPCR 2016) and suggest distal (targeting the branches) RDN may be effective. Augmented sympathetic activity can also exert deleterious effects on the heart and the potential benefits of RDN in this setting are promising. Watanabe et al. [15] showed that, in hypertensive rats, RDN suppressed the development of left ventricular hypertrophy and was protective against renal damage, resulting in prolonged survival.

Renal anatomy and the sympathetic nerve distribution in the proximity of the renal vasculature was the pathophysiological basis for the development of RDN. Particularly important was the distance of the renal nerves to the vessel lumen and how they were distributed around the artery. Atherton et al. [9] demonstrated that there were fewer but larger nervous bundles in the proximal segment, which were distributed throughout the artery and became progressively smaller but more numerous in the distal segments. Therefore, it has been hypothesized that if

the ablation points are closer to the ostium and directed to the superior side of the artery, the effectiveness of RDN is increased [16]. These recommendations were difficult to implement with the first-generation single-electrode devices, as this region is associated with higher catheter instability, which makes contact with the arterial wall difficult to achieve. However, more recent findings and an improved understanding of the renal anatomy refined the original technique. Roy et al. [17] examined three sections of post-mortem human renal arteries and found that although 77% and 22.5% of the nerves are located between 0.5–2.5mm and 2.5–5mm from the intimal layer, respectively, variations do occur, and in larger arteries with thicker parenchyma, the nerves are concentrated even further away from the lumen. Additionally, as nerve bundles have a three-dimensional distribution along the vessel, the authors suggest that a circumferential denervation pattern is preferential to an interrupted one in order to produce tissue damage beyond 2.5 mm. Sakakura et al. [10] showed that >75% of sympathetic nerves are located within 4.28mm from the lumen and less frequently in a dorsal location compared to the ventral, superior and inferior regions. Furthermore, the authors found that even though there are fewer nerves in the distal segments compared to the proximal and middle segments, they are closer to the lumen. They proposed a diagram that reflects the circumferential peri-arterial nerve location. In pigs, Mahfoud et al. [18] demonstrated that delivering RF to the branches resulted in greater NE reductions than treating the main renal artery alone; the later significantly reduced NE concentrations, but without a clear dose response to the increasing number of RF lesions. The greatest decline in NE concentrations and reduction of axon density observed in this analysis was obtained from a combined treatment of the main artery and branches, an approach currently being investigated by two major clinical trials [19, 20]. The 3-month results of the proof-of-concept, sham-controlled randomized trial SPYRAL HTN-OFF MED were recently published [21] and revealed a significant decrease in office and 24-h blood pressure in the RDN group compared to the sham control, suggesting RDN is effective in the treatment of hypertension.

NE, a neurotransmitter of the sympathetic nervous system, has been used in several human (NE spillover) and experimental (renal tissue quantification) studies to evaluate the efficacy and magnitude of RDN. Henegar et al. [22] evaluated whether RDN performed in different segments of the renal artery in pigs led to variable NE renal tissue concentrations and concluded that RDN performed in the branches, closer to the kidney, produced the greatest NE reductions. However, this study did not evaluate procedural safety.

RDN appears to be safe, although concerns regarding the rare occurrence of renal stenosis during follow-up have been raised by a few published case reports [23, 24]. In the last 15 years, OCT has become an important technology in the evaluation of coronary artery structure, by overcoming the limited spatial resolution and drawbacks of Intravascular ultrasound (IVUS). Not only is it utilized in the assessment of coronary vulnerable plaques, lumen geometry, and to guide coronary intervention, but it can also evaluate intraluminal repercussions of RDN, particularly the presence of spasm, intimal injury, or thrombus formation. The evaluation of human renal arteries with OCT is clearly more challenging; due to vessel size, a complete removal of blood cells is difficult to achieve, and proper visualization of layers is limited by an insufficient depth penetration. Nevertheless, a few small observational studies have disclosed some data concerning this subject. Templin et al. [25] performed RDN with two different denervation systems (Symplicity® and EnligHTNTM) in 16 patients, with OCT evaluation pre- and post procedure; spasm and edema occurred with both devices, although the greatest amount of thrombus formation occurred with the EnligHTNTM and one dissection with the Symplicity®.

In this study, we used a multi-electrode device to achieve a more circumferential ablation pattern. Preclinical anatomical studies performed in pigs [26], whose anatomical similarities with the human cardiovascular system have proven to be useful for the development of several techniques, revealed a much higher concentration of nervous bundles in the proximal arterial segments, and predominantly in the right side. Several studies have concentrated on determining the exact position of periarterial nerves, on identifying the ones involved in the pathophysiology of resistant HT and on determining the depth range of ablation devices. Vink et al. [27] evaluated histopathologically a human patient and determined the RF damage did not penetrate deeper than 2 mm. Therefore, an incomplete denervation potentially leads to a lack of renal tissue catecholamine reduction. Our study demonstrated similar NE and Ep concentrations between both sides, which we hypothesize was due to the following explanations: (1) there was not enough contact with the arterial wall, and RF energy was not delivered properly. Evidence against this is that the OCT analysis showed a clear edematous swelling in this segment, with a reduction in the area and diameter acutely (spasm) and thrombus formation evident in most of the cases. Additionally, the histological analysis revealed a significant increase in collagen concentration in the denervated sections, a finding consistent with the intra-arterial imaging. (2) The RF energy was adequately delivered (TH immunostaining showed a near absence of nervous terminals in the denervated side), but the distance from the

larger nerves to the arterial lumen was higher than previously described. (3) The number of ablations (mean 6.3 per artery) was not enough and was therefore unable to achieve a circumferential pattern in the artery, leading to incomplete denervation. In SPYRAL HTN-OFF MED, a mean of 43.8 ablations were performed. (4) Afferent sensory nerve fibers communicate with the contralateral kidney to maintain diuresis and natriuresis despite unilateral disturbances (renorenal reflex) [28]. Since RF energy was only delivered to one renal artery, after 1 month of follow-up, adaptive mechanisms could have brought NE and Ep levels back to normal, which validates the need to denervate both sides systematically. (5) A combination of the described mechanisms is probably the most likely explanation.

Implications of the current evidence in daily practice

RDN with a multi-electrode device leads to fibrosis and reduced nerve terminals and appears to have a favorable medium-term safety profile. The authors hypothesize, according to the current study and the available evidence, that a higher number of ablations, with a more even and circumferential distribution throughout the artery, should be performed to achieve a favorable hemodynamic outcome. These results provide imaging and histopathological evidence of the effect of unilateral RDN, in a small group of subjects, with the previously described method.

Limitations

Several limitations are present in this study. First, this was an observational study in which RDN was performed in a small number of animals. The distance between nerve fascicles to the arterial lumen was not evaluated. NE spillover was not assessed but could have been useful to compliment renal tissue measurements. Regarding safety, even though microthrombi were present peri-procedurally in most of the cases, the impact of this finding on renal function is unknown, as it was not assessed. Our study was performed in healthy, normotensive pigs, and hence, clinical outcomes were not analyzed.

Conclusions

Our study shows that RDN, when performed in the proximal/medial segment of the renal artery (prebifurcation) of a swine model, is associated with clear intraluminal disruptions, as assessed with optical coherence tomography, which are not present 1 month after the procedure. A statistically significant reduction in nervous terminals and an increase in areas of fibrosis were demonstrated by histological analysis, suggesting an efficacious application of the radiofrequency energy. No differences in NE or Ep renal tissue levels were found, probably due to an insufficient number of ablations and to the study design (unilateral treatment). Current research is very promising and suggests that a circumferential ablation pattern in both the main renal artery and the branches may yield more advantageous clinical/hemodynamic consequences.

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Original Article Number 2

Activated double-negative T cells (CD3+CD4−CD8−HLA-DR+) define response to renal denervation for resistant hypertension

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Conflict of interest: none.

Abstract

Purpose: To explore the cellular immune response of patients with resistant hypertension treated with renal denervation (RDN).

Methods and results: Twenty-three patients were included and blood samples were obtained in six timings, pre and post procedure. Response was evaluated at six-months and one year and was observed in 69.6% and 82.6% of patients, respectively. Absolute values of HLA-DR+ double negative (DN) T cells were significantly lower in the group of 'responders' at one year, and interaction between the timings were found in three T cell subsets (T CD4, T CD8 and naïve T CD8 cells), with the 'responders' tending to present with lower absolute values and little inter-timing variation.

Conclusions: 'Responders' significantly present with lower absolute values of activated DN T cells and have lower and more stable values of total T CD8+, CD4+, and naïve T CD8+ cells. These cell types may be able to predict response to RDN.

Keywords: Hypertension; Renal denervation; Double-negative T cells; Biomarker; Immune system; Inflammation.

Abbreviations: RDN: renal denervation; BP: blood pressure; HT: hypertension; DN: double negative; SNS: sympathetic nervous system; ABPM: ambulatory blood pressure monitoring; PRR: pattern recognition receptors; TCR: T cell receptors.

1. Introduction

Arterial hypertension (HT) remains one of the most important risk factors for the development of cardiovascular disease and one of the leading causes of cardiovascular mortality and hospitalizations [1]. It is now common knowledge that blood pressure (BP) reduction has a favorable prognostic impact through the reduction of major cardiovascular events like myocardial infarction, stroke and cardiovascular death, and hence, total cardiovascular risk burden [2]. The true prevalence of the so-called 'resistant hypertension' is still unknown but apparently revolves around 5–15% [3]. The etiology of a chronically uncontrolled BP is poorly understood but is believed to be due to genetic, epigenetic and environmental factors in which, the sympathetic nervous system (SNS) hyperactivation, the requirement of lifelong treatment and poor medication compliance, are contributing elements [4].

Catheter-based renal denervation (RDN) has emerged as a potential minimally invasive therapy for the treatment of resistant HT and has been the subject of extensive investigation since the first RDN was performed in a human, in 2007. The first publication describing its effects arose in 2009 and revealed significant office systolic and diastolic BP reductions in patients taking a mean of five anti-hypertensive drugs [5]. In the following years several studies were disclosed which confirmed the apparent BP reduction effect of RDN [6–8], and were followed by a larger trial, developed in the U.S., that included, for the first time, a sham control group [9]. Even though the trial failed to show the superiority of RDN, when compared to medical therapy, in BP control, several confounding factors, such as the Hawthorne effect, the use of a single-tip catheter (which led to incomplete denervation) and insufficient operator experience, could potentially explain this lack of efficacy [10]. RDN enthusiasm fainted but did not die and since then, three rigorously designed sham-controlled trials were developed and provided evidence that RDN effectively lowers BP (including a proof-of concept trial) and has no safety concerns [11–13].

Patient selection, technical aspects and operator experience have been thoroughly discussed in the literature in order to determine which factors could potentially identify the 'responder' to RDN, aspect that would help redefine the indications to this therapy and improve efficacy. A blood-circulating biomarker could be of particular interest to identify the 'responders' and could be a marker of renal function, hemodynamics, vascular stiffness or inflammation.

The participation of inflammation and, in particular, adaptive immunity, in the elevation of BP is known to be an elaborate interaction between activated immune cells, oxidative stress and angiotensin II activity, promoting an inflammatory status in the kidney, arteries and central nervous system [14,15]. T lymphocytes are one of the most important effector cells of the adaptive immune system and specific proof of their involvement in the pathogenesis of angiotensin II induced HT was obtained by Guzik et al. [16], using mice lacking T and B cells (RAG1−/−). These mice had blunted HT and did not develop the vascular alterations usually present in angiotensin II induced HT, such as T cell infiltration of perivascular tissue, oxidative stress, expression of specific inter-cellular adhesion molecules and cytokines, and endothelial-dependent vasodilation, even during the infusion of angiotensin II or desoxycorticosterone acetate (DOCA)-salt. All these features, including HT, were restored after the transfer of T (but not B) cells. Since then, experimental studies were conducted and showed that the presence of mutations leading to T lymphocytes deficiency are associated with blunted salt-induced [17] or angiotensin II-induced HT [18].

The SNS is the major link between the central nervous system and the immune system and its stimulation leads to an augmented BP due to an increase in renal sodium reabsorption, cardiac output and peripheral vascular resistance. The SNS is associated with several immunological alterations, most of them identified in experimental studies using angiotensin infusions. Adrenergic stimulation may induce renal immune cell infiltration and the production of noradrenaline and proinflammatory cytokines by T lymphocytes, macrophages and dendritic cells. [19] A potential effect of RDN is the reduction of renal inflammation through the modulation of the SNS but the true impact of this technique on the immunological response of patients with resistant HT is largely unknown.

The main purpose of this study is to evaluate the immunological response in cohort of patients with resistant HT and submitted to RDN, especially the behavior of T-cell population, and to potentially identify a cellular marker to predict response.

2. Methods

2.1. Study design and patients

This is a prospective, non-randomized, single-center study, which included 23 consecutive patients aged 38 to 77 years old, treated with RDN due to resistant HT, between May 2014 and October 2017. A comprehensive medical history and a thoroughly revision of the medication was undertaken. Ninety-six patients with HT were evaluated, with 73 being excluded from the study. Inclusion criteria were the following: age over 18 years-old, presence of idiopathic resistant HT confirmed by ambulatory blood pressure monitoring (ABPM) (total systolic BP over 135 mmHg in spite of a stable medication regimen of maximum tolerated dosages of 3 or more anti-hypertensive drugs, including a diuretic), glomerular filtration rate over 45 ml/min/1.73m2 (Modification of Diet in Renal Disease Formula) and compatible renal anatomy (absence of atherosclerotic stenosis over 50%, prior renal artery revascularization or fibromuscular dysplasia). Patients with HT due to secondary causes (screening with biochemical and imaging assessment and polysomnography), with hemodynamically significant valvular disease, with stroke or acute coronary syndromes in the past 6 months or who refused to sign the informed consent, were excluded. Baseline evaluation included routine blood work, an electrocardiogram and a transthoracic echocardiogram. To confirm the presence of 'true' resistant HT, all patients were admitted to the hospital two days prior to the procedure in order to assess medication adherence (drug-intake was observed). Discharge from the hospital was on the following day and follow up visits were performed at 7, 30, 90, 180 and 365 days in all patients. Renal angiogram via the radial artery, to confirm safety, was performed in all patients at 180 days. Every follow-up visit included a clinical evaluation (BP measurement in both arms). The occurrence of adverse events and treatment compliance was recorded. Routine blood work, an electrocardiogram, transthoracic echocardiogram and ABPM were performed in all patients at 6 months and one year follow-up. The study was approved by the Faculty of Medicine of the University of Coimbra and the Coimbra's Hospital and University Centre Ethics Committees, and all patients signed an informed consent.

2.2. Procedure

Renal denervation was performed via the femoral artery, using the single-tip radiofrequency Symplicity Flex catheter (Medtronic Inc., Santa Rosa, CA, USA), the EnligHTN system (St. Jude Medical, MN, USA) or the Symplicity Spyral catheter (Medtronic Inc., Santa Rosa, CA, USA). One Interventional Cardiologist performed all cases in order to minimize procedural variability. An anesthesiologist was present in all cases and patients were deeply sedated with propofol, midazolam and/or remifentanil. Arterial access hemostasis was achieved using a vascular closure device.

2.3. Responders vs. non-responders

A patient was considered 'responder' if the mean change in total and daytime ambulatory systolic blood pressure between baseline and 6 months dropped over 5 mmHg. 'Late responders' (patients considered non responders at six-months but with drops in systolic ABPM over 5 mmHg at one year) [20] were also identified. Mean difference between systolic and diastolic office BP between baseline, 6 months and one year was also assessed, even though it was not considered for the determination of 'responders', as ABPM monitoring is generally considered superior to office measurement, allows for the identification of patients with 'white coat' hypertension and more reliably predicts the future occurrence of cardiovascular events and target organ damage [21]

2.4. Immune profiling

Venous blood samples were obtained in several timings: D-1 (patient accepted RDN and signed informed consent), D0 (immediately before RDN), D1 (24h after RDN), D7 (one week after RDN), D30 (one month after RDN) and D180 (six months after RDN).

Peripheral blood cells were counted with hematological counter (Coulter AcT diff, Beckman Coulter, Pasadena, CA, USA). An extended 10-parameter panel was used for flow cytometry analysis of T cells. Additionally, activation and maturation profile, as well as memory status was investigated. Briefly, 100uL or up to 1 × 106 cells were incubated with the surface antibodies for

15 min, in the dark at room temperature. After incubation red blood cells were lysed with BD Lysing Solution, for 10 min. Next, cell suspensions were centrifuged at 453g for 5 min and washed one time with 1× PBS (phosphate-buffered saline) in same conditions. Lastly, cell suspensions were acquired in a BD FACSCanto II eight colour flow cytometer (BD Biosciences, San Jose, CA, USA). The antibodies used to cellular staining are described in Table 1 and Supplementary Table S1. These antibodies allowed discriminating major and minor populations of T cells.

Plasmatic levels of IFN-γ, IL-4, IL-10, TGF-β and IL-17A were measured using a ProcartaPlex™ multiplex xMAP-based immunoassay (eBioscience, San Diego, California, USA) according to manufacturer instructions.

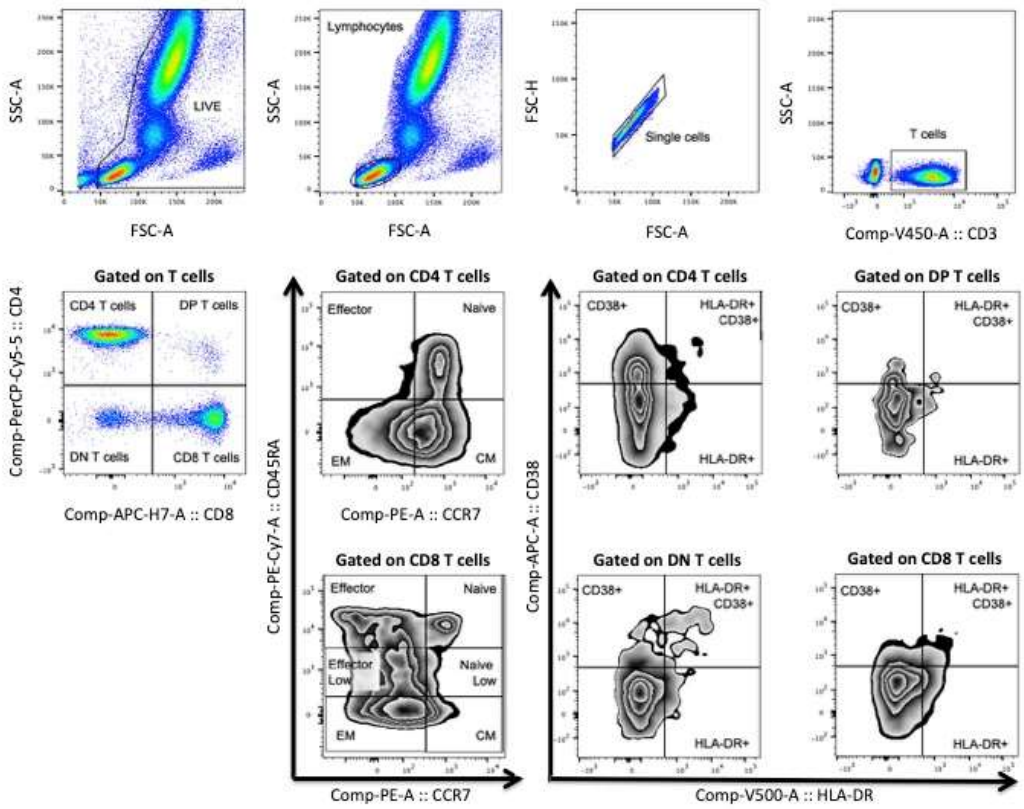
2.5. Data analysis

Flow cytometry data was acquired using BD FACSDiva™ v.6.1.3 (BD Biosciences, San Jose, CA, USA). All samples were then analyzed with FlowJo v.10.7 software (Tree Star Inc., Ashland, OR, USA). Gating strategy is present in Fig. 1. Multiplex xMAP cytokine data acquisition was performed in a Luminex® 200™ System (Luminex Corporation, Austin, Texas, USA) and data analysis was performed using the ProcartaPlex ™ Analyst 1.0 software immunoassay (eBioscience, San Diego, California, USA).

Table 1. Panel of fluorochrome-conjugated antibodies used for flow cytometry analysis.

Antibody	Conjugate	Clone	Brand	Cat#
CD3	V450	ICHT1	BD Biosciences	561416
CD4	PerCP-Cy5.5	OKT4	Biolegend	317428
CD8	APC-H7	HIT8a	BD Biosciences	641400
CD38	APC	HIT2	BD Biosciences	555462
CD45RA	PE-Cy7	5H9	BD Biosciences	561216
CD197 (CCR7)	PE	150503	BD Biosciences	560765
HLA-DR	V500	G46-6	BD Biosciences	561224

Figure 1: Gating Strategy for the analysis of T cells in peripheral blood samples. For peripheral blood samples, the discrimination of lymphocytes were based on SSC-A and FSC-A, then T cells were selected by positivity to CD3 antibody. T cells were plotted in a CD4 versus CD8 diagram to identify CD4 and CD8 T cells, as well as double positive (CD4 + CD8+) and negative (CD4-CD8) cells. T CD4 and T CD8 subpopulations were identified with CD45RA versus CCR7 plot, naïve cells (CD45RA+ CCR7+), central memory cells (CD45RA- CCR7+), effector memory cells (CD45RA-CCR7-) and effector cells (CD45RA+ CCR7-). The presence of CD38+, HLA-DR+ and HLA-DR+ CD38+ cells was also assessed within the CD4 T, CD8 T, DP T (CD4+ CD8+) and DN T (CD4- CD8-) populations. *DP – Double positive; DN – Double negative.*



Supplementary Table 1: Panel of fluorochrome-conjugated antibodies used for pilot study flow cytometry analysis.

Antibody	Conjugate	Clone	Brand	Cat#
CD3	V450	ICHT1	BD Biosciences	561416
CD3	APC-H7	SK7	BD Biosciences	560176
CD4	PerCp-Cy5.5	OKT4	Biolegend	317428
CD8	APC-H7	HIT8a	BD Biosciences	641400
CD11c	PE/Cy7	B-ly6	BD Biosciences	561356
CD14	V450	MOP9	BD Biosciences	560349
CD19	PerCp-Cy5.5	HIB19	Biolegend	302230
CD24	FITC	ML5	BD Biosciences	555427
CD25	PE	M-A251	BD Biosciences	555432
CD27	PE/Cy7	M-T271	BD Biosciences	560609
CD38	APC	HIT2	BD Biosciences	555462
CD45RA	PE/Cy7	5H9	BD Biosciences	561216
CD45RO	APC-H7	UCHL1	BD Biosciences	561137
CD56	PE	B159	Biolegend	318306
CD123	PerCp-Cy5.5	6H6	Biolegend	306016
CD127	AF647	HIL-7R-M21	BD Biosciences	558598
CD183 (CXCR3)	PE	IC6/CXCR3	BD Biosciences	550633
CD194 (CCR4)	PE/Cy7	IG1	BD Biosciences	577864
CD196 (CCR6)	PE/Cy7	11A9	BD Biosciences	560620
CD197 (CCR7)	PE	150503	BD Biosciences	560765
HLA-DR	V500	G46-6	BD Biosciences	561224
IgD	V500	IAS6-2	BD Biosciences	561490

2.6. Sample size calculation and statistical analysis

Sample size was calculated on 22 subjects to be able to reject the null hypothesis. The Type I error probability associated with this test of this null hypothesis is 0.05.

Categorical variables were characterized by determining the absolute and relative frequencies, and numerical variables the means and standard deviations. Comparisons between groups with regard to the categorical variables were conducted using the Chi-Square Test. Regarding the continuous variables, Mann-Whitney U Test was used to compare two groups, and Kruskal-Wallis Test between more than two groups. A general linear model for repeated measures was applied to analyze variance of each laboratorial parameter, measured several times on each subject from two different groups, ‘responder’ and ‘nonresponder’. Two different groups

definition were considered, responders at six months and at one year. Statistical analyses were conducted using SPSS 19.0®, at a 5% significance level for hypothesis-testing.

3. Results

3.1. RDN response (6 months) and late response (1 year)

A total of 23 consecutive patients (34.8% female, mean age 59.2 ± 11.4 years) were included in this study. Clinical and procedural characteristics are shown in Table 2. Response to RDN was evaluated at 6 months (group R vs. NR) and at one year (group R1Y vs. NR1Y), as described. Sixteen patients responded to treatment at 6 months and an additional three patients responded after one year ('late-responders'). In the studied population, there were no differences in most baseline characteristics (active smoking was more frequent in NR1Y group, p = .021), including office BP, ABPM, number of taken anti-hypertensive drugs or number of total ablations. There was no change in the anti-hypertensive medication in the first 6 months, being allowed afterwards. None of the 'late-responders' had any change in the medication in the first year after the procedure.

Six months after RDN, a drop of 21 ± 13 mmHg in ABPM mean systolic BP (155 ± 12 at baseline vs. 134 ± 12 mmHg at 6 months, p < .001) was observed in the R group (69.6% of the patients). At this time point, seven patients were classified as 'non-responders', with a drop of minus 7 ± 10 mmHg in ABPM mean systolic BP (153 ± 13 at baseline vs. 161 ± 10 mmHg at 6 months). Even though office systolic BP was not considered for evaluating response, a drop of 41 ± 34 mmHg (vs. 14 ± 13 mmHg, p =.047) was observed in the R group (191 ± 27 at baseline vs. 149 ± 21 mmHg at 6 months).

One year after RDN, three patients crossed over to the R group due to a significant drop in ABPM systolic BP at this time point. A drop of 20 ± 13 mmHg (vs. 1 ± 7 mmHg, p < .001) in ABPM systolic BP was observed in the R group (82.6%, nineteen patients) at one year (155 ± 12 vs. 135 ± 16 mmHg).

3.2. Pilot study

In a preliminary assay including samples of 4 RDN 'responders' and 3 'non-responders', collected in the previously described time points (D-1, D0, D1, D7, D30 and D180), we performed an extended analysis of T, B and NK cells, monocytes and dendritic cells (data not shown). Activated, memory, T helper, cytotoxic and regulatory T cell subsets were evaluated. Seemingly, relevant subset populations of other major leucocytes were analyzed. These results guided the forthcoming study in order to select T cells (CD4 and CD8, memory and activated subsets) as possible biomarkers of response in RDN.

Table 2: Clinical and procedural characteristics between 'responders' and 'non-responders' at 6 months and one year.

	R (n=16)	NR (n=7)	p value	R1Y (n=19)	NR1Y (n=4)	p value
Age in Y (mean±SD)	61±10.3	55±13.5	ns	59.9±11.8	55.5±9.5	ns
Female sex (%)	25	57,1	ns	36.8	25	ns
Dyslipidemia (%)	87,5	100	ns	89.5	100	ns
Type 2 diabetes (%)	37,5	57,1	ns	42.1	50	ns
Active smoking (%)	12,5	42,9	ns	10.5	75	**0.021**
Sleep apnea (%)	62,5	57,1	ns	63.2	50	ns
Number of HT drugs (n±SD)	5.1±1.3	5.4±0.5	ns	5.2±1.2	5.2±0.5	ns
- On spironolactone (%)	62.5	42.9	Ns	57.9	50	ns
Isolated HT (%)	12,5	14,3	ns	15.8	0	ns
BMI (Kg/m^2)	29.8±3.8	30.3±4.3	ns	30.3±3.7	27.8±4.3	ns
Baseline						
Office systolic BP (mmHg)	191±27	189±20	ns	191±27	186±11	ns
Office diastolic BP (mmHg)	107±15	108±26	ns	107±20	107±11	ns
Office HR (bpm)	69±10	69±10	ns	70±10	65±11	**0.026**
ABPM systolic BP (mmHg)	155±12	153±13	ns	155±12	150±12	ns
ABPM diastolic BP (mmHg)	87±13	93±16	ns	89±14	89±13	ns
6 Months						
Office systolic BP (mmHg)	149±21	175±26	**0.026**	154±26	172±14	**0.027**
Office diastolic BP (mmHg)	82±14	102±16	**0.014**	86±16	100±19	ns
Office HR (bpm)	63±11	72±14	ns	66±13	65±11	ns
ABPM systolic BP (mmHg)	134±12	161±10	**<0.001**	139±16	158±7	ns
ABPM diastolic BP (mmHg)	77±11	95±16	**0.008**	81±15	90±14	ns
One year						
ABPM systolic BP (mmHg)	135±16	149±20	ns	135±16	160±15	**0.016**
ABPM diastolic BP (mmHg)	76±11	88±14	ns	77±10	94±16	**0.044**
Total ablations (n)	25.6±9.2	22.4±7.3	ns	25.5±9.2	20.7±4	ns
Drop in office systolic BP at 6 months (mmHg)	41±34	14±13	**0.047**	37±32	14±16	ns
Drop in ABPM systolic BP at 6 months (mmHg)	21±13	-7±10	**<0.001**	17±16	-8.5±12	**0.004**
Drop in ABPM systolic BP at 1 year (mmHg)	20±12	4±21	ns	20±13	-11±7	**<0.001**

R: responder; NR: non-responder; R1Y: responder at one year; NR1Y: non-responder at one year; Y: years; HT: hypertension; BMI: body mass index; HR: heart rate; BP: blood pressure; ABPM: Ambulatory pressure monitoring. Results are displayed in mean± standard deviation (SD).

3.3. T cell memory and activation subsets help to define RDN responders

The analysis of T cells was performed before the procedure, at the time of informed written consent (D-1) and in the morning before RDN, during hospital staying (D0), and at four different time points after the procedure (D1: 24 h after; D7: one week after; D30: one month after; D180: six months after). The 'responders' were compared to 'non-responders' both at six months and at one year, in order to include the 'late responders'.

The analyzed cell populations are described in Table 3: absolute values of HLA-DR double negative (DN) T cells were significantly lower in the R1Y group at all timings (9.83% vs 11.48%, p = .032), but this difference is clearer at timing D-1 (Fig. 2). ROC curve at D-1showed an area under de curve (AUC) of 0.974 (p = .013, 95% CI 0.9–1). The ROC curve at all timings showed an AUC of 0.703 (p = .003, 95% CI 0.6–0.8). A HLA-DR DN T cells cut-off value of ≤9.635% showed the best overall sensitivity and specificity and was associated with RDN response (Fig. 3). To assess the activation status of DN T cells, IFN-γ, IL-4, IL-10, TGF-β and IL-17A were quantified at four timings. No statistically significant differences were found between groups but there was significant inter-timing variability regarding IL-17A (p = .034). Absolute values of IL-17A are similar between R and NR groups at D0, D1 and D7 but at D30, an increase is noted in the NR patients, a finding that could correlate with the described higher values of activated DN T cells in this group (Fig. 4).

Interactions between the timings, but not between the groups, were found in CD4 T cells (p = .03), CD8 T cells (p = .045), and naïve CD8 T cells (p = .035) when comparing R1Y vs. NR1Y, with the 'responders' tending to present with lower absolute values and little inter-timing variation, when compared to 'non-responders'. Fig. 5 illustrates the estimated marginal means of the referred cell types, within each group, at six timings.

Fig. 6 represents the heatmaps for absolute frequencies of DN, DP, CD4 and CD8 T cell subsets, activated (HLA-DR and CD38) DN, DP, CD4 and CD8 T cell subsets and naïve and memory CD4 and CD8 T cell subsets, in 'responders' at 6 months and one year. The heatmaps demonstrated that the 'non-responder' group shows a pattern distinct from the 'responder' group.

Table 3: Comparison of total lymphocytes and T cell populations between responders and non-responders at 6 months (R vs NR) and one year (R1Y vs NR1Y).

Cell populations	R (n=16)	NR (n=7)	ITVar p value	R vs NR p value	R1Y (n=19)	NR1Y (n=4)	ITVar p value	R1Y vs NR1Y p value
Absolute counts (cells/µl)								
CD8 T cell								
D 0	582.4±289.6	491.3±281.5			591.2±280.3	395.5±267.9		
D 1	579.1±357	668.1±381.6			606.1±350	619.4±415.1		
D 30	611.8±8.8	705.4±384.3	ns	ns	605.2±339.3	794.1±434.4	**0.045**	ns
D 180	564.4±319.7	614.7±293			568.9±304.4	630.4±348.8		
Relative frequencies (%)								
CD4 T cells								
D -1	57.9±13.4	57±10.9			57.7±13.3	57.1±9.1		
D 0	57.5±12.8	62±9.4			57.6±12.6	64.7±4.1		
D 1	55.7±13	58.2±8.5	ns	ns	55.8±12.7	59.5±4.7	**0.03**	ns
D 7	56±14.8	57±10.9			55.6±14.4	59.2±8.4		
D 30	58±14.5	58.1±11.8			57.5±14.5	59.9±8.1		
D 180	56.1±13.3	54.1±14.7			56.6±13.4	50.7±14.1		
Naïve CD8 T cells								
D -1	11.3±7.4	19.1±13.7			14.4±11.1	12.9±8.5		
D 0	11.9±10.5	26.4±15			15.3±14.3	23.6±10.9		
D 1	11.1±9.2	21.9±14.3	ns	ns	14.2±13.2	17.5±7.7	**0.035**	ns
D 7	9.9±5.9	17.4±10.5			11.9±8.7	15.1±8		
D 30	11.1±8.8	22.4±17.1			13.6±11.7	21±18.8		
D 180	9.7±6.5	22.4±17.7			14.2±13.1	14.1±14.1		
HLA-DR DN T cells								
D -1	5.7±2.5	14.21±12.7			5.7±3.2	19.7±13.4		
D 0	4.6±2.3	9.4±4.5			5.3±3.7	10±1.6		
D 1	5.7±4.6	9±6.1	ns	ns	6.4±5.7	8.3±1.8	ns	**0.032**
D 7	5.7±4	6.1±3.3			5.2±3.9	8.4±0.4		
D 30	6.6±3.9	9.2±4.8			6.4±4	12±1.2		
D 180	10.6±13.9	8±10.5			9.6±12.8	10.5±13.8		
Not statistically significant	*Lymphocytes, T cells, DN T cells, CD38 DN T cells, HLA-DR CD38 DN T cells, DP T cells, HLA-DR DP T cells, CD38 DP T cells, HLA-DR CD38 DP T cells, Naïve CD4 T cells, Central memory CD4 T cells, Effector memory CD4 T cells, Effector CD4 T cells, HLA-DR CD4 T cells, CD38 CD4 T cells, HLA-DR CD38 CD4 T cells, Naïve CD45RA low CD8 T cells, Central memory CD8 T cells, Effector memory CD8 T cells, Effector CD8 T cells, Effector CD45RA low CD8 T cells, HLA-DR CD8 T cells, CD38 CD8 T cells, HLA-DR CD38 CD8 T cells*							

ITVar: inter-timing variability; DN: double negative; DP: double positive. Results are displayed in mean± standard deviation.

Figure 2: Comparison between activated CD8+, double-positive, double-negative and CD4+ T cells, in 'responders' at one year (R1Y) vs 'non-responders' (NR1Y). HLA-DR+ double-negative T cells are significantly lower in R1Y vs NR1Y. Samples were obtained at six timings (D-1, D0, D1, D7, D30 and D180 – see text). The red line represents NR1Y (n =4) and the blue line R1Y (n=19). Statistical analysis was performed using the Kruskal-Wallis Test and a general linear model was applied to analyze variance of each laboratorial parameter. Data are presented using mean ± standard deviation

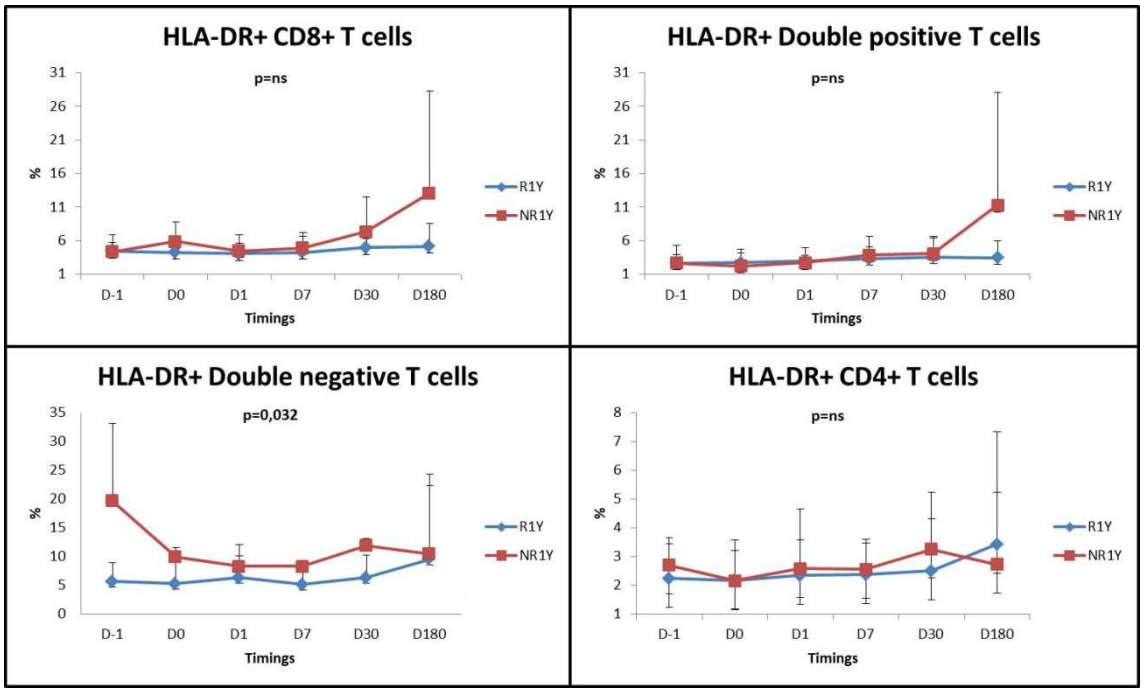


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Figure 3: A HLA-DR+ double-negative T cells cut-off value of ≤9.635% showed the best overall sensitivity and specificity for determining renal denervation response (A) ROC curve: HLA-DR+ double negative T cells at timing D-1. (B) ROC curve: HLA-DR+ double negative T cells at all timings. *DN – double negative; AUC – area under the curve.*

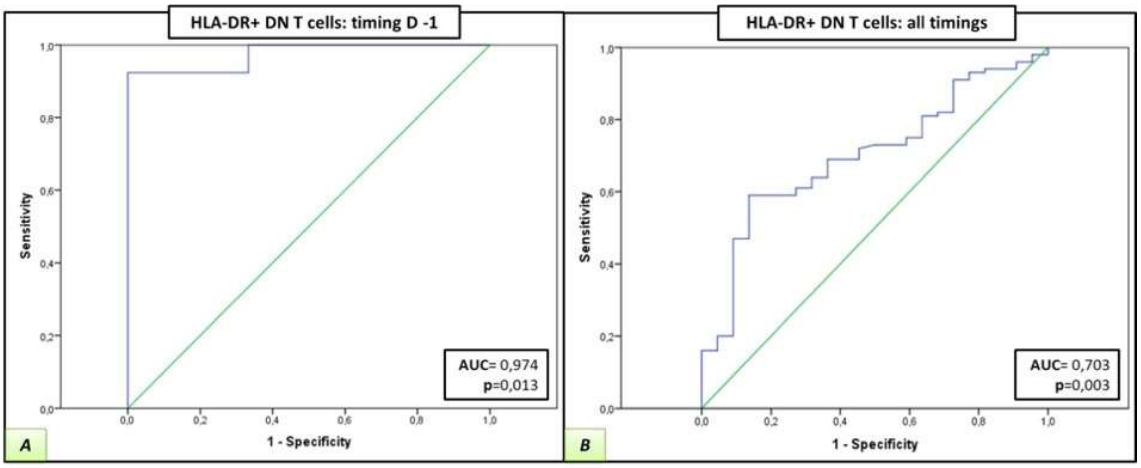


Figure 4: Quantification of IL-17A at four timings (D0, D1, D7 and D30 – see text). No statistically significant differences were found between groups but there was significant inter-timing variability (ITVar) in the response at six months. At D30 there is a tendency for increased values of IL-17A in the NR and NR1Y groups. *R – responders at six months; NR – non responders at six months; R1Y – responders at one year; NR1Y – non responders at one year.*

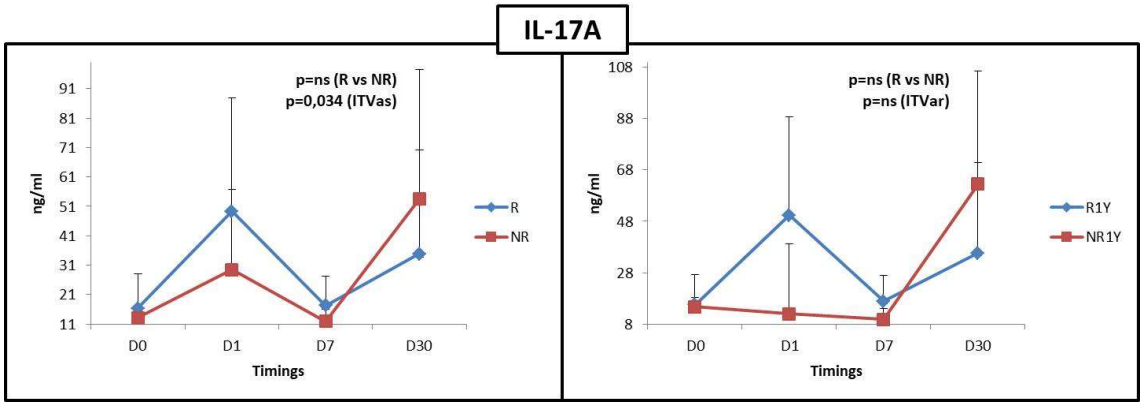
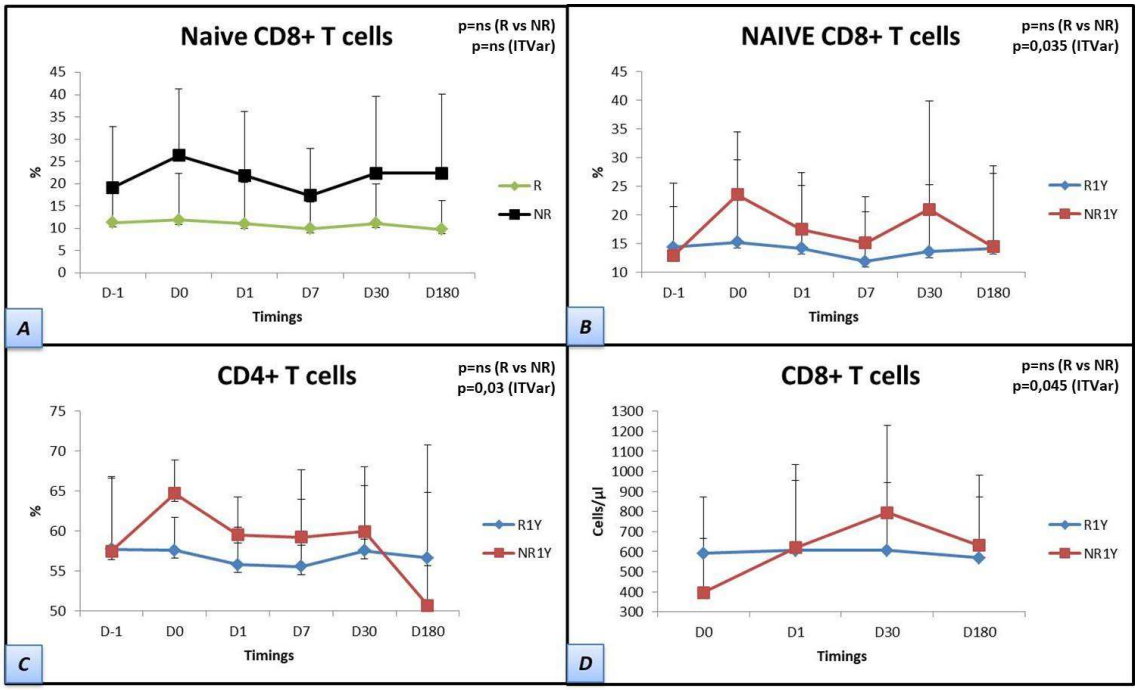


Figure 5: 'Responders' to renal denervation tend to have lower absolute values and lower variability of CD4, CD8 and naïve CD8 T cells throughout the time. Intertiming variability is significant within the groups. Samples were obtained at six timings (D-1, D0, D1, D7, D30 and D180 – see text). (A) Estimated marginal means of naïve CD8+ T cells in 'responders' at six months (R, green line) vs 'non-responders' (NR, black line). (B) Estimated marginal means of naïve CD8+ T cells in 'lateresponders' (R1Y, blue line) vs non-responders (NR1Y, red line). (C) Estimated marginal means of total CD4+ T cells in R1Y (blue line) vs NR1Y (red line). (D) Estimated marginal means of total CD8+ T cells in R1Y (blue line) vs NR1Y (red line). Statistical analysis was performed using the Kruskal-Wallis Test and a general linear model was applied to analyze variance of each laboratorial parameter. Data are presented using mean ± standard deviation. *ITVar – inter-timing variability.*



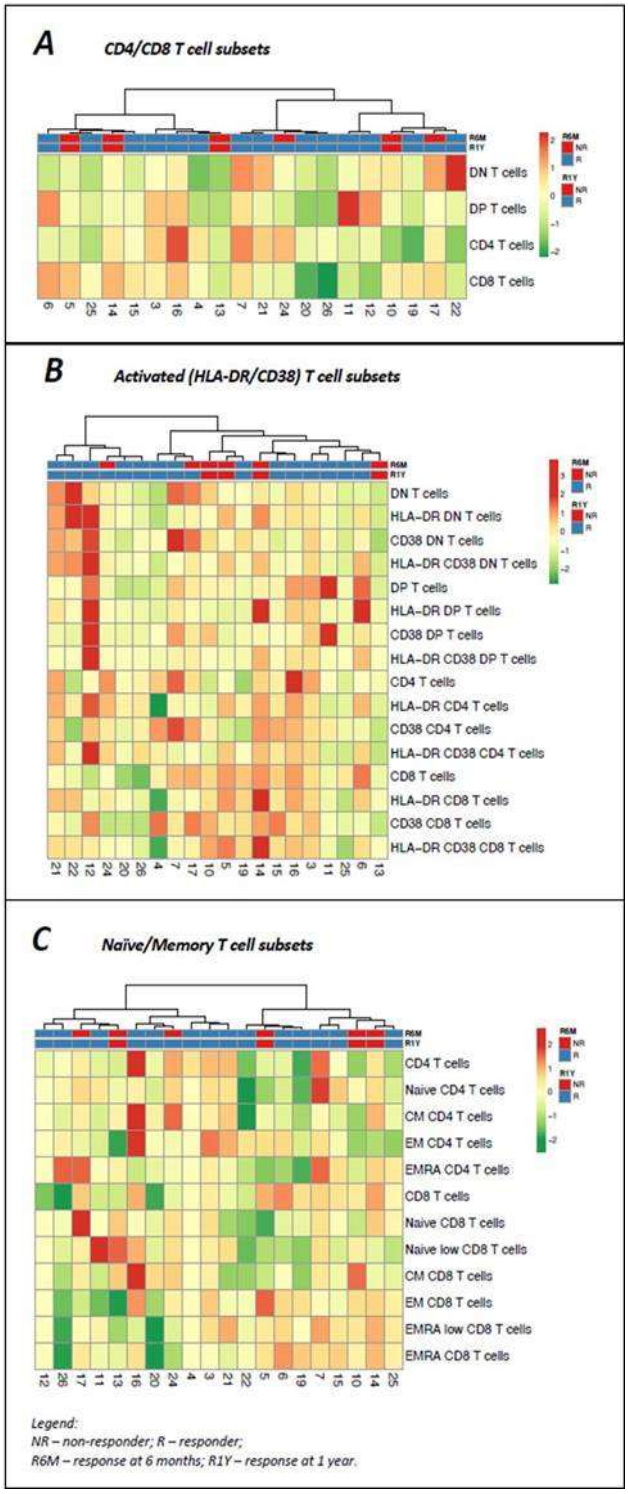


Figure 6: Renal denervation response at 6 months and one year, according to CD4/ CD8, activation and memory T cell subsets. (A) Heatmap for absolute frequencies of DN, DP, CD4, CD8 T cell subsets in 'responders' and "non-responders" at 6 months (R6M) and one year (R1Y). (B) Heatmap for absolute frequencies of activated (HLA-DR and CD38) DN, DP, CD4, CD8 T cell subsets in 'responders' and "non-responders" at 6 months (R6M) and one year (R1Y) after renal denervation. (C) Heatmap for absolute frequencies of naïve and memory CD4 and CD8 T cell subsets in 'responders' and "non-responders" at 6 months (R6M) and one year (R1Y) after renal denervation. Blue and red bars in the top of heatmaps represent 'responders' and "non-responders", respecti vely, for response at 6 months (R6M) and response at 1 year (R1Y), according to legend in the right of figure. Numbers at the bottom of the heatmaps represent patient case numbers identifiers. *DN – double negative; DP – double positive.*

4. Discussion

The latest research on RDN showed that the procedure effectively lowers BP more than sham-control groups [22]. However, not all patients respond to RDN and the reasons for this lack of efficiency are altogether unknown [23]. Many questions remain to be answered regarding the physical, hemodynamic, humoral or immunological profile of the responder. A biomarker that could potentially predict the BP response to RDN has not yet been found even though it has been the focus of research teams worldwide [24–26].

Essential HT is a complex disease characterized by a complex interaction between (1) the heart, which pumps blood to the body, (2) the central and peripheral nervous system, which regulates all organs, (3) the kidney, that regulates sodium and water excretion and hence blood volume, and (4) the vasculature, which regulate the blood flow by vasoconstriction and –dilation. However, the specific contributors and the exact manner of the disturbance in the interaction between these systems, is unknown. Two major systems are overactivated in HT, the renin-angiotensin-aldosterone system and the SNS [4]. Increased sympathetic outflow is a constant finding in HT and is well known that this hyperactivation leads to vasoconstriction, vascular smooth muscle cell proliferation and vascular remodeling [27]. Emerging evidence in the last years, suggest that a significant part of these alterations originate from a baseline inflammatory milieu, which leads to the activation of innate and adaptive immunity and consequent end-organ damage, ensuing with cellular infiltration and production of several cytokines and chemokines [28]. The innate immunity is an immediate response to threatening signals, identified as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). These signals are recognized by pattern recognition receptors (PRR) that will lead to the appearance of inflammasomes, which secret various proinflammatory cytokines [29]. The Toll-like receptors are a type of PRR and the only ones, until the present time, that have been shown to be involved in the inflammation process associated with HT [19], being expressed by T and B cells, among others. Besides offering an immediate response, the inflammasome effectively presents antigens to naïve T cells and therefore promotes a subsequent specific adaptive immune response [30]. The adaptive immune system is characterized by a unique response to endogenous or exogenous antigens and the most important effector cells are T and B lymphocytes [19].

As the contribution of the immune system to the development of HT is well known, basically, every cell type of the innate and adaptive immunity has been implicated in its pathogenesis. There is a large amount of evidence stating that immune cells infiltrate organs responsible for the regulation of BP, and that the depletion of specific immune cells types, or inhibiting their function in animal models, protects against HT. Activated T cells, through the promotion of various levels of inflammatory response, tissue damage, cytokine production and chemoattraction of other inflammatory cells, and the promotion of autoantibody production by B cells, have a central role in different autoimmune diseases and inflammatory states [31]. In humans, approximately 95% of T cells express T cell receptor (TCR) αβ and the remaining 5% of T cells express TCR γδ [32]. CD4 and CD8 define the most common T cell subsets. The so-called 'double-negative' (DN) T cells are approximately 1–10% of all CD3 T cells in the peripheral blood and are linked to the pathophysiology of autoimmune/inflammatory conditions characterized by increased levels of this cell type [33–35]. In spite of all the ongoing investigation, origin, differentiation and function of DN T cells both in healthy individuals and in disease, remains poorly understood.

This study identified, for the first time, a potential cellular biomarker and a set of parameters that may be the jump-start to the characterization of the immunological behavior of the 'responder' to RDN.

First, 'non-responders' at one year showed significantly elevated levels of activated DN T cells at all timings, when compared to 'responders', a difference not observed when response is evaluated at six months, eventually because 'late-responders' show a similar immunological pattern to earlier 'responders', and were part of the 'non-responder' group at that time. The mechanisms leading to an early, vs. a late response are not clarified. Several studies report the involvement of self-reactive, pro-inflammatory DN T cells in systemic inflammation and tissue damage, and their role in the pathogenesis, symptom onset and disease activity in autoimmune diseases such as autoimmune lymphoproliferative syndrome [36], systemic lupus erythematosus [37], Sjögren's syndrome [38] and psoriasis [39] is established. HT is associated with a deregulated immune response but the precise factors leading to immune cell activation are not defined.

Second, in this study, 'non-responders' have much higher baseline levels of activated DN T cells, a finding that can be related to a much more exuberant inflammatory systemic response and a major contribution of other mechanisms, other than the SNS, to the perpetuation of HT.

Third, DN T cells levels were reduced after RDN even in 'non-responders', notwithstanding the levels in 'responders' were significantly lower. Several studies report that DN T cells secret several cytokines and chemokines such as IL-10 and IFN-γ, which may exhibit both regulatory and pro-inflammatory effects, and also IL-1, IL-3 and IL-17, which are pro-inflammatory [40]. As the SNS is a major intercommunicating pathway between the central nervous system and the immunological system, and adrenergic stimulation leads to increased pro-inflammatory cytokine production by several immune cells, one possible explanation for the observed decrease in activated DN T cells after RDN is the blunted sympathetic activity, much more significant in 'responders' but most certainly also present in 'non-responders', leading to a decrease in the stimulation of DN T cells and therefore their absolute levels. ROC curve analysis revealed that a HLA-DR DN T cells value of ≤9.635% offered the best overall sensitivity and specificity for predicting response. IFN-G, IL-4, IL-10, TGF-B and IL-17A quantification was similar between both groups, even though a separation of the curves is noted at D30, regarding IL-17A, which is consistent with the perpetuation of an inflammatory milieu in non-responders.

A fourth finding in this study is the inter-timing variability in some T cell subsets such as CD4 and CD8 T cells and naïve CD8 T cells. Even though statistically significant differences in absolute values were not found between the R/NR or the R1Y/NR1Y groups, a tendency for 'nonresponders' to exhibit higher and more variable values of this cell types is evident, suggesting the presence of an active inflammatory milieu.

Our group is the first to potentially identify an immunological cellular biomarker to predict response to RDN and complement clinical practice, and to characterize the cellular immunological behavior of the 'responder'. So far as we know, this is the first study to assess the immune cell population in patients with resistant HT submitted to RDN.

5. Limitation

This is a small single center study, and one of the major limitations is the small number of 'non-responders'. Large-scale studies/trials are necessary to confirm these results.

6. Conclusions

RDN is an option for patients with resistant hypertension, even though it is not effective in all subjects. The reasons for this lack of response are unknown and research is currently focusing on identifying a laboratory biomarker, which would help to better select the patients referred to this technique. Our study assessed the immune system in patients submitted to RDN and demonstrated, for the first time, that low absolute values of activated DN T cells and that lower and more stable values of total CD4, CD8, and naïve CD8 T cells, are cellular biomarkers able to potentially predict the response to RDN.

7. Impact on daily practice and take-home message

• A biomarker to predict the response to renal denervation has not yet been identified and many questions remain to be answered regarding physical, hemodynamic, humoral or immunological profile of the responder.

• 'Non-responders' presented with significantly elevated levels of activated double-negative T cells, when compared to 'responders', a difference more evident before performing renal denervation.

• 'Non-responders' tend to exhibit higher and more variable levels of specific T cell subsets, such as total CD4 and CD8 and naïve CD8 T cells.

• This cell types can potentially predict the response to renal denervation and help to better select patients to perform this minimally invasive technique.

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Original Article Number 3

Low plasmatic levels of RANTES/CCL5 are associated with a positive response to renal denervation in patients with resistant hypertension

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Abstract

The role of the immune system and hence inflammation, in the pathophysiology of hypertensive patients, is not clear. Up to this time, most clinical and biochemical parameters failed to predict a positive response to renal denervation (RDN). Our aim was to evaluate the immune response in a cohort of patients submitted to RDN, through the analysis of cytokine, chemokine and growth factor behavior.

A population of 21 resistant hypertension patients, submitted to RDN, was evaluated at six-months and one year. Response was defined as a drop ≥ 5mmHg in ambulatory blood pressure monitoring. Sixty-seven percent of patients clinically responded after 6 months and eighty-one percent at one year. There were no complications or safety issues. Blood samples were obtained at four timings, pre- and post-procedure and, plasmatic levels of 45 cytokine, chemokine and growth factors were quantified.

Baseline characteristics were similar between groups, except active smoking that was more frequent in non-responders at one year. Regulated on activation, normal T cell expressed and secreted (RANTES/CCL5) levels were significantly lower in responders, both at baseline and at 30 days ($p=0.037$), and a level ≤15496 pg/ml was the optimal cutoff, for prediction of response. IL-15, IL-17A, IL-27 and leukemia inhibitory factor (LIF) varied significantly in time, with an acute rise being observed 24h after RDN.

Low plasmatic levels of the chemokine RANTES/CCL5 was the most significant result associated to RDN response and may help to identify the best candidates among patients with true resistant hypertension.

Keywords: *Hypertension; Renal denervation; RANTES; Cytokines; Chemokines; Immune system; Inflammation.*

Abbreviations: *RDN - renal denervation; BP - blood pressure; HT - hypertension; SNS - sympathetic nervous system; ABPM - ambulatory blood pressure monitoring.*

1. Introduction

The identification of specific patient subsets which derive the most benefit from renal denervation (RDN) has been the focus of investigators in the last years. Following the unexpected outcomes of HTN-3 trial [1], three second generation randomized trials on RDN [2-4] were carefully elaborated and have shown a significant decrease of blood pressure (BP) in a wider cohort of patients, with and without anti-hypertensive drugs, demonstrating not only efficacy but also safety. The role of the immune system and hence inflammation in the pathophysiology of hypertensive patients, submitted to RDN, is not clear and, up to this time, most clinical and biochemical parameters failed to predict response to RDN. [5]

Several mechanisms contribute to the pathogenesis and perpetuation of hypertension (HT), in which the renin-angiotensin-aldosterone system (RAAS) has major implications. Angiotensin II binds to angiotensin 1 receptors (AT1), usually present in several immune cells such as T cells, dendritic cells and macrophages, and determine their differentiation and subsequent pro-inflammatory cytokine production. [6] Pro-inflammatory stimuli trigger endothelial expression of adhesion molecules and increase leucocyte migration, promoting fibrosis and hypertrophy, with reduction of vascular luminal diameter. [7] Additionally, angiotensin II also contributes to inflammation as it increases reactive oxygen species (ROS) production (including superoxide), by stimulating nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, leading to reduced levels of nitric oxide (NO) and development of oxidative stress. Mitochondrial oxidative stress (main source of ROS) may lead to both end-organ dysfunction and HT, by enhancing more NADPH production, the RAAS system and pro-inflammatory cytokine secretion which, in turn, stimulate oxidase activity and superoxide production, creating a never-ending process. [8-10]

Dörr et al aimed to study the impact of endothelial adhesion molecules, indicators of endothelial dysfunction, on RDN response, and concluded that responders may be more affected by HT-related endothelial dysfunction and shear stress. [11] The same research group studied the impact of brain-derived neurotrophic factor (BDNF) on RDN response, a neurotrophin directly involved in the regulation of neurotransmitter production by the sympathetic nervous system (SNS). The authors found a significant decrease in BDNF levels immediately after RDN that correlated with

systolic BP reduction at 6-month follow-up (FU), which is in accordance with the previous knowledge stating that a decrease in BDNF expression is associated with an impaired density of sympathetic activity and transmitter release. [12] Eikelis et al assessed the bioavailability of NO in patients submitted to RDN, hypothesizing it could function as a potential mechanism to the lowering BP effects. In this study, none of the plasma cytokines measured had a predictive value to differentiate responders from non-responders but the authors found a significant reduction in soluble fms-like tyrosine kinase-1 (sFLT-1) and interestingly, NO elevation was only seen in responders, with a possible explanation being the disassemble of vascular endothelial growth factor (VEGF)-NO pathway, which was preventing generation of NO and therefore limiting the BP lowering effect in non-responders. [13]

Previously, our group explored the cellular immune profile in patients with resistant HT and treated with RDN. Levels of HLA-DR+ double-negative T cells were significantly higher, both at baseline and post-procedure, in the non-responders, a finding that is aligned with the current knowledge stating the presence of a more profuse inflammatory milieu in this group, which could contribute to the perpetuation and severity of HT. [14] In continuance of the described research, the aim of the current study was to evaluate the behavior of a subset of cytokines, chemokines and growth factors in a cohort of patients with resistant HT, treated with RDN.

2. Material and Methods

2.1 Patients

The study population in this prospective, non-randomized, single center study included 21 patients with resistant HT and treated with RDN between May 2014 and October 2017. Patients were included if they presented with idiopathic resistant HT (treated with three or more anti-hypertensive drugs in maximum tolerated dosages, including a diuretic), measured by ambulatory BP monitoring (ABPM), with mean systolic BP > 135mmHg. Patients with renal dysfunction (glomerular filtration rate <45 ml/min/1.73m2), recent major cardiovascular events, fibromuscular dysplasia, previous renal angioplasty or untreated secondary HT were excluded. Basal assessment consisted of routine hematology and biochemistry profile, ABPM monitoring, electrocardiogram, transthoracic echocardiogram, 24h-hour rhythm monitoring and renal doppler or CT scan. Adherence was assessed in all patients by witnessed drug-intake. Patients were considered 'responders' (R) if a drop ≥ 5mmHg in mean ambulatory BP was observed at six-months (vs NR). Additionally, 'late-responders' included 'responders' at six months and patients who responded after one-year FU (R1Y vs NR1Y).

The study was approved by the Faculty of Medicine of the University of Coimbra and the Coimbra's Hospital and University Centre Ethics Committees, and all patients signed an informed consent.

2.2 Renal denervation

Patients received peri-procedural analgesia and conscious sedation with propofol/midazolam and remifentanil i.v. perfusion. Anti-thrombotic treatment included aspirin and a bolus of weight-adjusted unfractioned heparin (70-100U/Kg). Femoral arterial access was obtained with 6F or 8F sheaths. Radiofrequency energy was delivered using the single-tip radiofrequency Symplicity Flex catheter (Medtronic Inc, Santa Rosa, CA, USA), the EnligHTN system (St. Jude Medical, MN, USA) or the Symplicity Spyral catheter (Medtronic Inc, Santa Rosa, CA, USA), in 9.5%, 38.1% and 52.4% of the patients, respectively. Energy was applied to the main artery and branches, when feasible and according to the characteristics of each device, with the goal of

achieving a maximum number of circumferential ablation points. Administration of intra-renal nitrates and a final renal angiogram was performed. All procedures were conducted by one interventional cardiologist dedicated to the field. Hemostasis was accomplished with a vascular closure device. All patients were monitored for 24h before discharge.

2.3 Follow-up

Patients were evaluated at 7, 30, 90, 180 and 365 days after the procedure. Renal angiogram through the radial artery was performed at 180 days to assess safety. An electrocardiogram, transthoracic echocardiogram and ABPM were performed after six-months and one year. Venous blood samples were obtained at four timings: D0 (before RDN), D1 (24h after RDN), D7 (one week after RDN) and D30 (one month after RDN).

2.4 Plasmatic Cytokine profiling

Plasma of RDN patients was freshly isolated and stored at -20 °C until thawed before analysis. Plasmatic levels of cytokines, chemokines and growth factors were quantitatively analyzed by commercially available single well Luminex® xMAP assay (ProcartaPlex, Invitrogen). For this study we used a broad human factor panel consisting of 45 protein targets comprising in five modular subpanels: Th1/Th2 [GM-CSF, IFN gamma, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-18, TNF alpha], Th9/Th17/Th22/Treg [IL-9, IL-10, IL-17A (CTLA-8), IL-21, IL-22, IL-23, IL-27], Inflammatory cytokines [IFN alpha, IL-1 alpha, IL-1RA, IL-7, IL-15, IL-31, TNF beta], Chemokines [Eotaxin (CCL11), GRO alpha (CXCL1), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 alpha (CCL3), MIP-1 beta (CCL4), RANTES (CCL5), SDF-1 alpha] and Growth factors [BDNF, EGF, FGF-2, HGF, NGF beta, PDGF-BB, PlGF-1, SCF, VEGF-A, VEGF-D]. Ninety-six well plates were read in a Luminex® 200 System (Luminex Corporation, Austin, Texas, USA) and plasmatic concentrations were calculated applying a five-parameter logistic (5PL) regression non-linear model using the Luminex xPONENT v3.1 software.

2.5 Statistical analysis

Categorical variables were characterized by determining the absolute and relative frequencies, and numerical variables the means and standard deviations. Comparisons between groups with regard to the categorical variables were conducted using the Chi-Square Test. Regarding the continuous variables, Mann-Whitney U Test was used to compare two groups, and Kruskal-Wallis Test between more than two groups. A general linear model for repeated measures was applied to analyze variance of each laboratorial parameter, measured several times on each subject from two different groups, 'responder' and 'non-responder'. Two different groups definition were considered, responders at six months and at one year.

Statistical analyses were conducted using SPSS 19.0®, at a 5% significance level for hypothesis-testing. The Type I error probability associated with this test of this null hypothesis is 0.05.

3. Results

Twenty-one consecutive patients were included in this study (mean age 59 ± 11.3 years, 33.3% female). Fourteen patients were 'responders' at six months (R-66.7%) and 17 were 'late-responders' (R1Y-81%). There were no significant differences between responders and non-responders regarding comorbidities such as dyslipidemia, type 2 diabetes or presence of sleep apnea, but active smoking was more frequent in NR1Y (p=0.008). The overall group was overweight (BMI 29.7 ± 3.9). Baseline mean office systolic and diastolic BP was 189.5 ± 26 and 106.1 ± 18.6mmHg and ABPM mean systolic and diastolic BP was 153.8 ± 12.2 and 89 ± 14.6mmHg, respectively (p=ns). 28.6 and 41.2% of the patients in the R and R1Y groups were non-dipper (vs 71.4 and 50% in NR and NR1Y, p=ns). An ABPM systolic BP mean drop of 21.1 ± 13.3 and 16.2±16.6mmHg was observed in the R and R1Y groups, respectively (vs -7.6 ± 10.6 and -8.5 ± 11.9 mmHg in the non-responders) (p<0.02). Baseline characteristics, number of ablations and BP evolution during follow-up are shown in Table 1.

As described, absolute levels of 45 protein cytokines, chemokines and growth factors were quantified at four timings, pre and post procedure. Levels of RANTES (regulated on activation, normal T cell expressed and secreted) were significantly lower in responders, both at baseline (12.821 ± 3646 vs 15102 ± 3069 pg/ml) and at 30 days (13941 ± 3098 vs 16108 ± 2209 pg/ml) (p=0.037), without significant variation throughout the time. ROC curve analysis showed an area under the curve of 0.701, 95% CI=0.567-0.834, p=0.01). A RANTES level ≤ 15496 pg/ml showed the best overall sensitivity (57.1%) and specificity (85.7%) for determining response to RDN. Even though late-responders (R1Y) continued to present with lower levels of RANTES at every time point, this difference did not reach statistical significance. (Figure 1) Figure 2 illustrates the principal component analysis for the distribution of cytokines, chemokines and growth factors at the four analyzed timings (panel A) and at baseline (panel C). Panels B and D illustrate the overall and baseline heatmaps representing clustering of multivariate data.

Even though, absolute levels of other analyzed cytokines were not statistically different between groups, a pattern was evident when analyzing their behavior. IL-15, IL 17A, IL 27 and leukemia inhibitory factor (LIF) had significant variability through time, with responders presenting with an acute rise at D1 (24h after RDN). (Figure 3)

Furthermore, other biomarkers known to be associated with cardiovascular disease 15-16 were also quantified. Both NR ($p=0.02$) and NR1Y ($p<0.001$) had higher levels of baseline glycated hemoglobin and, in NR, levels of NT pro-BNP were significantly higher ($p=0.005$), a difference not observed at one-year follow-up. No other significant differences were found regarding renal function, LDL levels, C-reactive protein, lipoprotein A and fibrinogen. (Table 1)

Table 1: Baseline clinical, biochemical and procedural characteristics. Ambulatory blood pressure monitoring behavior, from baseline to one-year follow-up.

	R (n=14)	NR (n=7)	p value	R1Y (n=17)	NR1Y (n=4)	p value
Age in Y (mean±SD)	61±10	55±13.5	ns	59.8±11.8	55.5±9.5	ns
Diagnosis of HT in Y (mean±SD)	16.3±9.3	17±5.9	ns	16.6±8.6	16.2±7.5	ns
Female sex (%)	21.4	57.1	ns	35.3	25	ns
Dyslipidemia (%)	85.7	100	ns	88.2	100	ns
Type 2 diabetes (%)	42.9	57.1	ns	47.1	50	ns
Active smoking (%)	14.3	42.9	ns	11.8	75	**0.008**
Sleep apnea (%)	57.1	57.1	ns	58.8	50	ns
Number of HT drugs (n±SD)	5.1±1.4	5.4±0.5	ns	5.2±1.3	5.2±0.5	ns
- On spironolactone (%)	57.1	42.9	ns	52.9	50	ns
Isolated HT (%)	14.3	14.3	ns	17.6	0	ns
BMI (Kg/m^2)	29.4±3.8	30.2±4.3	ns	30.1±3.8	27.8±4.3	ns
Biochemical profile at baseline						
HBA1c (%)	5.9±0.75	7±2.5	**0.02**	5.9±0.7	7.7±3.4	**<0.001**
NT pro-BNP (pg/ml)	165±265	1037±2478	**0.005**	541±1594	94.7±62.2	ns
LpA (mg/dl)	42.3±46	30±45.2	ns	37.6±42.7	40.6±60.9	ns
Fibrinogen (mg/dl)	278.1±59.1	271.2±76.5	ns	282±65	243.7±44.8	ns
Creatinin (mg/dl)	0.97±0.2	0.76±0.2	ns	0.9±0.2	0.76±0.2	ns
Cystatin C (mg/l)	0.97±0.2	0.85±0.2	ns	0.95±0.2	0.8±0.1	ns
LDL col (mg/dl)	98±23	118±26	ns	102±25	116±28	ns
PCR (mg/dl)	0.83±0.97	1.2±2.2	ns	1.1±1.6	0.4±0.3	ns
Baseline						
ABPM systolic BP (mmHg)	154±12.3	153.4±12.9	ns	154.8±12.5	149.7±11.6	ns
ABPM diastolic BP (mmHg)	87.1±14.3	92.7±15.6	ns	89±15.3	88.7±13.3	ns
HR (bpm)	70.6±9.1	73.3±13.9	ns	72.3±9.8	68±15	ns
E/E' ratio	10.6±4.8	15±3.6	ns	11.4±5.2	13.8±3.5	ns
6 Months						
ABPM systolic BP (mmHg)	133±11	161±9.7	**<0.001**	138.6±16.6	158.2±7.4	**0.004**
ABPM diastolic BP (mmHg)	77.1±11.2	95.1±15.8	**0.007**	81.3±15	90.5±13.7	ns
HR (bpm)	68.8±7.3	75.8±10.8	0.09	71.3±8.7	70.5±11.7	ns
E/E' ratio	12.8±6.3	12.3±2.8	ns	12.6±5.2	12.5±3.4	ns
One year						
ABPM systolic BP (mmHg)	135.9±16.2	149.3±20.2	ns	135.6±15.8	160.5±15	**0.03**
ABPM diastolic BP (mmHg)	77.1±11.2	88.4±14.3	ns	77.5±10.5	94.5±15.8	ns
HR (bpm)	67.8±8.1	74.4±12	ns	69.4±8.3	72.5±16.3	ns
Total ablations (n)	23.8.6±8.3	22.4±7.3	ns	23.9±8.5	20.7±4	ns
Drop in ABPM systolic BP at 6 months (mmHg)	21.1±13.3	-7.6±10.6	**<0.001**	16.2±16.6	-8.5±11.9	**0.013**
Drop in ABPM systolic BP at 1 year (mmHg)	18.1±11.5	4±21.5	ns	19.2±12.2	-10.7±6.7	**<0.001**

Legend - R: responder; NR: non-responder; R1Y: responder at one year; NR1Y: non-responder at one year; Y: years; HT: hypertension; BMI: body mass index; ABPM: Ambulatory pressure monitoring; BP: blood pressure; HR: heart rate; HBA1c: glycated hemoglobin; LpA: lipoprotein A; Col: cholesterol; PCR: C-reactive protein. Results are displayed in mean ± standard deviation (SD).

Figure 1: Quantification of RANTES at four time-points (D0, D1, D7 and D30 – see text), before and after renal denervation. (A) Plasmatic levels of RANTES are significantly lower in 'responders' (blue line) at six months. (B) After one year follow-up, levels of RANTES are still lower, even though statistically non-significant. (C) ROC curve: A RANTES cut-off value of ≤15496 pg/ml showed the best overall sensitivity and specificity for determining renal denervation response. Data are presented using mean ± standard deviation. *RANTES - regulated upon Activation, Normal T cell Expressed, and Secreted; FU – follow-up; RDN – renal denervation; AUC – area under the curve*

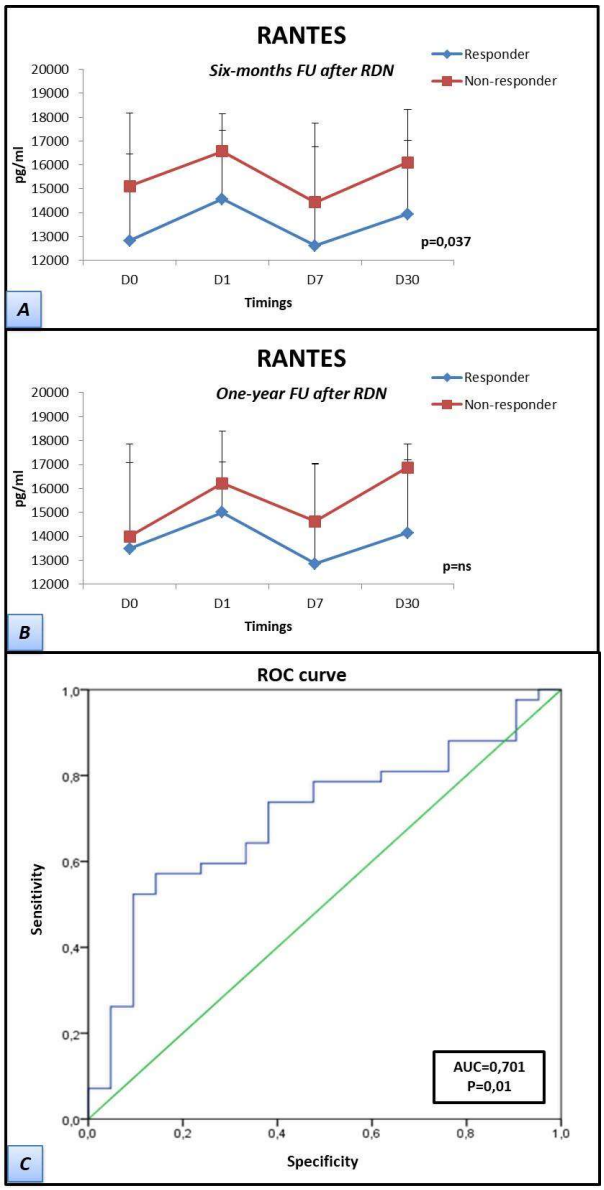


Figure 2: Distribution of cytokines, chemokines and growth factors, according to response to renal denervation response, at 6 months and one year. (A) Principal component analysis for the distribution of cytokines, chemokines and growth factors in R/NR and R1Y/NR1Y at the four analyzed time-points (D0, D1, D7 and D30 - see text). (B) Heatmap for plasmatic cytokines, chemokines and growth factors in R/NR and R1Y/NR1Y, at the four time-points. (C) Principal component analysis for the distribution of cytokines, chemokines and growth factors in R/NR and R1Y/NR1Y, at baseline (D) Heatmap at baseline for cytokine, chemokine and growth factor quantification in R/NR and R1Y/NR1Y. Bars at the top of the heatmaps represent each patient individualized (panels B and D), the time-point of the analysis (panel D) and the groups according to response (R/NR – 6 months; R1Y/NR1Y – one year), in consonance with the legend on the right side of the figure. Numbers at the bottom of the heatmaps represent patient case numbers identifiers. *RDN - renal denervation*

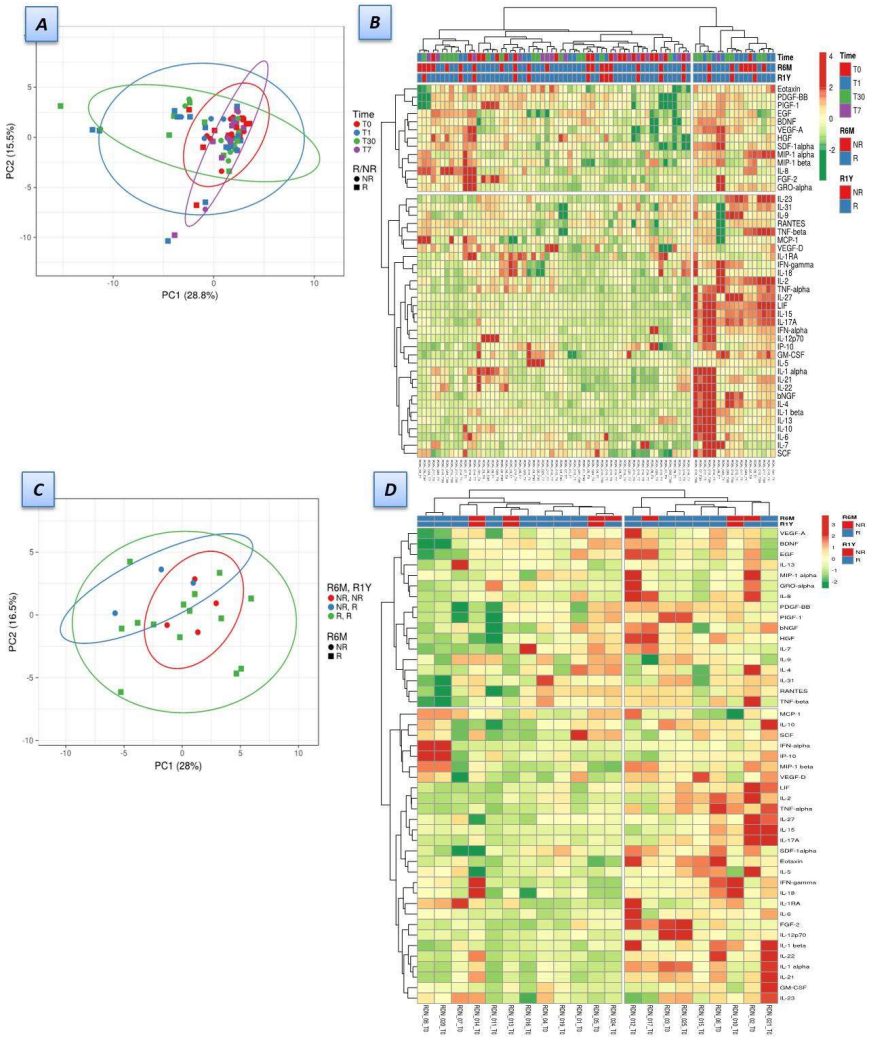
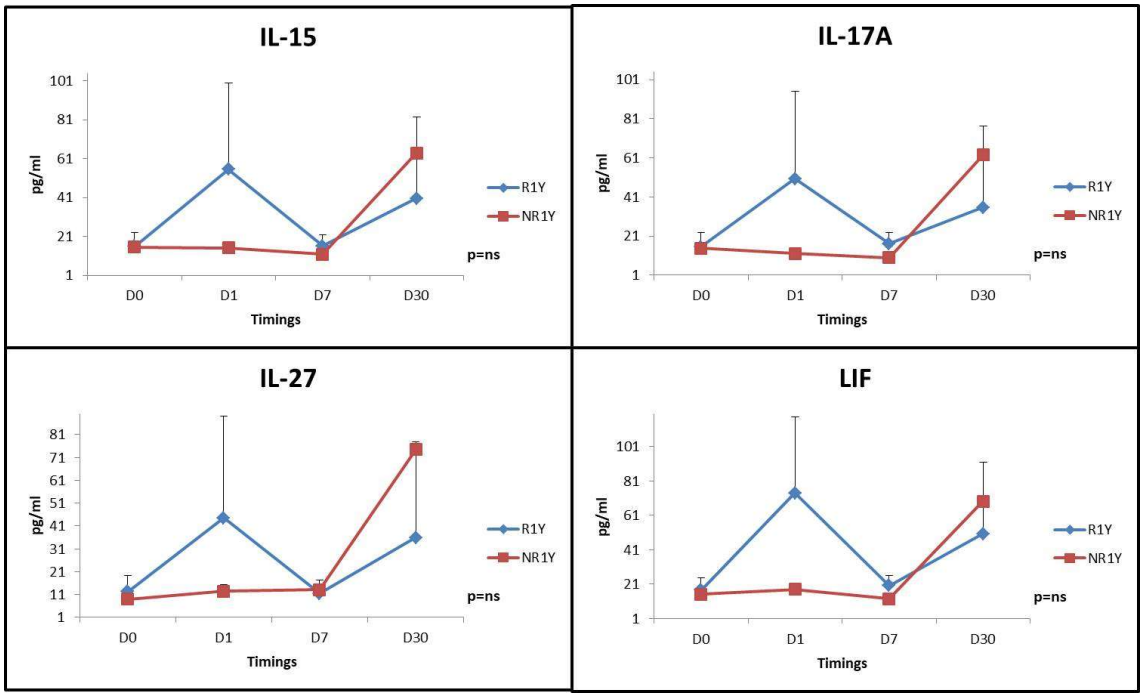


Figure 3: Significant variation in time was observed when analyzing IL-15, IL-27, IL-17A an LIF at four time-points (D0, D1, D7 and D30), with responders presenting an acute rise 24h after RDN. Panels indicate values between R1Y and NR1Y. The same pattern maintains when analyzing response at six-months (not shown). Data are presented using mean ± standard deviation. *LIF – leukemia inhibitor factor; R1Y – responders at one year; NR1Y – non-responders at one year.*



4. Discussion

The mechanisms that contribute to the development of HT are complex and involve renal deregulation, endothelial dysfunction and unbalanced central and autonomic nervous systems. Inflammation is directly linked to the development of HT and is a complicated process implicating multiple cell types and secreted pro and anti-inflammatory substances. Monocyte and macrophage are one of the cell types directly involved in the activation of the inflammatory cascade. [17] The inflammasomes are protein complexes activated through the detection of inflammatory particles, including ROS, by danger-associated molecular patterns (DAMPs). It has been hypothesized that DAMPs may be formed due to initial non-immune mechanisms, such as direct vasculature and renal damage caused by the activation of the SNS and RAAS, thus leading to mechanical and oxidative stress. [18] Inflammasomes have enzymatic activity and participate in the cleavage and activation of numerous pro-inflammatory cytokines, some of them directly involved in the pathogenesis of HT. [19] The most studied inflammasome is the NLRP3, which contains the NLRP3 protein. Lee et al [20] investigated the changes of early inflammatory biomarkers following RDN and verified that several pro-inflammatory cytokines (IL-1β, IL-18, IL-6, tumor necrosis factor (TNF)-α and also, caspase-1 activity and NLRP3 expression, increased immediately after RDN and then recovered two weeks after, suggesting a self-limited inflammatory response to RDN. The cytokine RANTES (also called CCL5, CC-motif ligand 5) is a soluble pro-inflammatory chemokine secreted by several cell types such as activated T cells, fibroblasts, endothelial cells, smooth muscle cells, glial cells, mesangial cells and platelets [21-22], which is highly expressed in the atheroma and has been implicated in the pathophysiology of atherosclerosis. [23] Activated platelets are thought to have a crucial role in the pathogenesis of atherosclerosis and there is evidence linking RANTES with metabolic syndrome and IL-6, which is an activator of platelets. [24] Some researchers have linked the pro-inflammatory cytokine IL-6 to endothelial dysfunction, to increased risk for cardiovascular events and to HT, possibly having an important role in the progression of atherosclerosis. [25] Lang et al examined several renal and inflammatory parameters in resistant HT, treated with RDN, and observed that IL-6 and white blood cell count significantly decreased 6 and 12 months after the procedure. At baseline, even though a trend towards higher IL-6 values was evident, the difference was not statistically significant and thus, not useful to predict response in the studied cohort. [26] Dörr et al had also

determined IL-6 levels in sixty patients submitted to RDN and observed that IL-6 diminished significantly after the procedure, hypothesizing a beneficial effect of RDN on cardiovascular inflammation. [27] Some other studies investigated the effects of RDN on pro-inflammatory cytokine environment. Hilderman et al verified that TNF and IL-1b values were significantly lower 1 day after RDN and that IL-10, an anti-inflammatory cytokine, rose significantly after the procedure, vs a control group. These differences however were less evident in de medium-term follow-up, suggesting a transitory immuno-modulatory effect. [28] SNS hyper-activation associated with profuse inflammatory response is also triggered by ischemia/reperfusion (I/R) injury. This association has been examined by Sun et al. [29] in a translational research involving a group of patients with ST-elevation acute myocardial infarction (MI), and a group of mice, submitted to surgical RDN and I/R injury through left anterior descending coronary artery suture or ligation. They observed that MI patients had elevated norepinephrine and leucocyte plasma levels. Denervated mice showed a reduction in neutrophils and macrophages in blood and myocardium, associated with a significant decrease in IL-1, IL-6 and TNF-α. The authors concluded that it appears to exist a link between the SNS and the inflammatory response following I/R injury, identifying RDN as a potential therapeutic strategy in this setting.

In the present study, we demonstrated that responders presented with lower levels of RANTES, than non-responders. Yun et al [30] observed that angiotensin II, mediated by 12-lipoxygenase, inhibited RANTES expression in spontaneous hypertensive rat's vascular smooth muscle cells, through the activation of AT1 and AT2 receptors. As the SNS is directly linked to RAAS [31], we could deduce that lower levels of RANTES are associated with higher levels of angiotensin II and therefore to an over activated SNS, turning these patients more prone to a RDN response. Our study also demonstrated that, even though lower levels of RANTES persisted at one-year follow-up, this difference was not statistically significant, probably due to the low number of non-responders at this stage. Data regarding the role of RANTES in atherosclerosis and plaque vulnerability is controversial. Studies that included patients with acute coronary syndromes found elevated RANTES levels [32], while others have shown that low RANTES levels were independently predictive of adverse outcomes in chronic stable disease. [33] RANTES is known to be a very potent chemo-attractant of T-cells, monocytes and macrophages and is possible that its higher levels may be associated with cellular infiltration and hence end-organ damage. However, a protective role of RANTES over the kidney, that appears tissue specific, has been described by Rudemiller et al. [34] The authors observed that RANTES deficiency led to

exaggerated hypertensive renal damage through macrophage accumulation, up-regulation of TNF and IL-1β and renal parenchyma matrix deposition and fibrosis. In our study, even though responders had lower levels of RANTES, they were not suppressed, in fact, were above 10.000pg/ml. We could hypothesize RANTES continued to exert its protective role in the kidney, in both responders and non-responders, as renal function was within normal values in the overall cohort.

A second finding in our study was that several cytokines had significant variability in the evaluated time points. It was possible to depict a pattern regarding the absolute values of IL-15, IL-27, IL-17A and LIF, as responders presented peak levels 24 hours after RDN. Radiofrequency energy is directly applied to the renal wall and the vessel temperature is increased in order to destroy the nervous sympathetic terminals wrapped around the renal artery. Even though current research shows there is no luminal damage to the vessel at a medium-term follow-up, there is evidence of endothelial edema, vessel spasm and intraluminal thrombus formation, immediately after the procedure, that rapidly heals. [35] These alterations are likely to provoke an inflammatory reaction, probably directly related to the amount of induced nerve damage. As such, the acute rise of pro-inflammatory cytokines in responders, 24h after RDN, may indicate a successful nerve ablation. IL-15 is a pro-inflammatory cytokine which is a potent chemo-attractant for T cells and is expressed predominantly by macrophages. It has been shown that increased levels of IL-15 are associated with progression of atherosclerotic disease and more severe degrees of HT. [36] IL-27 belongs to the IL-12 family and is considered to have pro- and anti-inflammatory properties. It induces interferon-γ (IFN-γ), IL-1 and TNF-α production, but may also induce IL-10 production by T-cells. [37] IL-17A is a well-known effector cytokine produced by Th17 cells, and is involved in tissue inflammation in several chronic inflammatory diseases, including HT. [38] LIF is a member of IL-6 family and activates pathways that promote cardio-protection both in the acute and chronic settings, by protecting against oxidative stress and cell death and, by stimulating differentiation of cardiac stem cells into endothelial cells and neovascularization, post-MI. [39] The fact that, in our research, pro- and anti-inflammatory cytokines peaked after 24h is a very interesting finding and, we could hypothesize that the provoked numbness of the SNS allowed for anti-inflammatory pathways to fight the acute inflammatory process. This is in line with the findings by Lee et al 20, who observed an increase in inflammatory cytokines IL-1β, IL-18, IL-6 and TNF-α and anti-inflammatory cytokine IL-10, immediately after RDN and then a decrease in week 2 of the follow-up.

Finally, several parameters were evaluated to assess safety and for risk stratification. Glycated hemoglobin was significantly higher in non-responders, even though the number of diabetic patients was similar between groups, implying that poorly controlled glycaemia may adversely affect response to RDN.

5. Limitations

This is a single-center, non-randomized study, with a relatively small sample size and no control group. Larger cohort studies will be necessary to confirm these results.

6. Conclusions

Our study analyzed the behavior of several cytokines, in patients submitted to renal denervation, and identified RANTES as a potential predictor of a response. Renal denervation effectively lowered blood pressure, in the majority of treated patients, without safety concerns.

7. Highlights

- The role of the immune system, in the pathophysiology of hypertensive patients, submitted to renal denervation, is not clear.
- RANTES is a potential predictor of response to renal denervation.
- IL-15, IL-27, IL-17A and LIF increased 24 hours after renal denervation, in responders, and decreased one week after.
- Renal denervation effectively lowered blood pressure, in the majority of patients.

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Original Article Number 4

Risk stratification and cardiac sympathetic activity assessment using myocardial [^{123}I] MIBG imaging in renal denervation

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Conflict of interest: none.

Abstract

Hyperactivation of the sympathetic nervous system plays a central role in the pathophysiology of hypertension. The aim of this study was to assess cardiac sympathetic activity and investigate the role of myocardial 123I-labelled meta-iodobenzylguanidine ([123I] MIBG) scintigraphy in cardiovascular risk stratification of patients with resistant hypertension treated with renal denervation (RDN). Eighteen patients were included in this study (mean age 56 ± 10 years old, 27.8% female). Transthoracic echocardiogram, general blood analysis and myocardial ([123I] MIBG scintigraphy were performed before and six-months after RDN. A patient was considered a responder (R) if a drop ≥ 5mmHg on mean systolic ambulatory blood pressure (BP) monitoring was observed at six-month follow-up. 66.7% of patients were R (drop in systolic BP of 20.6 ± 14.5mmHg, vs minus 8 ± 11.6mmHg in non-responders (NR), p=0.001). Early heart-mediastinum ratio (HMR) was significantly lower at baseline in the R group (1.6 ± 0.1 vs 1.72 ± 0.1, p<0.02) but similar at six months. Putting together both instants in time, the R group had lower early HMR values than the NR group (p<0.05). Both the late HMR and the washout rate were identical and no significant correlation between response to RDN or any MIBG imaging index was found. Renal denervation effectively lowered blood pressure in the majority of patients but [123I] MIBG was not useful in predicting response. However, there was evidence of sympathetic overdrive and, both early and late HMR were reduced overall, probably putting this population at a higher risk of adverse events.

Keywords: *Resistant hypertension; Sympathetic nervous system; Renal denervation; Myocardial [123I] MIBG scintigraphy.*

Introduction

Hypertension (HT) has long been recognized as one of the leading causes of cardiovascular death and hospitalizations. [1] According to the current guidelines, HT is defined as resistant when optimized pharmacological therapy with three anti-hypertensive drugs, including a diuretic, is unable to effectively lower systolic and diastolic blood pressure (BP) to < 140mmHg and <90mmHg, respectively. Its prevalence is thought to revolve around 5-15%. [2] The sympathetic nervous system (SNS) and its involvement in circulatory regulation have first been demonstrated in the 19th century by showing that stimulation of renal nerves elevated BP. [3] According to this knowledge, invasive procedures targeting the SNS were developed in early/mid-20th century but were discontinued due to increased side effects and mortality. [4] Since then, clarification of the mechanisms by which the SNS leads to uncontrolled BP conducted to the development of a minimally invasive percutaneous procedure that has been shown to reduce renal and central sympathetic activity. [5] Renal denervation (RDN) has been the subject of extensive investigation in the past few years and, the latest second-generation randomized trials have demonstrated both efficacy in lowering BP, but also safety, in cohorts of patients at different levels of cardiovascular risk. [6] 123I-labelled meta-iodobenzylguanidine ([123I] MIBG) is an analogue of norepinephrine, labeled with iodine-123 which shares the same uptake mechanism into pre-synaptic nerves. After the uptake, it is transported to catecholamine storage vesicles and, as it is not metabolized, allows for the characterization of cardiac sympathetic activity and neuronal integrity, through planar imaging acquisition using gamma cameras. By analyzing the images, two semi-quantitative parameters are calculated, early and late heart-mediastinum ratio (HMR) and washout rate (WR). Increases in [123I] MIBG concentration in the synaptic cleft translated into augmented WR and diminished HMR.

The purpose of this study was to assess cardiac sympathetic activity and investigate the role of myocardial [123I] MIBG scintigraphy (MIBG-S) in cardiovascular risk stratification of patients with resistant HT treated with RDN.

Methods

We included, in this single-center study, 18 consecutive patients with resistant HT treated with RDN, from May 2014 to October 2017. A comprehensive medical history was recorded in all patients and untreated secondary HT was excluded. Adherence to drug therapy was confirmed by witnessed intake. Exclusion criteria included recent major adverse cardiovascular events, fibromuscular dysplasia, previous renal angioplasty and glomerular filtration rate < 45ml/min/1.73m2. Patients with a mean systolic BP >135mmHg (ambulatory blood pressure monitoring – ABPM) were included. All patients underwent a thorough clinical evaluation, electrocardiogram, transthoracic echocardiogram, standard hematologic and biochemistry profile, and MIBG-S, both at baseline and at six months follow-up. For the RDN procedure, the EnligHTN system (St. Jude Medical, MN, USA) was employed in 33.3% of the cases and the Symplicity Spyral catheter (Medtronic Inc., Santa Rosa, CA, USA) in 66.7%. All patients received conscious sedation and analgesia, and femoral artery hemostasis was achieved using a vascular closing device. Before MIBG-S, patients were pre-treated with Lugol's solution for thyroid blockade (equivalent to 130 mg of iodine for adults) or 500mg of potassium perchlorate if the patient was allergic to iodine. Afterwards, an intravenous injection of 185 MBq of [123I] MIBG was administered, and planar images of the thorax were acquired with a dual-headed gamma camera, fifteen minutes (early imaging) and four hours (late imaging) after the radiopharmaceutical administration. MIBG uptake was semiquantified by calculating HMR, after drawing ROIs over the heart (including the cavity) and the upper mediastinum (avoiding the thyroid gland) in the planar anterior view. Average counts per pixel in the myocardium were divided by average counts per pixel in the mediastinum. The myocardial WR from initial to late images was also calculated, and expressed as a percentage, being the rate of reduction in myocardial counts over time, between early and late imaging (normalized to mediastinal activity). None of the prescribed medications were stopped for the performance of MIBG-S, due to high probability of adverse events and hence ethical issues. Response to RDN was defined if a drop in mean ABPM systolic BP ≥ 5mmHg was observed at six months and patients were divided into two groups accordingly.

Categorical variables were characterized by determining the absolute and relative frequencies, and numerical variables the means and standard deviations. Normality of distribution was

checked and a p value of <0.05 was considered significant. Statistical analyses were conducted using SPSS 19.0®, at a 5% significance level for hypothesis-testing. This study was approved by the Coimbra's Faculty of Medicine Ethics Committee and all patients signed an informed consent.

Results

Eighteen patients (mean age 56 ± 10 years old, 27.8% female) were included in this study. Twelve patients were 'responders' (R, 66.7%) and six 'non-responders' (NR, 33.3%). No significant differences were observed between groups regarding baseline characteristics. RDN was well tolerated by all patients and no peri-procedural complications were detected. Fluoroscopy time was significantly higher in the R group (16.3 ± 5.5 vs 16.5 ± 18.6 minutes, p<0.04). At 6-month follow-up, one patient had an acute pulmonary edema, being diagnosed with renal stenosis, successfully treated with angioplasty. A drop of 20.6 ± 14.5mmHg in mean ABPM systolic BP was observed in the R group (vs -8 ± 11.6mmHg in NR, p=0.001). Even though office systolic BP was not considered for response, due to possible 'white-coat effect', a drop was also observed in the R group (29.2 ± 8.4mmHg) vs the NR group (13 ± 13.4mmHg) (p=0.09). No side effects such as orthostatic hypotension, electrolyte disturbances or renal failure were noticed in the medium-term follow-up. Findings in transthoracic echocardiography (regarding diastolic function, wall thickness or biventricular systolic function) did not differ significantly between the two groups, either at baseline or after 6 months assessment. Baseline and procedural-related characteristics of the overall, 'responders' and 'non-responders' groups, are shown in table 1.

Early HMR was significantly lower at baseline in the R group (1.6 ± 0.1 vs 1.72 ± 0.1, p<0.02) but was not statistically different from the NR group at six months. Putting together both time periods, the R group had lower early HMR values than the NR group (p<0.05). Regarding late HMR and WR, differences before and after RDN were not significant between groups. No significant correlation between response to RDN or any [123I] MIBG imaging index was found, either at baseline or at follow-up (table 1, figure 1 and supplementary table 1).

Table 1: Baseline and procedural-related characteristics, ABPM baseline and 6 months evolution and MIBG scintigraphy parameters at baseline and 6-month follow-up, in overall, 'responder' and 'non-responder' groups.

	Overall	R (n=12)	NR (n=6)	p value
General baseline features				
Mean age (Y)	56 ± 10	58.4 ± 9.8	51.3 ± 10.3	ns
Female sex (%)	27.8	16.7	50	ns
Diagnosis of HT (Y)	19 ± 7.9	19.8 ± 8.7	17.5 ± 6.2	ns
Dyslipidemia (%)	88.9	83.3	100	ns
Diabetes (%)	44.4	41.7	50	ns
Active smoking (%)	27.8	16.7	50	ns
BMI (Kg/m2)	29.7 ± 4.1	29.5 ± 4	30 ± 4.7	ns
Sleep apnea (%)	66.7	66.7	66.7	ns
Number of HT drugs (n±SD)	5.2 ± 1.2	5.2 ± 1.5	5.3 ± 0.5	ns
Baseline Creatinine (mg/dl)	0.88 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	ns
Baseline and 6M Echo				
Baseline ejection fraction (%)	59 ± 9	59 ± 9	59 ± 8	ns
6M ejection fraction (%)	58 ± 9	56 ± 9	62 ± 9	ns
Baseline IVS thickness (mm)	12.4 ± 3.7	13.4 ± 4.2	10.5 ± 1.4	ns
6M IVS thickness (mm)	12.9 ± 2.6	13.4 ± 3	12 ± 1.3	ns
Baseline PW thickness (mm)	10.9 ± 1.9	11.5 ± 2	9.7 ± 1.2	ns
6M PW thickness (mm)	10.2 ± 2.3	10.5 ± 2.5	9.7 ± 1.6	ns
Baseline LA volume (ml/m^2)	56.4 ± 19.5	53.8 ± 14.8	61.8 ± 11.3	ns
6M LA volume (ml/m^2)	56 ± 22.3	59 ± 23	51 ± 22	ns
Baseline E/E'	11.1 ± 3.5	9.9 ± 3	13.6 ± 3.2	**0.03**
6M E/E'	11 ± 3.5	10.9 ± 4	11 ± 2.4	ns
Baseline and 6M ABPM				
Baseline mean SBP (mmHg)	154.6 ± 11.7	154.5 ± 11.4	154.8 ± 13.5	ns
Drop in SBP 6M (mmHg)	11 ± 19.2	20.6 ± 14.5	-8 ± 11.6	**0.001**
Baseline mean DBP (mmHg)	90.7 ± 14	88.2 ± 13.5	95.7 ± 14.8	ns
Drop in DBP 6M (mmHg)	6.3 ± 9	10.4 ± 7.1	-1.8 ± 6.5	**0.004**
Baseline heart rate (bpm)	71 ± 10	70 ± 9	73 ± 14	ns
6M heart rate	70 ± 10	68 ± 9	76 ± 11	ns
Renal denervation				
Number ablations (n ± SD)	27.2 ± 7.7	28.7 ± 8.1	24.2 ± 6.3	ns
Fluoroscopy time (min)	19.3 ± 11.4	16.3 ± 5.5	26.5 ± 18.6	**<0.04**
Cardiac MIBG scintigraphy				
Baseline HMR 15 min	1.63 ± 0.11	1.59 ± 0.10	1.72 ± 0.08	**<0.02**
6M HMR 15 min	1.64 ± 0.12	1.61 ± 0.10	1.70 ± 0.14	ns
Baseline HMR 4 hours	1.60 ± 0.11	1.59 ± 0.10	1.64 ± 0.14	ns
6M HMR 4 hours	1.60 ± 0.16	1.59 ± 0.12	1.63 ± 0.24	ns
Baseline WR	22.7 ± 18.6	17.9 ± 10	32.2 ± 28.2	ns
6M WR	25.9 ± 16.4	25.4 ± 17.9	27 ± 14.2	ns

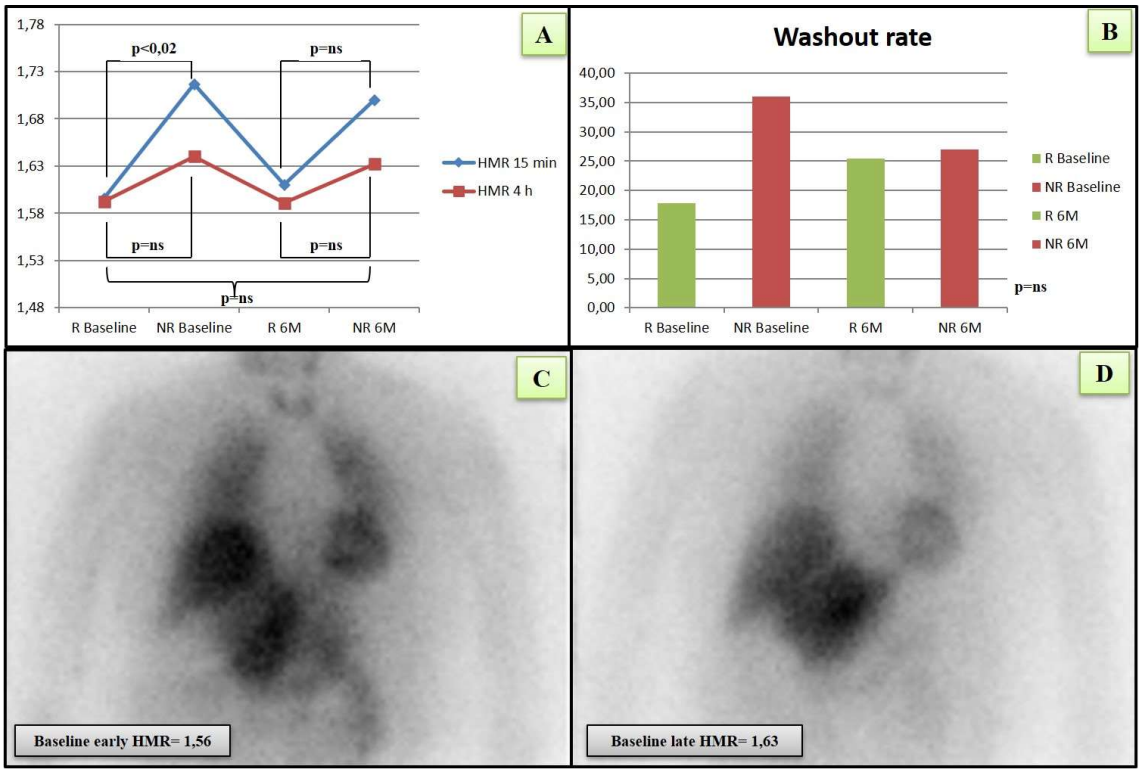
Y: years; HT: hypertension; BMI; body mass index; M: months; IVS: intraventricular septum; PW: posterior wall; LA: left atrium; ABPM: ambulatory blood pressure monitoring; SBP: systolic blood pressure; DBP: diastolic blood pressure; bpm: beats per minute; MIBG: 123I-labelled meta-iodobenzylguanidine; HMR: heart-mediastinum ratio; WR: washout rate. Results are displayed in mean ± standard deviation (SD).

Supplementary table 1: Descriptive results of myocardial MIBG scintigraphy, performed in the renal denervation cohort at baseline and 6 months after the procedure.

	Age (Y)	Gender	HMR 15 min		HMR 4h		Washout rate		Response to RDN
			Baseline	6 months	Baseline	6 months	Baseline	6 months	NR
Patient 1	68	M	1,80	1,73	1,81	1,7	13,28		R
Patient 2	71	M	1,67	1,62	1,71	1,63	15,77	16,68	R
Patient 3	62	M	1,75	1,71	1,67	1,64	23,81	24,96	NR
Patient 4	55	M	1,73	1,63	1,67	1,65	22,72	14,28	R
Patient 5	74	F	1,56	1,55	1,63	1,73	4,11	70,93	R
Patient 6	56	M	1,56	1,60	1,57	1,56	11,68	18,33	NR
Patient 7	45	F	1,58	1,63	1,52	1,47	83,54	40,84	R
Patient 8	55	M	1,51	1,49	1,43	1,38	31,61	33,92	NR
Patient 9	54	M	1,67	1,71	1,56	1,51	29,78	39,12	R
Patient 10	61	M	1,55	1,67	1,62	1,65	10,43	10,50	R
Patient 11	49	M	1,66	1,74	1,56	1,61	27,99	32,54	NR
Patient 12	38	F	1,78	1,96	1,88	2,04	3,64	9,91	R
Patient 13	54	M	1,52	1,57	1,52	1,43	25,06	37,86	R
Patient 14	51	M	1,50	1,50	1,52	1,58	18,73	7,53	R
Patient 15	73	M	1,43	1,45	1,48	1,46	11,24	19,85	R
Patient 16	46	M	1,72	1,77	1,81	1,83	3,11	4,12	NR
Patient 17	48	F	1,74	1,54	1,57	1,49	40,28	31,12	R
Patient 18	49	F	1,72	1,65	1,59	1,59	31,22	28,13	NR

Y: years; M: male; F: female; HMR: heart-mediastinum ratio; RDN: renal denervation; NR: non-responder; R: responder.

Figure 1: Myocardial MIBG scintigraphy in patients submitted to renal denervation (RDN). (A) Early (15 minutes) and late (4 hours) heart-mediastinum ratio (HMR) at baseline and six-months (6M) after RDN, in 'responders' (R) vs 'non-responders' (NR) – early HMR was significantly lower in R, at baseline; (B) Washout rate at baseline and six-months after RDN in R vs NR; (C) and (D) MIBG scintigraphy, thorax anterior projection, at baseline, in a responder, at 15 minutes (C) and at four hours (D).



Discussion

The aim of our study was to determine whether RDN had any impact in myocardial sympathetic activity, and also to assess safety of the procedure, as a significant decrease in HMR afterwards, could signify disruption of the sympathetic pathway. We verified a significant reduction of BP, six months after RDN, in 66.7% of the patients, which is aligned with the reported efficacy of the technique. No safety issues were reported, except for one patient who was diagnosed with renal artery stenosis six months after RDN, probably due to radiofrequency delivery next to a non-significant atherosclerotic plaque. We determined that responders had a significantly lower baseline early HMR, which could be due to decreased neuronal integrity, but no significant changes were observed after six months.

Late HMR was similar in both groups but reduced in comparison to values reported in normal subjects (normal reported values 2.2 ± 0.3 5, local reference values 1.9-2.8), both at baseline and at six-month follow-up, translating a maintained sympathetic hyperactivity even after RDN, and probably being associated with a higher risk of events. WR was also statistically similar in both groups. However, WR was significantly increased overall, in comparison to normal individuals (normal reported mean values 10 ± 9% [5], local reference values 8.5-9.6%), with this discrepancy being more evident in non-responders at baseline, due to a possible sympathetic overdrive.

The SNS is an extremely complex system, with clinical implications in both physiologic and pathological states. It is characterized by multiple levels of action that involve central regulation, ganglionic transmission, release and reuptake of norepinephrine and the response of adrenergic receptors. [7] As such, a precise method to evaluate global and regional sympathetic activity does not exist, with each technique having its strengths and limitations.

The effect of RND on sympathetic activity has been described previously. Krum et al [8] reported a 47% decrease in the release of noradrenaline from the renal sympathetic nerves bilaterally, after RDN, using the isotope dilution renal noradrenaline spillover method. MIBG-S has been performed in small cohorts of RDN patients in order to assess sympathetic activity, but results have been rather divergent, reporting decreases in WR [5], increases in late HMR [9] or no change at all. [10] This imaging method has also been considered useful to evaluate cardiac sympathetic

activity in the context of heart failure, being able to estimate both prognosis and response to treatment. Indeed, in the ADMIRE-HF trial, a significant lower event and cardiac death rate was observed in patients with a late HMR ≥ 1.6. [11]

What is not clear in our study is, given that non-responders had evidence of increased SNS activity, why did they not clinically respond to RDN? Were there other factors/systems superseding the contribution of the SNS in the pathophysiology of HT? Furthermore, none of the evaluated rates altered significantly at follow-up, translating an absence of deleterious sympathetic nerves disruption and, none of the evaluated MIBG parameters were useful to predict response to RDN.

Limitations

Our study has some limitations. First, the number of patients enrolled is small. Second, there was no control group. Third, the study was not randomized and, even though the nuclear medicine specialist was highly experienced, there was no internal or external validation of the results.

Conclusions

In this study we demonstrate that renal denervation significantly reduced blood pressure in a significant percentage of patients, but there was no evidence of reduced cardiac sympathetic activity visible by myocardial [123I] meta-iodobenzylguanidine scintigraphy. None of the imaging parameters were useful to predict response to renal denervation. However, both early and late heart-mediastinum ratio were found reduced/lower, compared to the general population, probably putting this population at a higher risk of events. Large-scale studies are needed to determine the validity of this method in the evaluation of cardiac renal denervation effects.

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Original Article Number 5

Renal Sympathetic Denervation in Resistant Hypertension: the association between Vitamin D and Positive Early Response in Systolic Blood Pressure

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Conflict of interest: none.

Abstract

Aims: Vitamin D deficiency is a common finding and has been suggested to be associated with hypertension. Resistant hypertension is a clinical problem seen in 5% to 30% of hypertensive patients. Renal denervation has been used for patients with resistant hypertension and has been proven to lower blood pressure. Our primary goal was to assess the serum vitamin D serum concentration as a predictor of blood pressure response to RDN in highly selected patients.

Methods: This prospective, nonrandomized, single-centre study included 24 patients treated with renal denervation (RDN). Based on their 1-year response after RDN, patients were classified as responders or non-responders. Based on their 1-year response after RDN, patients were classified as responders or non-responders at 6 months or at 12 months.

Results: The median follow-up was 52 months (range, 14-91 months). After RDN, 17 patients (70.8%) had a reduction >5 mmHg in the mean systolic blood pressure, at the first 6 months of follow-up. At 12 months, 20 patients (83.3%) were responders. Vitamin D levels at baseline (15.1±4.8 vs 24.2±8.8 ng/ml) and at 6 months (16.6±7.2 vs 25±9.2 ng/ml) were lower in early non-responders as compared to early responders (p = 0.008), without significant variation during the follow-up. Even though Vitamin D levels were lower in the total responder's group, no statistically significant differences were found (p=ns).

Conclusion: In patients with resistant hypertension, low vitamin D concentrations were associated with an absence of early response to RDN.

Keywords: *Renal denervation; Resistant hypertension; Vitamin D.*

Abbreviations: *ABPM - Ambulatory Blood Pressure Monitoring; BP – Blood Pressure; DBP – Diastolic Blood Pressure; SBP – Systolic Blood Pressure; HTN – Hypertension; RDN – Renal denervation.*

Introduction

Vitamin D deficiency is a common finding in 30-50% of the general population. [1] Although vitamin D deficiency consequences usually involve pathologies of musculoskeletal system, increasing evidence shows an association with cardiovascular risk factors, including hypertension (HTN). [2] Hypertension (HTN) is a major risk factor affecting the global burden of cardiovascular disease. [1,2] Despite the facts that lifestyle changes and pharmacological treatments reduce blood pressure (BP) and cardiovascular complications in hypertensive patients, the treatment of HTN remains suboptimal worldwide, and is insufficiently controlled in many patients. [3] It is now common knowledge that BP reduction has a favourable impact on prognosis through the reduction of major cardiovascular events such as myocardial infarction, stroke, and cardiovascular death; and hence, the total burden of cardiovascular risk. [4,5]

The true prevalence of so-called 'resistant HTN' is remains unknown, but is reported to range from 5% to 30%. [6-9] According to the current guidelines of the European Society of Cardiology and European Society of Hypertension, resistant hypertension is defined as the HTN of patients for whom the targeted BP values are not achieved despite triple antihypertensive therapy that includes a diuretic administered at the maximum tolerated dosage. [10] Catheter-based renal denervation (RDN) is one of the most frequently used invasive method for the treatment of resistant HTN. Some initial non-randomized studies revealed significant reductions of BP. Nevertheless, the controversial double-blind Symplicity HTN-3 trial did not confirm the superiority of renal denervation compared to a sham procedure and medical therapy. [11] This could be explained by several confounding factors, such as variations in the procedural methods as well as changes in drug regimens after randomization. [12–14] Therefore, the recently published Spyral HTN-OFF MED, and the ongoing Spyral HTN-ON MED could address these issues and show that RDN could be an effective approach to manage resistant HTN. [15,16]

Despite some epidemiological data describing the relationship between vitamin D deficiency and arterial HTN [17], evidence investigating the effect of vitamin D is conflicting. It is still under debate whether vitamin D status has an influence on therapeutical blood reduction. [18]

Here, our group reports the results of a single-centre study of percutaneous RDN applied to patients affected by resistant HTN in daily clinical practice. Our primary goal was to assess the

serum vitamin D serum concentration as a predictor of blood pressure response to RDN in highly selected patients. The secondary goals were to evaluate the safety and long-term effectiveness of RDN for reducing BP, as well as the echocardiographic response to RDN.

Methods

Study Design and Patients

This was a prospective, non-randomized, single-centre study that included 24 patients aged 38 to 77 years with resistant HTN, who were treated by RDN between May 2014 and October 2017.

A comprehensive medical history and a thorough revision of the medication was undertaken. The initial evaluation included 97 hypertensive patients, and 73 were excluded from the study because they did not satisfy the inclusion criteria (Figure 1). The inclusion criteria were as follows: > 18 years of age, presence of idiopathic resistant HTN confirmed by ambulatory blood pressure monitoring (ABPM) (mean BP > 135/85 mm Hg despite a stable medication regimen of maximum tolerated doses of 3 or more anti-hypertensive drugs, including a diuretic), glomerular filtration rate > 45 mL/min/1.73m2 (Modification of Diet in Renal Disease Formula), and compatible renal anatomy (atherosclerotic stenosis < 50%, prior renal artery revascularization, fibromuscular dysplasia, or accessory renal arteries as evaluated by either computed tomography angiography or duplex ultrasonographic scanning of renal arteries).

The exclusion criteria were as follows: HTN due to secondary causes (screening by biochemical and imaging assessments and polysomnography), hemodynamically significant valvular disease, history of stroke or acute coronary syndrome over the past 6 months, refusal to sign informed consent, life expectancy < 1 year, pregnancy, or presence of pseudo-resistant HTN.

All patients were admitted to the hospital 2 days prior to the procedure in order to confirm the presence of ‘true’ resistant HTN by assessing adherence to prescribed medications by witnessed intake of agents. Baseline evaluations included routine blood testing, including Vitamin D concentration, electrocardiography, transthoracic echocardiography, and 24-h Holter monitoring.

The study was approved by the Faculty of Medicine of the University of Coimbra and the Coimbra Hospital and University Centre Ethics Committees (reference CE-031/2014. Every study patient signed an informed consent.

Procedure

All RDN procedures were performed by a cardiologist with expertise in endovascular procedures. The standard percutaneous technique used a 6/8 F introducer sheath to enter the femoral artery. A standard JR catheter was used for a selective bilateral renal angiogram that was performed before and after the procedure. The following 3 treatment catheters were used (ordered according chronological use): Symplicity Flex™ catheter (Medtronic Inc, Santa Rosa, CA, USA [2 patients]), EnligHTN™ (St. Jude Medical, MN, USA [8 patients]) and Symplicity Spyral™ (Medtronic Inc, Santa Rosa, CA, USA [14 patients]) an. A minimum of 4 and a maximum of 24 ablations, that were separated both longitudinally and rotationally, were performed in each renal artery. During the ablation, the catheter system monitored the tip temperature and impedance, and altered the radiofrequency energy in response to a predetermined algorithm. An anaesthesiologist was present for all patients. Conscious sedation (via propofol, midazolam, and/or remifentanil) was commonly induced to prevent and manage visceral pain. Intra-arterial heparin and pre- and post-procedure nitrates were administered during the procedure. Haemostasis was achieved by a vascular closure device.

Follow-up

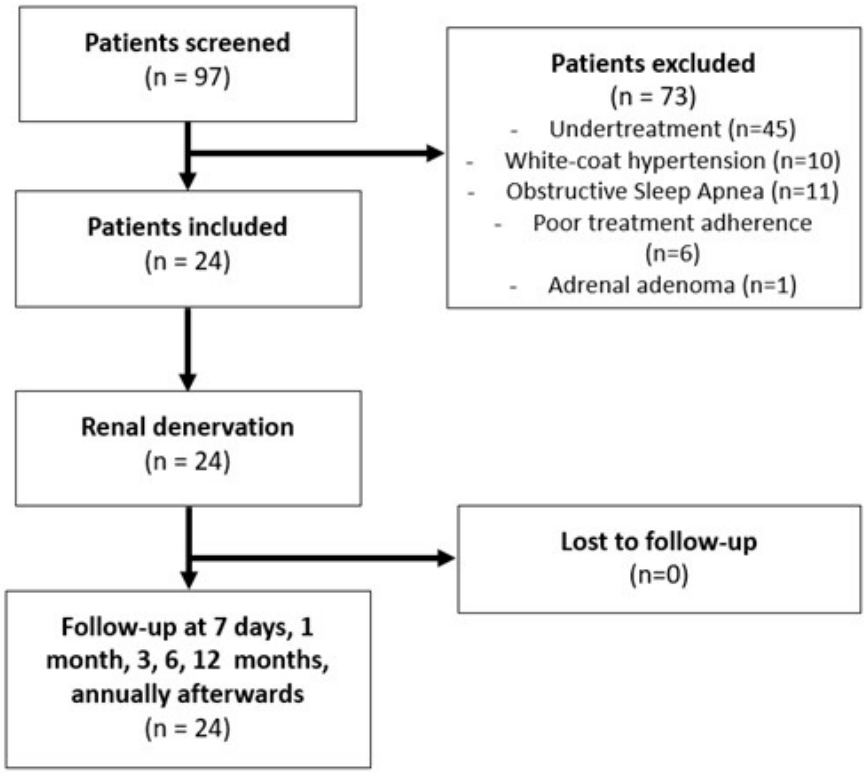
The patients were discharged from the hospital the day after the procedure unless there were immediate complications requiring medical attention. All patients underwent follow-up evaluations at 1 week, 1 month, and 3, 6, 12 months, followed by annual visits. The follow-up evaluations consisted of a clinical examination, BP measurements in both arms, and medication adjustment if deemed necessary. Information on adverse events and treatment compliance was recorded. Additionally, follow-up ABPM, electrocardiography, biochemical analysis, including Vitamin D levels, and transthoracic echocardiography were performed at 6 and 12 months. A renal angiogram was performed at 6 months in every patient to assess safety. The median

duration of follow-up was 52 (range, 14 to 91) months. According to the response to RDN, the patients were classified as responders or non-responders. A responder to RDN was defined as a patient who obtained > 5 mm Hg decrease in the mean systolic BP values determined by ABPM.

Sample calculation and statistical analysis

We planned a study of a continuous response variable, systolic blood pressure (mmHg), from matched pairs of study subjects. Prior data indicate that the difference in the response of matched pairs is normally distributed with standard deviation. If the true difference in the mean response of matched pairs is 13, we will need to study 22 pairs of subjects to be able to reject the null hypothesis that this response difference is zero with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. Categorical variables were characterized by determining the absolute and relative frequencies, and numerical variables the means and standard deviations. Comparisons between groups with regard to the categorical variables were conducted using the Chi-Square Test. Regarding the continuous variables, MannWhitney U Test was used to compare two groups, and Kruskal-Wallis Test between more than two groups. A general linear model for repeated measures was applied to analyse variance of each laboratorial parameter, measured several times on each subject from two different groups, 'responder' and 'non-responder'. Two different group definitions were considered, responders at six months (early) and at one year. Statistical analysis was conducted using SPSS 19.0®, at a 5% significance level for hypothesis-testing.

Figure 1: Flowchart of study design.



Results

Patients' characteristics

A total of 24 patients underwent RDN in our department between May 2014 and October 2017. Based on BP values following the procedure, 20 patients (80.3%) were found to be responders to RDN, of whom 17 (70.8%) were responders at 6 months. Table 1 shows the baseline demographics and clinical characteristics of the responders and non-responders at 6 and 12 months.

Parameters characterizing the RDN procedures

Analysis of the catheter systems used for RDN show that differences between the systems were not significant. The mean number of ablations performed per each RDN was 25.3 ± 9.10 ablations (a minimum of 10 ablations and a maximum of 41 ablations). Table 2 shows the procedure-related parameters. The differences between procedure-related parameters such as the diameters and lengths of renal arteries, number of renal artery branches, and number of ablations in the responders and non-responders at 6 and 12 months were not significant.

Vitamin D Concentration and BP response

The patients who responded by 6 months after RDN had a significantly higher baseline vitamin D concentration than the baseline concentration of patients who were nonresponders at 6 months after RDN (15.1±4.8 vs 24.2±8.8 ng/mL, respectively), which was also observed at the 6-month follow-up (16.6±7.2 vs 25±9.2 ng/mL, respectively; p = 0.008). The differences between the vitamin D concentrations of responders vs. non-responders at the 12-month follow-up were not significant. However, the responders at 12 months had higher mean vitamin D levels than those of the non-responders at 12 months both at baseline (15.6 ± 5.1 vs. 23.0 ± 9.0 ng/mL, respectively) and at 6 months after RDN (17.3±7.6 vs 23.8±9.5 mL, respectively). The Receiver Operating Characteristic curve-derived optimal cut-off for determining response to RDN was ≥ 19.5 ng/mL (sensitivity 63.3%, specificity 81.8%; p=0.011) (Figure 2).

Renal Denervation Efficacy

We observed a significant decline in the mean 24 h-systolic BP (SBP) (+10.2 mmHg vs -22.3 mmHg; p=0.007) and 24 h-diastolic BP (DBP) (+5.7 mmHg vs. -13.0 mmHg; p=0.026) in the total responders group, with significantly lower values compared to the non-responders group (Figure 3 and Figure 4). The same findings were detected in both diurnal and nocturnal SBP and DBP values. The differences between the number of antihypertensive agents used in each group at baseline and at 6 and 12 months of follow-up were not significant.

Regarding long-term follow-up, for a median period of 52 months, seventeen patients (70,8%) remained responders after RDN, one patient died (previously non-responder; cause of death unknown - 4,2%), two responders required a new renal denervation, due to BP severe re-elevation after 18 months follow-up, with a positive BP response (8,3%) and four patients remained non-responders (16,7%).

Complications

In terms of safety, there was a new renal artery stenosis reported in one patient at 6-month follow-up. He was a 46-year-old male patient, who had severe refractory hypertension with target-organ lesion (hypertensive nephropathy, stage 3 chronic kidney disease) and baseline atherosclerotic plaques were identified during baseline renal angiography. The Spyral system was used and a total of 30 ablations were applied (11 on the left renal artery and 19 on the right). No vasospasm was reported post-procedure. At 6-month follow-up, the patient presented with flash pulmonary oedema, which was treated with stent angioplasty following new renal artery stenosis identification (right renal artery, proximal portion, 75% stenosis). The patient had a significant BP-reduction after treatment. No other complications were observed.

Impact on Echocardiographic Variables

The differences between the variables of the non-responders at baseline and 6 months after RDN were significant as follows: E/e' septal (17.7±2.3 vs 13.8±1.2, respectively; p=0.045), E/e' lateral (13.3±2.4 vs 10.6±1.9, respectively; p=0.044), and E/e' mean (14.6±2.6 vs 11.8±1.8,

respectively; p=0.033). No differences between variables were observed between the responders, vs the non-responders. Furthermore, a non-significant decline was detected in the non-responders at 12 months group (Table 3).

Table 1: Clinical baseline characteristics of studied patients according to responsiveness to renal denervation.

		6-month follow-up			1-year follow-up		
Characteristics	**Total (N=24)**	**Responders (N=17)**	**Non-responders (N=7)**	**p-value**	**Responders (N=20)**	**Non-responders (N=4)**	**p-value**
Age - yr	59±11	61±10	55±13	0,247	60±11	56±9	0,482
Male sex - n (%)	15 (63)	12 (71)	3 (43)	0,356	12 (60)	3 (75)	1
Time since arterial hypertension diagnosis -yr	17±7,9	17,1±8,8	17±5,9	0,898	17,2±8,2	16,3±7,5	0,907
Body-mass index	29,7±3,9	29,5±3,8	30,2±4,5	0,691	30,1±3,8	27,9±4,4	0,491
Diabetes - n (%)	10 (42)	6 (35)	4 (57)	0,393	8 (40)	2 (50)	1
Insulin therapy- n (%)	4 (40)	2 (33)	2 (50)	1	2 (25)	2 (100)	0,133
Smoking - n (%)	5 (21)	2 (12)	3 (43)	0,126	2 (10)	3 (75)	0,018
Hypercholesterolemia - n (%)	22 (92)	15 (88)	7 (100)	0,569	18 (90)	4 (100)	1
Coronary artery disease – n (%)	3 (13)	2 (12)	1 (14)	1	2 (10)	1 (25)	1
Previous myocardial infarction – n (%)	2 (8)	2 (12)	0 (0)	0,569	2 (10)	0 (0)	1
Previous stroke or TIA – n (%)	5 (21)	4 (24)	1 (14)	1	5 (25)	0 (0)	0,544
Obstructive sleep apnea – n (%)	15 (63)	11 (65)	4 (57)	1	13 (65)	2 (50)	1
CPAP – n (%)	10 (67)	9 (82)	1 (25)	0,077	9 (69,2)	1 (50)	1
Chronic Kidney Disease – n (%)	2 (8)	1 (6)	1 (14)	1	1 (5)	3 (75)	0,312
Atrial fibrillation – n (%)	24 (100)	17 (100)	7 (100)	NA	20 (100)	4 (100)	NA
Pacemaker – n (%)	24 (100)	17 (100)	7 (100)	1	20 (100)	4 (100)	1
Symptomatic – n (%)	17 (71)	11(65)	6 (86)	0,384	14 (70)	3 (75)	1
Number of antihypertensive medication – n (%)	5,3±1,1	5,2±1,3	5,4±0,5	0,084	5,25±1,2	5,5±0,5	0,032
Spironolactone – n (%)	14 (59)	11 (65)	3 (43)		12 (60)	2 (50)	
Calcium antagonist – n (%)	24 (100)	17 (100)	7 (100)		20 (100)	4 (100)	
Angiotensin-receptor blocker – n (%)	17 (71)	11 (65)	6 (86)		14 (70)	3 (75)	
Angiotensin-converting-enzyme inhibitor - no(%)	10 (42)	8 (47)	2 (29)		8 (40)	2 (50)	
Thiazide - no(%)	18 (75)	13 (77)	5 (71)		16 (80)	2 (50)	
Loop Diuretic - no(%)	6 (25)	4 (24)	2 (29)		4 (20)	2 (50)	
Beta-Blocker - no(%)	19 (79)	13 (77)	6 (86)		16 (80)	3 (75)	
Alpha2-adrenergic agonist - no(%)	15 (63)	10 (59)	5 (71)		12 (60)	3 (75)	

Table 2: Characteristics of renal denervation procedure, according to blood pressure response.

Characteristics	Total (N=24)	6-month follow-up			1-year follow-up		
		Responders (N=17)	Non-responders (N=7)	p-value	Responders (N=20)	Non-responders (N=4)	p-value
Radio-frequency renal denervation system							
EnligHTN™- no(%)	8 (33)	6 (35)	2 (29)		6 (30)	2 (50)	
Spyral™- no(%)	14 (58)	10 (59)	4 (57)		12 (60)	2 (50)	
Symplicity Flex™- no(%)	2 (8)	1 (6)	1 (14)		2 (10)	0	
Maximal diameter right renal artery (mm)	6,3±1,5	6,3±1,6	6,2±1,4	0,975	6,2±1,5	6,6±1,8	0,51
Minimal diameter right renal artery (mm)	4,9±1,4	4,8±1,5	5±1,2	0,484	4,8±1,4	5,5±1,4	0,188
Length right renal artery (mm)	49,9±20	47,7±18,8	55±23,6	0,431	50,3±19,4	47,4±26,2	0,796
Right renal artery ablations – (n)	12,3±4,3	12,8±4,6	11±3,5	0,374	12,5±4,6	11±2,7	0,538
Maximal diameter left renal artery (mm)	6,3±1,5	6,3±1,6	6,1±1,5	0,634	6,1±5,1	6,2±5,6	0,583
Minimal diameter left renal artery (mm)	5±1,5	4,8±1,5	5,5±1,3	0,27	4,8±1,4	6,1±1,5	0,113
Length left renal artery (mm)	42,3±17	44,1±17	39,3±17,8	0,542	44,5±16,9	33,7±16,1	0,254
Left renal artery ablations – (n)	13,2±5,6	13,8±5,8	11,7±5,1	0,426	13,8±6,0	10,3±1,5	0,293
Total ablations (n)	25,3±9,10	26,5±9,67	22,4±7,35	0,326	26,2±9,61	20,7±4,03	0,088
EnligHTN™ (n)	21,1±5,8	20,7±6,7	22,5±2,1		20,7±6,7	22,5±2,1	
Spyral™ (n)	28,9±7,6	30,7±7,3	25±7,8		30,1±6,7	15,8±1,6	
Symplicity Flex™ (n)	11±1,41	10	12		11±1,4	NA	

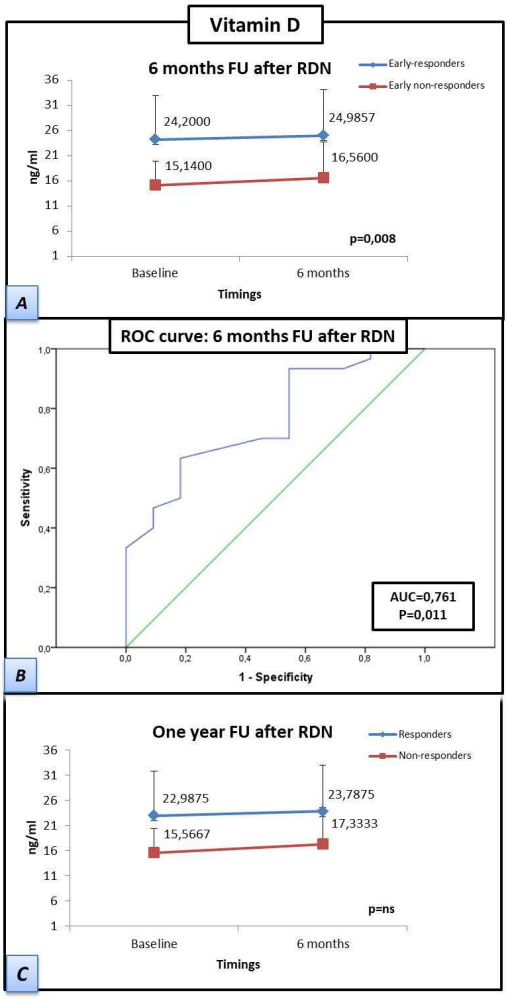


Figure 2: Early-responders to renal denervation have significant higher levels of vitamin D than early non-responders. Samples were obtained at two timings (baseline and at 6-month follow-up). (A) Estimated marginal means of vitamin D levels in 'responders' at six months (early-responders, blue line) vs 'early non-responders' (red line). (B) Operating characteristic curve (ROC): A vitamin D cut-off value of ≥19.5 ng/ml showed the best overall sensitivity and specificity for determining renal denervation response. (C) Estimated marginal means of vitamin D levels in 'responders' at one year (blue line) vs 'non-responders' (red line). Statistical analysis was performed using the Kruskal-Wallis Test and a general linear model was applied to analyze variance of each laboratorial parameter. Data are presented using mean ± standard deviation. *FU: follow-up; RDN: renal denervation; AUC: area under the curve.*

Figure 3: 24-hour systolic blood pressure (SBP) in 6-month responders and non-responders at baseline, 6 months and 12 months after renal denervation.

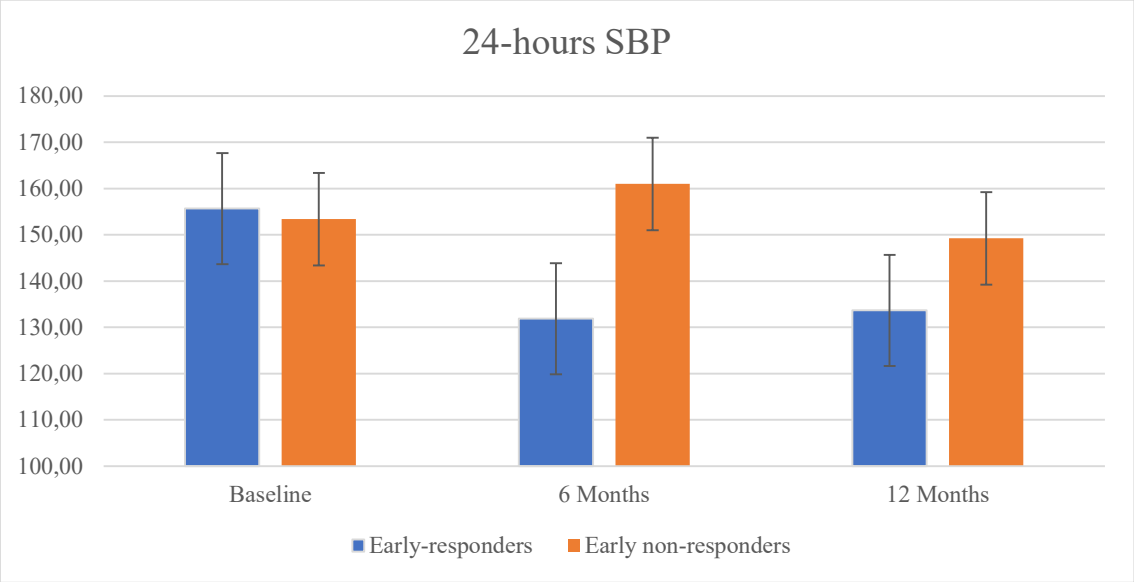


Figure 4: 24-hour systolic blood pressure (SBP) in 12-month responders and non-responders at baseline, 6 months and 12 months after renal denervation.

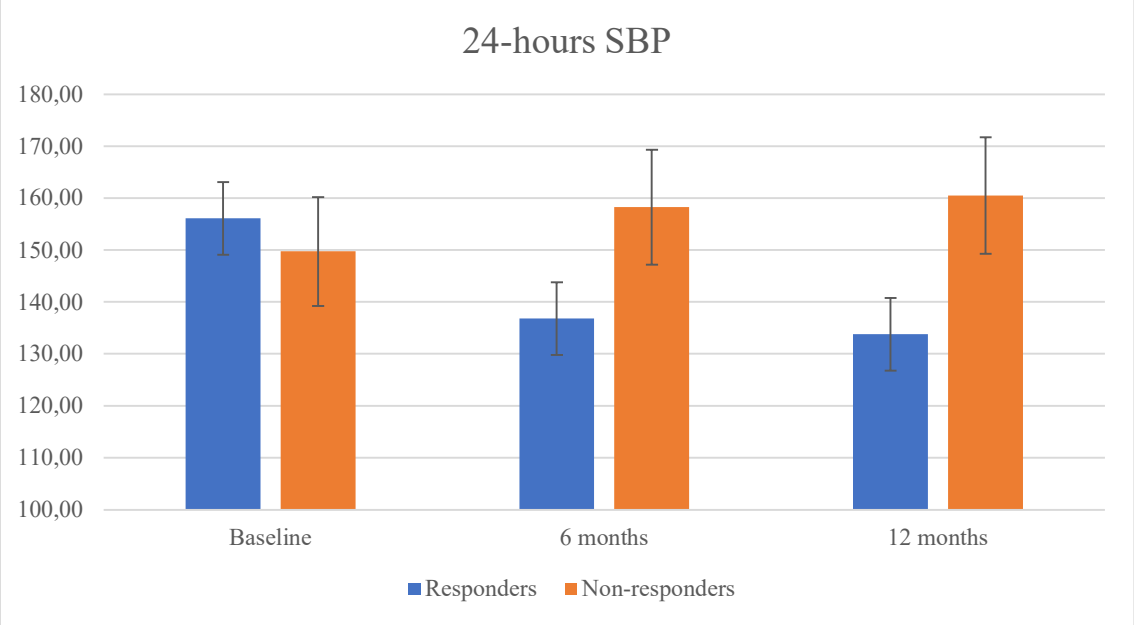


Table 3: Echocardiogram characteristics, according to responsiveness to renal denervation at baseline and 6-month follow-up.

Characteristics	Early- Responders (N=17)	Early non-responders (N=7)	p-value	Total Responders (N=20)	Non-responders (N=4)	p-value
E/e' septal at baseline	12.9±5.4	17.7±3.8	ns	14.0±5.6	16.5±4.4	ns
E/e' septal at 6 months	12.9±7.6	13.8±1.2	ns	13.1±7.0	13.5±3.4	ns
p-value	ns	**0.045**		ns	ns	
E/e' lateral at baseline	9.7±2.9	13.3±2.4	ns	10.7±3.6	11.2±2.6	ns
E/e' lateral at 6 months	9.8±3.8	10.6±1.9	ns	10.3±3.6	9.2±2.8	ns
p-value	ns	**0.044**		ns	ns	
E/e' mean at baseline	10,6±3,9	14,6±2,6	ns	11,4±4,3	13,8±3,5	ns
E/e' mean at 6 months	11,1±4,8	11,8±1,8	ns	11,3±4,5	11,3±3,1	ns
p-value	ns	**0,033**		ns	ns	

Discussion

In this study, we performed a prospective analysis of the BP-reducing effect of RDN in our centre, evaluating the predictor value of vitamin D in RDN response. We observed a reduction in the 24-h mean SBP and mean DBP in 83% of the 24 patients. The reduction in BP was comparable with the findings of recent randomized sham-controlled trials. We also observed a significantly higher baseline vitamin D level in the patients who responded within 6 months after RDN group compared to the patients who did not respond by 6 months after RDN non-responders. At 12 months after RDN, higher vitamin D values continued to be observed in the patients who responded to RDN, but the difference between the vitamin D levels of the groups was not significant.

Low vitamin D levels have been found to be associated with arterial HTN in cross-sectional studies. [19–21] A prospective study found that vitamin D deficiency was associated with an increased risk of HTN, independent of age, body mass index, and other covariates. [22] Likewise,

observational studies have supported the association between low levels of vitamin D and the presence of resistant HTN. [22–24] Various underlying mechanisms to explain this relationship have been discussed. The vitamin D receptor is broadly expressed by cardiovascular tissues that include endothelial cells, cardiomyocytes, and vascular smooth muscle cells. [25–27] Vitamin D also supresses the expression of the renin gene, inhibits the proliferation of vascular smooth muscle cells, and is associated with increased endothelial-dependent vasodilation and reduced cytokine release from lymphocytes. Thus, an association between vitamin D deficiency and HTN may be plausible. Likewise, a recent cross-sectional study found a statistically significant association between vitamin D deficiency and resistant HTN. [28] Although there have already been several randomized controlled trials that have examined the effect of vitamin D supplementation on HTN [29–31], there is only a single randomized controlled trial that has assessed the effects of vitamin D supplementation on individuals with resistant HTN. The result was negative. [28] However, the study only included 68 patients (34 in each arm), including a few patients with type 2 diabetes. Previous studies have revealed that patients with type 2 diabetes who were administered vitamin D obtained reduced BP. Interestingly, in our cohort, the patients who responded after RDN had lower haemoglobin A1C levels than the patients who did not respond to RDN. The results of several studies have suggested an association between the lack of vitamin D and changes in the levels of blood glucose and insulin and the sensitivity of cells to insulin. [32] Moreover, an inverse linear relationship between HbA1C levels and vitamin D levels has been reported. [32]

In our study, the patients who responded by 6 months after RDN showed a significantly higher baseline vitamin D level compared to the patients who did not respond by 6 months, which was not significant 1 year after RDN, although the patients who had responded continued to show a higher mean concentration of vitamin D at 1 year. The association between low vitamin D concentrations with a decreased response to RDN was previously reported by Poss et al in a single retrospective study. [33] Nevertheless, they only reported a follow-up of 6 months, with no further evaluations. To our best knowledge, ours in the first study to report a lower vitamin D level in patients responding by 6 months than in patients not responding by 6 months after RDN. To date, it remains unclear whether vitamin D supplementation of patients with resistant HTN can affect the magnitude or timing of responsiveness to renal RDN. Our findings were observed without the potential effects of calcium supplements that were neither introduced nor

discontinued during this study. Our findings must be considered preliminary only. Additional prospective studies with a larger population of patients are warranted.

We also reported that the patients who did not respond by 6 months after RDN showed a decrease in the E/e' septal, E/e' lateral, and E/e' mean values, whereas the patients who did respond by 6 months after RDN did not show a decrease. Most echocardiographic studies find evidence of improved diastolic function after RDN, with divergent results in the diastolic parameters, while others have not been able to find differences between diastolic function over time. [34] Additionally, some studies have revealed the beneficial impact of sympathetic modulation on diastolic function without significant association with changes in BP over time. [35,36] Interestingly, these studies only considered the patients who were responders, who obtained reduced BP 6 months after RDN. A recent multicentre study found significantly improved ventricular global longitudinal strain, which is a surrogate for diastolic myocardial function, in patients with heart failure with preserved ejection fraction who underwent RDN. [37] Therefore, additional studies are needed to determine if RDN could be a treatment option for patients with heart failure with preserved ejection fraction independent of BP response. Likewise, several studies confirmed reduction in the left ventricular mass after RDN, independent from changes in BP. [35,38] Similar beneficial effects are also suspected for glucose metabolism, obstructive sleep apnoea, heart failure, and cardiac arrhythmias. [39,40] In our cohort, the LV mass index was not measured. In another Portuguese single centre registry of renal denervation, which included 65 patients submitted to RDN, de Sousa Almeida et al. reported a reduction in LV mass evaluated in both responders and non-responders at 1-year follow-up. [41] However, in non-responders, no statistical significance was reached most probably due to small sample size. Regarding diastolic echocardiographic parameters, no significant changes were reported, specifically mitral E/E' ratio. Nevertheless, the authors did not differentiate between responders and non-responders. Therefore, the data presented in our study about RDN impact on diastolic function should be considered hypothesis generating.

Limitations

Our study has several limitations. It was a single centre prospective study with a small sample, there was no control group, and it was not blinded, neither for RDN (no control group) nor for the physicians performing the follow-up echocardiograms. Additionally, different devices were used for RDN, and no specific techniques were used to control patient adherence to medication.

Conclusion

This single-centre study of patients with resistant hypertension found that renal denervation was associated with significant reduction in both the systolic and diastolic 24-h ABPM blood pressure. There was evidence suggesting a link between vitamin D levels and blood-pressure response within 6 months after RDN. Randomized trials should further clarify if the normalisation of low vitamin D levels by vitamin D supplementation could play a role in the responsiveness of BP to RDN.

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Part III: Discussion and Future Perspectives

Abbreviation List

HT: Hypertension

SNS: Sympathetic nervous system

BP: Blood pressure

RDN: renal denervation

ABPM: Ambulatory blood pressure monitoring

RF: Radiofrequency

OCT: Optical coherence tomography

MIBG: ^{123}I-labelled meta-iodobenzylguanidine scintigraphy

WR: Washout rate

HMR: Heart-mediastinum ratio

DN: Double-negative

RANTES: Regulated on activation, normal T cell expressed and secreted

RAAS: Renin-angiotensin-aldosterone system

ATR1: Angiotensin type 1 receptor

RNA: Ribonucleic acid

Discussion

Hypertension (HT) is one of the most prevalent cardiovascular risk factors and has been the focus of many researchers is the last century, in order to elucidate its pathophysiologic background. The vasoconstrictor effects of the sympathetic nervous system (SNS) and its implication in the pathogenesis of HT have long been known and were the basis to develop surgical techniques in the early 20th century [1], as malignant HT, if left untreated, was associated with near 100% 5-year mortality. However, these aggressive procedures were discontinued due to severe side effects (severe orthosthatic hypotension, intestinal disturbances, urinary incontinence, anydrosis and erectile dysfunction) and high mortality.

Elevated blood pressure (BP) is a known cause for cerebrovascular disease, coronary artery disease, heart failure, chronic kidney disease and peripheral artery disease. Despite the wide availability of anti-hypertension medication and significant advances in the management of HT, many patients, without an evident secondary cause, have persistent uncontrolled HT with consequent end-organ damage. The development of device-based interventional therapies, such as renal denervation (RDN), was based on the same pathophysiological concept as the abandoned surgical sympathectomy, and consists on the ablation of afferent and efferent renal nerves that conglomerate around the renal vasculature. Stimulation of renal nerves raises BP through vasoconstriction and volume/sodium mechanisms [2] and thus, the disruption of this nervous network has been shown to reduce BP and assessed in several randomized trials. [3-5]

However, in 2014, the clinical development of RDN was interrupted due to the unexpected findings of the SYMPLICITY HTN-3 trial. [6] This sham-controlled trial included 535 patients with resistant HT who were randomly assigned to undergo RDN with a single-tip electrode catheter or a sham procedure. Six-month results showed no significant differences between RDN and the sham groups regarding both systolic office BP and mean systolic ambulatory BP monitoring (ABPM). Several pitfalls were identified in the trial which included the Hawthorne effect, non-adherence to medication, inadequate patient selection, unidentified secondary HT, insufficient interventionist experience and lack of a standardized treatment procedure leading to incomplete denervation. In the following year, the DENERHTN trial was published and revealed opposite results. [7] This French randomized study included 106 patients and added a

standardized stepped-care antihypertensive treatment to RDN, which was performed with a single electrode catheter. At 6 months, patients in the RDN group had a modest but significant decrease in daytime systolic ABPM with a mean baseline-adjusted difference between the two groups of -5.9 mm Hg, p = .0329. Similar results were observed for nocturnal and 24-hour mean BP (without reaching statistical significance). Multivariate analysis was performed and revealed that male gender, high adherence to medication, high baseline daytime systolic ABPM, large changes in daytime ambulatory heart rate from baseline to 6 months, but not race, were independently and significantly associated with greater changes in daytime systolic ABPM. Interestingly, the number of ablations was not a predictor of response.

Current scientific and clinical evidence

We published in 2016, in the *Journal of the American Society Hypertension*, a Review Article which gave an overview of the status of RDN at the time and summarized available results of randomized trials. Since then, important randomized and non-randomized data have been published.

The RDN technique has been revised due to anatomical considerations. The penetration depth of commercially available RDN devices ranges from 2-5mm. Human anatomical findings showed that that the mean distance from renal artery lumen to nerve location is least in the distal segments compared to proximal and middle segments of the artery, even though nerve density is higher at this location. [8] Additionally, the renal ganglionic plexus is anatomically interconnected with the superior mesenteric ganglion, the contralateral aorticorenal ganglion, the lumbar sympathetic chain, and the thoracic splanchnic nerves,[9] which may lead to undesirable side effects if the radiofrequency (RF) energy is applied solely at this region. According to this knowledge, several preclinical studies identified RDN distal targeting to be superior when comparing to main artery treatment only. [10]

Recently, three carefully designed second generation randomized trials found a meaningfull reduction both in office and ABPM after device-based RDN. The Spyral HTN Global Clinical trial program was a series of studies designed to investigate the multielectrode Spyral catheter (Medtronic) in hypertensive patients with (SPYRAL HTN-ON MED [11]) and without (SPYRAL HTN-OFF MED [12]) anti-hypertensive medication. Both studies included patients with mild to moderate

HT (patients with office systolic BP > 180mmHg and systolic ABPM > 170mmHg were excluded). A toxicology analysis was performed to monitor drug adherence and BP-lowering efficacy was based on ABPM. The first 80 patients enrolled in the SPYRAL HTN OFF-MED trial had a significant 3-month reduction in systolic ABPM (5.5mmHg, p= 0.0031), when compared to the sham group. Furthermore, a higher 24-hour heart rate at baseline was a predictor of a greater BP reduction after RDN, potentially being a predictor of response in untreated individuals. In the SPYRAL HTN ON-MED trial, 80 patients treated with one to three anti-hypertensive drugs were randomly assigned to receive RDN vs a sham procedure. The RDN group had a significant reduction of systolic ABPM and an interesting finding, similar to the DENERHTN trial [7], was that only 60% of the patients was fully adherent to the prescribed anti-hypertensive therapy. The RADIANCE-HTN trial investigated the superiority of an ultrasound-based RDN device and found a significant daytime systolic ABPM reduction in the RDN group, vs the sham group, in the absence and presence of anti-hypertensive therapy, both at 6 and 12-month follow-up. [13]

The Global Symplicity Registry is the largest prospective, open-label, single-arm, observational registry to date and includes patients with uncontrolled HT and/or conditions associated with SNS activation. At 3-year follow-up, 2237 patients had been enrolled at 196 active sites in 45 countries worldwide. Systolic BP reduction after 3 years was sustained both regarding office BP (-16.5 ± 28.6mmHg, p<0.001) and ABPM (-8 ± 20mmHg, p<0.001). The impact on BP reduction after RDN was more pronounced in patients with severe resistant HT (with baseline systolic office BP ≥ 160mmHg and systolic ABPM ≥ 135mmHg, on three or more antihypertensive drugs). In multivariate analysis, a higher baseline systolic BP was consistently associated with BP decrease at 12, 24 and 36 months after RDN. Regarding safety, at 3 years, mortality was 4% (2% cardiovascular death), 3.2 % of the patients had a stroke, 2.6% were hospitalized due to a hypertensive crisis and 1.6% developed end-stage renal disease. At one year, 3 patients (0.1%) were diagnosed with renal artery stenosis and two of them were treated with stent angioplasty. [14]

Heart failure is characterized by a chronically active SNS. In the early stages of the disease, SNS activation increases sodium and volume retention in the kidneys, activates renin-angiotensin-aldosteron system (renal efferent sympathetic pathways) and causes peripheral vasoconstriction, in order to increase stroke volume and maintain mean arterial pressure. However, these compensatory mechanisms progressively lead to a downregulation of cardiac

beta-adrenoreceptors and myocardial injury and therefore contribute to the development of cardiac dysfunction. By interfering with both efferent and afferent sympathetic signals, RDN may be beneficial in heart failure, through the suppression of sympathetic outflow to the heart, kidneys and vessels. A recent metanalysis studied the effect of RDN on heart failure and comprised 5 single-center, open-label, randomized studies which included 177 patients with reduced ejection fraction and receiving standard heart failure therapy. A significant improvement in NYHA class, left ventricular function and exercise capacity was noted in the RDN arm, vs control, without significant impact on BP or renal function. The fact that BP was not significantly reduced is benefical as most heart failure patients have normal/low BP and hypotension may adversely affect prognosis. [15]

Renal nerves distribution and anatomical considerations

Post-hoc analysis of the procedural data of HTN-3 trial revealed that only a small fraction of renal arteries showed evidence of fully circunferencial 4-quadrant ablations. [16] Early investigations revealed that sympathetic fibers are mainly located in the adventitia of renal vessels with larger bundles in the proximal segment, compared to the thinner fibers found distally. [17] However, more recent research on renal nerve distribution showed that even though sympathetic nerve fibers are concentrated in the proximal and middle arterial segments, the distance from the lumen is significantly higher than that of distal fibers, which are fewer but probably more vulnerable to RF injury. [8] Second-generation systems use multiple electrodes to simultaneously ablate multiple areas along the renal artery. Each of these devices provide unique ablation patterns, different from that of single-electrode first generation systems, and offer a more controlled and effective ablation of renal fibers.

We first performed RDN, using a multielectrode device, in a swine model, in order to evaluate the effect of RF energy application on the proximal/middle segment of the renal artery (prebifurcation), through histological analysis of the arterial wall and periadventitial tissue. We compared the levels of fibrosis with the untreated contralateral side (control) and assessed the disruption of the arterial wall acutely (pre procedure/post procedure/one month follow-up) using intrarenal optical coherence tomography (OCT). Levels of renal tissue epinephrine and norepinephrine were quantified at both sides. Our study demonstrated several main findings:

(1) RDN causes acute vessel wall changes, visualized by OCT, such as intimal disruption (edema/spasm) and intraluminal thrombus formation. (2) RDN was safe in the medium term, as OCT revealed a completely healed vessel and the absence of *de novo* stenosis at one month follow-up. (3) The histological analysis revealed nearly absent tyrosine hydroxylase immunostaining and a statistically significant increase in the amount of collagen fibers in the denervated artery, compatible with a decrease in nerve terminals and an increase in fibrosis, compared to the control, suggesting an efficacious delivery of RF energy to the vessel wall. No differences were found in the norepinephrine or epinephrine renal tissue levels between the treated and contralateral kidney, which could be due to contralateral adaptive mechanisms and the renorenal reflex. This finding validates the need to denervate both sides systematically. In accordance to our study, Mahfoud et al [18] demonstrated that delivering RF to the branches resulted in greater norepinephrine reductions than treating the main renal artery alone and that the greatest decline in norepinephrine concentrations was obtained from a combined treatment of the main artery and branches. Our results provided imaging and histopathological evidence of the effect of unilateral RDN, perfomed with a multielectrode device, in a swine model.

Assessing the effects of renal denervation with myocardial ^{123}I-labelled meta-iodobenzylguanidine scintigraphy

The function of several organs, like the heart, vasculature, and the kidneys, is controlled by the SNS, through an efferent nerve stream. The SNS activity is not constant or predictable and is modulated by an afferent sympathetic outflow and also by the contralateral kidney. The SNS is involved in the pathogenesis of multiple clinical entities associated with marked sympathetic cardiovascular drive, such as advanced heart failure, obesity, and sleep apnea syndrome, and increases the risk of life-threatening cardiac arrhythmias and sudden death. [19-20] Sympathetic activity is highly complex as it functions at several levels of the body. Therefore, there is no single method to obtain an overall activity assessment.

In the second part of our investigation, we assessed cardiac sympathetic activity in eighteen patients with resistant HT and submitted to RDN, using myocardial ^{123}I-labelled meta-iodobenzylguanidine scintigraphy (MIBG-S). Cardiac MIBG-S has been performed in small cohorts of RDN patients with resistat HT but results have been rather divergent. Donazzan et al

evaluated eleven RDN patients and reported a borderline decrease in the washout rate (WR) both in responders and non-responders, a parameter which is independent of the number of neurons avalilable and in close relation with sympathetic activity. [21] Berukstis et al [22] investigated sixteen RDN patients and observed a significant increase in the delayed heart-mediastinum ratio (HMR), indicating a reduction in the sympathetic overdrive. Ziakas et al [23] performed myocardial MIBG-S in seven RDN patients and observed that none of the imaging indexes (HMR and WR) were different between *responders* and *non-responders*. MIBG-S has been employed to evaluate patients with heart failure undergoing cardiac ressynchronization therapy and has shown that HMR is both an independent predictor of response [24] and a predictor of clinical outcomes, in this cohort of patients. [25]

The aim of our study was to determine whether RDN had any impact in myocardial sympathetic activity and also to assess safety of the procedure, as a significant decrease in HMR afterwards, could signify disruption of the sympathetic pathway. We determined that *responders* had a significantly lower baseline early HMR, which could be due to decreased neuronal integrity, but no significant changes were observed after six months. Late HMR was statistically similar in both groups but reduced in comparison to values reported in normal subjects (normal mean values 2.2 ± 0.3), both at baseline and at six-month follow-up, translating a maintained sympathetic hyperactivity even after RDN and a probable higher risk of events. The WR was also statistically similar in both groups. However, WR was significantly increased, in comparison to normal individuals (normal reported mean values 10 ± 9%), with this discrepancy being more evident in *non-responders* at baseline, due to a possible sympathetic overdrive. What is not clear is, given that *non-responders* had evidence of increased SNS activity, why did they not clinically respond to RDN? Are there other factors/systems superseding the contribution of the SNS in the pathophysiology of HT? The fact is that the sympathetic network is only a part of the SNS and there may be sympathetic compensatory mechanisms which could prevent a clinical response in particular patients. Furthermore, none of the evaluated rates altered significantly at follow-up, suggesting an absence of deleterious sympathetic nerves disruption. We concluded that none of the evaluated MIBG-S parameters were useful to anticipate clinical outcomes after RDN and, up to current day, there is no single method sensitive and specific enough to predict response to this technique.

Immune response in renal denervation

Patient selection, technical aspects and operator experience have been thoroughly discussed in the literature in order to determine which factors could potentially identify the *responder* to RDN, aspect that would help redefine the indications to this therapy and improve efficacy. A blood-circulating biomarker could be of particular interest to identify the *responders* and could be a marker of renal function, hemodynamics, vascular stiffness or inflammation.

In the third part of our research, we aimed to evaluate the cellular immune response in a cohort of 23 patients with resistant HT and submitted to RDN. The participation of inflammation and, in particular, adaptive immunity, in the elevation of BP is known to be an elaborate interaction between activated immune cells, oxidative stress and angiotensin II activity, promoting an inflammatory status in the kidney, arteries and central nervous system. [26-27] T lymphocytes are one of the most important effector cells of the adaptive immune system and are known to be directly involved in the pathogenesis of angiotensin II induced HT. [28] Adrenergic stimulation may induce renal immune cell infiltration and the production of noradrenaline and proinflammatory cytokines by T lymphocytes, macrophages and dendritic cells. [29] A potential effect of RDN is the reduction of renal inflammation through the modulation of the SNS but the true impact of this technique on the immunological response of patients with resistant HT is unclear.

To study the behavior of immune cells, venous blood samples were obtained at six timings (pre and post procedure). We performed a preliminary assay including samples of four *responders* and three *non-responders*, and executed an extended analysis of T, B and natural killer cells, monocytes and dendritic cells. These results guided the forthcoming study in order to select T cells (CD4 and CD8, memory and activated subsets) as possible biomarkers of response in RDN. We included twenty-three patients and response at six months and one year was determined, in order to include *late-responders*.

Our research identified, for the first time, a potential immunological cellular biomarker and a set of parameters that may be the jump-start to the characterization of the immunological behavior of the *responder* to RDN. So far as we know, this is the first study to assess the immune cell population in patients with resistant HT submitted to RDN. There were several main findings: (1) *Non-responders* at one year showed significantly elevated levels of activated double-negative (DN) T cells at all timings, when compared to *responders*, a difference not observed when

response was evaluated at six months, eventually because *late-responders* show a similar immunological pattern to earlier *responders*, and were part of the *non-responder* group at that time. The mechanisms leading to an early vs. a late response are not clarified. Several studies report the involvement of self-reactive, pro-inflammatory DN T cells in systemic inflammation and tissue damage, and their role in the pathogenesis, symptom onset and disease activity in autoimmune diseases is established; (2) *Non-responders* presented with much higher baseline levels of activated DN T cells, a finding that could be related to a much more exuberant inflammatory systemic response and a major contribution of other mechanisms, other than the SNS, to the perpetuation of HT; (3) DN T cells levels were reduced after RDN overall, with the levels in *responders* being significantly lower. Several studies report that DN T cells secret several cytokines and chemokines such as IL-10 and IFN-γ, which may exhibit both regulatory and pro-inflammatory effects, and also IL-1, IL-3 and IL-17, which are pro-inflammatory. [30] As the SNS is a major intercommunicating pathway between the central nervous system and the immunological system, and adrenergic stimulation leads to an increased pro-inflammatory cytokine production by several immune cells, one possible explanation for the observed decrease in activated DN T cells after RDN is the blunted sympathetic activity, much more significant in *responders* but most certainly also present in *non-responders*, leading to a decrease in the stimulation of DN T cells and therefore their absolute levels; (4) Finally, there was a tendency for *non-responders* to exhibit higher and more variable values in some T cell subsets such as CD4 and CD8 T cells and naïve CD8 T cells, suggesting the presence of an active inflammatory milieu.

To continue our analysis of the immune response in the same cohort of patients, we subsequently quantified a subset of cytokines, chemokynes and growth factors in a subgroup of 21 RDN patients. Venous blood samples were obtained at four timings, one before the procedure and three after RDN, up to 30 days. A broad human factor panel was used, which consisted of 45 protein targets. We demonstrated that *responders* presented with significantly lower levels of regulated on activation, normal T cell expressed and secreted (RANTES), both at baseline and at six months follow-up. Even though *responders* continued to present with lower levels of RANTES when response was evaluated at one year, this difference was not statiscally significant, probably due to the reduced number of *non-responders* at this time. Data regarding the role of RANTES in atherosclerosis and plaque vulnerability is controversial. This chemokyne is known to be a very potent chemo-attractant of T-cells, monocytes and macrophages and is

possible that its higher levels may be associated with cellular infiltration and hence end-organ damage. However, a protective role of RANTES over the kidney, that appears tissue specific, has been described by Rudemiller et al [31], whose findings showed that RANTES deficiency led to severe hypertensive renal damage. In our study, even though responders had lower levels of RANTES, they were not suppressed, in fact, were above 10.000pg/ml. We could hypothesize RANTES continued to exert its protective role in the kidney, in both *responders* and *non-responders*, as renal function was within normal values in the overall cohort.

Another interesting finding in our study was that IL-15, IL-27, IL-17A and leukemia inhibitory factor (LIF) peaked 24 hours after RDN, constituting an intriguing pattern. The fact that both pro- and anti-inflammatory cytokines reached its highest value immediately after RDN suggest that the induced numbness of the SNS allowed for anti-inflammatory pathways to fight the acute inflammatory process provoked by RDN. This is in line with the findings by Lee et al [32], who observed an increase in inflammatory cytokines IL-1β, IL-18, IL-6 and TNF-α and anti-inflammatory cytokine IL-10, immediately after RDN, and then a decrease in week 2 of the follow-up. Additionally, several biochemical parameters were evaluated and were similar between groups, except for glycated hemoglobin which was significantly higher in non-responders, even though the number of diabetic patients did not differ in *responders* vs *non-responders*, implying that poorly controlled glycaemia may adversely affect response to RDN.

Several research groups have aimed to identify a specific biomarker able to anticipate response to RDN and potential predictors are endothelial adhesion molecules, [33] brain-derived neurotrophic factor [34] and, potentially, IL-6. [35-36] The cytokine RANTES is a soluble pro-inflammatory chemokine secreted by several cell types such as activated T cells, fibroblasts, endothelial cells, smooth muscle cells, glial cells, mesangial cells and platelets, which is highly expressed in the atheroma. Activated platelets are thought to have a crucial role in the pathogenesis of atherosclerosis and there is evidence linking RANTES with metabolic syndrome and IL-6, which is an activator of platelets. [37] Our research was able to link RANTES to a potencial response to RDN and therefore contribute significantly to the ongoing search for a selective biomarker, which would help to a better selection of patients referred to RDN.

Overrall response to renal denervation, long-term follow-up and impact of Vitamin-D levels

In the final stage of our investigation, we aimed to evaluate overall efficacy and safety of RDN, the echocardiographic evolution and the predictor value of vitamin D. Vitamin D was selected as a potential target of response as its deficiency is described as being present in 30 to 50% of the general population, and increasing evidence shows an association with cardiovascular risk factors, including HT, even though it is conflicting whether vitamin D status has any influence in BP variation. [38]

Ninety-seven patients were screened for our study and thoroughly evaluated in the outpatient clinic. Seventy-three patients were excluded due to nonconformity with the inclusion criteria (undertreatment, pseudo-resistant HT, secondary HT, non-adherence to medication). Twenty-four patients were diagnosed with *true* resistant HT and submitted to RDN. 70.8% of the patients were *responders* at six-months and 83.3% at one year, with a significant drop in mean systolic ABPM being observed in *responders,* vs *non-responders*. Our results are consistent with the latest trials published in the field of RDN.

Regarding the long-term follow-up of our study, for a mean period of 52 months (14-91 months), seventeen patients (70,8%) remained *responders* to RDN, one patient died (*non-responder*, severe HT with target-organ damage, cause of death unknown), two patients who were *responders* up to 18 months required a re-RDN, due to severe re-elevation of BP, and presented with a positive BP response (8,3%), and four patients remained *non-responders* (16.7%).

In terms of safety, there was a new renal artery stenosis reported in one patient at six-month follow-up. He was a 47-year-old male patient, who had severe refractoty HT with target-organ lesion and non-significant atherosclerotic plaques were identified during baseline renal angiography. The RF energy was applied at least 5 mm distant from the atherosclerotic plaque. At 6-month follow-up, the patient presented with flash pulmonary oedema, which was treated with stent angioplasty following new renal artery stenosis identification. The patient had a significant BP-reduction after treatment. No other complications were observed. Three cases of renal artery stenosis were reported in the Global Symplicity Registry but were considered not to be a direct consequence of RDN but rather the progression of atherosclerotic disease [39], which could be the case of our patient. A recent meta-analysis of 50 RDN trials including over 5700

patients with a median follow-up of 11 months estimated the incidence of new renal artery stenosis leading to revascularization to be 0.2% per year, a value lower than previous reports of the natural history incidence rate in a hypertensive population. [40]

Responders at six-months showed significantly higher baseline vitamin D levels, compared to *non-responders*, a difference also observed at 6 months follow-up. After one year, no significant differences were found between *responders*, vs *non-responders*, even though higher mean vitamin D levels, both at baseline and at 6 months, continued to be observed in the *responders* group. Vitamin D deficiency has been shown to be associated with increased cardiovascular risk, specifically risk of sudden death, HT and dysfunction of the cardiac autonomic system. [41-43] Mann et al [44] demonstrated that vitamin D deficiency may be associated with a decline in cardioprotective vagal tone in response to an acute vascular stressor (angiotensin II challenge) in healthy individuals. In another study, Tønnesen et al [45] verified that vitamin D supplementation in young healthy individuals with low serum vitamin D modulates the SNS, with the insufficient group having significantly higher mean heart rate and systolic BP at rest. Vitamin D receptor is known to be broadly expressed by cardiovascular tissues such as endothelial cells, cardiomyocytes and vascular smooth muscle cells, also suppressing renin gene expression, inhibiting proliferation of vascular smooth muscle cells and being associated with an increase of endothelial-dependent vasodilation and reduction of cytokine release from lymphocytes. Thus, association of vitamin D deficiency and an absent response to RDN may be plausible and directly related to a global autonomic dysfunction.

Limitations

In this thesis, we conducted an extensive translational research to better understand the effects and the benefits of RDN. However, there are several limitations that ought to be acknowledged.

Regarding the animal study, RDN was performed in a small number of animals. The levels of fibrosis in the denervated artery were quantified and compared to the contralateral side but the distance between nerve fascicles to the arterial lumen was not evaluated. Norepinephrine spillover was not assessed but could have been useful to compliment renal tissue measurements. Regarding safety, even though intra-renal OCT was performed, the impact of

RDN on renal function is unknown, as it was not assessed. Our study was performed in healthy, normotensive pigs, and hence, clinical outcomes were not analyzed.

Regarding the human study, it is a single-center, prospective, non-randomized analysis. Given that *true* resistant HT is uncommon and even rarer is the indication for RDN, 97 patients were sreened but only 24 were submitted to RDN and included in the final study. Also, there was no control group (sham procedure was not performed) and there was no blinding neither for the physicians nor the patients. Additionally, even though drug-intake was observed at baseline in all patients, to confirm adherence to medication, no specific tecnhiques were used to assess adherence at the follow-up. With respect to the procedure itself, every technique was performed by the same interventional cardiologist. However, three different RDN devices were used and notwithstanding that most of the procedures were performed with a multielectrode device, which allows for a circumferential and more predictive ablation pattern, in the two first procedures a single-electrode catheter was employed.

Future Perspectives

The variability of individual response to RDN remains considerable. Future research should continue to focus on identifying patients with a high likelihood to derive benefit from the treatment. Until the present day, there is no marker of technical success, neither intraprocedurally or afterwards. Our research identified two potential predictors of response but given the reduced number of patients, these findings should be corroborated by other groups of investigators for feasibility and reference values. Our group will pursue research in this field and one future plan is to engage in collaboration with another Portuguese center with high volume in RDN, to confirm our findings and mantain investigation in this area.

Non-adherence to pharmacological treatment is another complex problem in patients with HT. When performing RDN, it is of the utmost importance to identify individuals who do not comply with prescribed drugs, in order to better understand the clinical response to the technique. Overall patient non-adherence to medication in resistant HT trials is around 50% [46] and additionally, individual behavior in the follow-up is completely unpredictable, with studies showing different levels of adherence in various timings of the follow-up. As such, offering the choice of a device-based therapy to individuals not willing to be medically treated may be an option in the future, based on shared-decision making and on weighing the risks, vs the benefits of devices.

It is well known that the renin-angiotensin-aldosterone system (RAAS) interacts with the autonomic nervous system at both central and peripheral levels, and has a role in the regulation of both the cardiac function and the BP. Angiotensin II, which is a potent vasopressor and aldosterone-stimulating, is involved in the augmentation of the sympathetic tone and increases the release of cathecolamines from the cardiac sympathetic nerve terminals, via the angiotensin type 1 receptor (ATR1). This SNS hyperactivation is present in several pathological conditions such as resistant HT, chronic heart failure and obstructive sleep apnea. Genetic polimorphisms in the RAAS components (angiotensinogen, angiotensin converting enzyme and AT1R genes) have been investigated in the last decades for their etiological role in cardiovascular disease and HT. As such, a future line of investigation from our group would be to search for mutations directly linked to the RAAS and the SNS, both retrospectively, as ribonucleic acid (RNA) isolation

kits were obtained from each patient for a posterior gene expression analysis, and prospectively, in patients submitted to RDN in the future.

Finally, the autonomic nervous system in involved in the pathophysiology of several cardiovascular diseases, such as heart failure, atrial fibrillation and ventricular arrhythmias. Moreover, proven therapies are based on autonomic modulation with beta-blocker therapy. The mechanisms underlying the potential favorable effects of RND in these patients is not clear and further research is needed in the field.

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Part IV: Supplements

Supplement Number 1

Protocol Approval: ***Comissão de Ética e Bem-Estar Animal (CEBEA), Faculdade de Medicina Veterinária de Lisboa***

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FACULDADE DE MEDICINA VETERINÁRIA
Comissão de Ética e Bem-Estar Animal (CEBEA)

Exma. Senhora
Dra. Joana Delgado Silva

Lisboa, 28 de Abril de 2016

Assunto: Avaliação projecto de investigação

Vimos pela presente informar V.Exa. que a CEBEA, após ter avaliado as actividades que envolvem manipulação de animais, no âmbito do projecto de investigação "Segurança e eficácia da desnervação renal no tratamento da hipertensão: avaliação clínica, imagiológica, imunológica e histológica" considerou que estão salvaguardados os princípios éticos e de bem-estar animal exigidos pela legislação vigente e pelo código de boas práticas, pelo que aprovou a execução do protocolo experimental nas instalações e serviços da FMV, conforme requerido por V.Exa.

Agradecemos que nos informe com a maior brevidade se o projecto já está concluído ou se teve continuidade.

Com os melhores cumprimentos,

Luis Telo da Gama
Presidente da Comissão de Ética e Bem-Estar Animal

Avenida da Universidade Técnica – Pólo Universitário do Alto da Ajuda – 1300-477 – PORTUGAL
☎ (351) 213 652 800 🖷 (351) 213 652 896

Supplement Number 2

Protocol Approval: ***Faculdade de Medicina da Universidade de Coimbra***

· U C ·

FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

COMISSÃO DE ÉTICA DA FMUC

Of. Refª **035-CE-2014**
Data 30/6/**2014**

C/conhecimento ao aluno

Exmo Senhor
Prof. Doutor Joaquim Neto Murta
Presidente do Conselho Científico

Assunto: Projecto de Investigação no âmbito do Programa de Doutoramento em Ciências da Saúde. (refª CE-031/2014)

Candidato(a): Joana Delgado Silva

Título do Projecto: *"Segurança e eficácia da desnervação renal no tratamento da hipertensão resistente: avaliação clínica, imagiológica e imunológica".*

A Comissão de Ética da Faculdade de Medicina, após análise do projecto de investigação supra identificado, decidiu emitir o parecer que a seguir se transcreve: ***"Parecer Favorável".***

Queira aceitar os meus melhores cumprimentos,

O Presidente,

Prof. Doutor João Manuel Pedroso de Lima

GC

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Supplement Number 3

Protocol Approval: ***Centro Hospitalar e Universitário de Coimbra (CHUC)***

CHUC

Unidade de Inovação e Desenvolvimento
Centro de Ensaios Clínicos

06-11-2014

Exm.º Senhor
Dr. José Martins Nunes
Presidente do Conselho de Administração
Centro Hospitalar e Universitário de Coimbra,

Dr. José Martins Nunes EPE
Presidente do Conselho de Administração
C.H.U.C. - EPE

29/10/2014

Ref.ª: **CHUC-085-14**

A pedido de ***Dr.ª Joana Silvia Delgado Silva,*** recebeu esta Unidade um pedido de autorização de um Projecto de Investigação sobre ***"SEGURANÇA E EFICÁCIA DA DESNERVAÇÃO RENAL NO TRATAMENTO DA HIPERTENSÃO RESISTENTE: AVALIAÇÃO CLÍNICA, IMAGIOLÓGICA E IMUNOLÓGICA"*** ao qual não se aplicam as normas previstas na Lei n.º 46/2004 de 19 de Agosto e colheu parecer **favorável** da Comissão de Ética da Faculdade de Medicina de Coimbra e o parecer em anexo da CES deste hospital.

Informa-se V. Exª. que este projecto não acarreta qualquer encargo financeiro adicional para o CHUC.

Solicita-se assim a autorização do Conselho d e Administração para este Projecto.

Com os mais respeitosos cumprimentos,

Pl' O Director da Unidade de Inovação e Desenvolvimento

(Prof. Doutor José Saraiva da Cunha)

CONSELHO DE ADMINISTRAÇÃO
Reg. N.º 8602 PCA
Origem
Data 29 10 2014

CHUC Centro Hospitalar e Universitário de Coimbra, E.P.E.
Unidade de Inovação e Desenvolvimento
Centro de Ensaios Clínicos

AUTORIZAÇÃO DE PROJECTO DE INVESTIGAÇÃO

CHUC	085	14

NOME DO PROJECTO:

SEGURANÇA E EFICÁCIA DA DESNERVAÇÃO RENAL NO TRATAMENTO DA HIPERTENSÃO RESISTENTE: AVALIAÇÃO CLÍNICA, IMAGIOLÓGICA E IMUNOLÓGICA

INVESTIGADOR PRINCIPAL:

Dr.ª Joana Silvia Delgado Silva

Tendo por base o parecer da Comissão de Ética, é autorizada a realização, no Centro Hospitalar e Universitário de Coimbra, do Projecto de Investigação supracitado.

DATA: 06/1/2014

Presidente do Conselho de Administração

Pedro Roldão
Vogal Executivo do Conselho de Administração
C.H.U.C. - EPE

(Dr. José Martins Nunes)

Supplement Number 4

Informed Consent: Final Version

CHUC

CARDIOLOGIA
CHUC-HG

FORMULÁRIO DE INFORMAÇÃO E CONSENTIMENTO INFORMADO

TÍTULO DO PROJECTO DE INVESTIGAÇÃO: SEGURANÇA E EFICÁCIA DA DESNERVAÇÃO RENAL NO TRATAMENTO DA HIPERTENSÃO RESISTENTE: AVALIAÇÃO CLÍNICA, IMAGIOLÓGICA E IMUNOLÓGICA.

PROTOCOLO Nº

PROMOTOR: Joana Delgado Silva (Faculdade de Medicina da Universidade de Coimbra)

INVESTIGADOR COORDENADOR: Joana Delgado Silva
CENTRO DE ESTUDO: Serviço de Cardiologia B, CHUC-HG
INVESTIGADOR PRINCIPAL Joana Delgado Silva
CONTACTO TELEFÓNICO 239 800093

NOME DO DOENTE
(LETRA DE IMPRENSA) _____

É convidado(a) a participar voluntariamente neste estudo porque tem hipertensão arterial resistente à medicação que se encontra a fazer e poderá benefeciar da realização de um tratamento percutâneo intitulado desnervação renal.

Este documento é chamado Consentimento Informado e descreve a finalidade do estudo, os procedimentos, os possíveis benefícios e riscos. A sua participação poderá contribuir para melhorar o conhecimento sobre esta patologia e identificar parâmetros que nos permitam identificar mais facilmente os doentes que beneficiarão desta técnica e que, consequentemente, terão uma redução significativa do risco de mortalidade cardiovascular.

Receberá uma cópia deste Consentimento Informado para rever e solicitar aconselhamento de familiares e amigos. O Investigador ou outro membro da sua equipa irá esclarecer qualquer dúvida que tenha sobre o termo de consentimento e também alguma palavra ou informação que possa não entender.

QUINTA DO VALES - S. MARTINHO
DO BISPO • 3040 COIMBRA
Tel.: 239-800093 • Fax: 239-445737

Depois de compreender o estudo e de não ter qualquer dúvida acerca do mesmo, deverá tomar a decisão de participar ou não. Caso queira participar, ser-lhe-á solicitado que assine e date este formulário. Após a sua assinatura e a do Investigador, ser-lhe-á entregue uma cópia. Caso não queira participar, não haverá qualquer penalização nos cuidados que irá receber.

1. INFORMAÇÃO GERAL E OBJECTIVOS DO ESTUDO

Este estudo irá decorrer no Centro Hospitalar e Universitário de Coimbra, Hospital Geral, em colaboração com a Faculdade de Medicina da Universidade de Coimbra.

Trata-se de um estudo observacional, pelo que não será feita nenhuma alteração na sua medicação ou tratamentos habituais. Terá a duração máxima de 4 anos.

Este estudo foi aprovado pela Comissão de Ética da Faculdade de Medicina da Universidade de Coimbra (FMUC) de modo a garantir a protecção dos direitos, segurança e bem-estar de todos os doentes ou outros participantes incluídos e garantir prova pública dessa protecção.

Como participante neste estudo beneficiará da vigilância e apoio do seu médico, garantindo assim a sua segurança.

Na Europa, aproximadamente 4.6 milhões de pessoas morrem, anualmente, de doença cardiovascular, sendo a hipertensão arterial (HTA) um factor de risco muito importante. Apesar da existência de vários medicamentos antihipertensores, 5-30% dos doentes não estão controlados. A hipertensão resistente está associada a um alto risco de eventos cardiovasculares e renais.

A desnervação renal é uma nova opção no tratamento da hipertensão arterial resistente. Os nervos que existem nos rins (nervos simpáticos renais) contribuem para a HTA. Os estudos demonstram que a interrupção destes sinais nervosos reduz a pressão arterial. A desnervação renal é um procedimento que interrompe de modo eficaz os impulsos gerados pelos nervos renais. Realiza-se numa sala de hemodinâmica, por um médico especializado em procedimentos cardiovasculares. Antes do tratamento irão ser-lhe administrados sedativos para o ajudar a manter-se relaxado e analgésicos a fim de aliviar qualquer desconforto que possa sentir. O tubo para a desnervação será introduzido pela virilha ou pelo braço (zona que será anestesiada). Depois, com o auxílio de RX e a administração de contraste, o catéter será introduzido na artéria renal onde serão aplicados impulsos de energia aos nervos. O tratamento será repetido na outra artéria renal. Depois de concluída a desnervação, o médico removerá cuidosamente todo o material da sua virilha/braço e encerrará o ponto de inserção com um tampão cirúrgico (virilha) ou será feita apenas compressão

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(braço). Deverá permanecer deitado durante um período de 6-24h para evitar complicações como hemorragias; poder-se-á levantar de acordo com as indicaçoes das equipas médica e de enfermagem. A maioria dos doentes terá alta no dia seguinte ao procedimento, podendo regressar à sua vida normal em poucos dias.

Este estudo tem por objectivos a avaliação da segurança e eficácia da desnervação renal no tratamento de doentes com hipertensão resistente.

Serão incluídos cerca de 60 doentes.

2. PROCEDIMENTOS E CONDUÇÃO DO ESTUDO

2.1. Procedimentos

Na primeira consulta, a Médica responsável pelo estudo realizará uma revisão da sua história clínica, registará a sua medicação e avaliará os seus exames complementares de diagnóstico mais recentes. Serão avaliados a sua pressão arterial, pulsação, peso, altura e perímetro abdominal.

As colheitas de sangue serão feitas previamente ao procedimento, após 24h, ao 1º mês e após 6 meses.

Previamente ao procedimento realizará os seguintes exames: Holter das 24h e Monitorização Ambulatória da Pressão Arterial (MAPA) durante 24h.

Poder-lhe-á ser pedido que realize uma cintigrafia cardíaca (apenas um subgrupo de doentes selecionados aleatoriamente a irá realizar) antes da desnervação renal e após 6 meses, para avaliar os nervos do coração; ser-lhe-á administrado um marcador inócuo e as imagens serão obtidas através de RX (é um exame não invasivo). Terá uma duração de 4-5 horas.

Será admitido(a) no internamento 48h antes da desnervação renal e realizará electrocardiograma, ecocardiograma transtorácico, estudo do sono e análise da urina.

Será efectuada uma técnica de imagem intravascular intitulada tomografia de coerência óptica (OCT) num subgrupo de 30 doentes; o objectivo principal da sua realização é a avaliação da segurança da desnervação renal e avaliar eventuais preditores de sucesso.

Realizará uma angiografia renal 6 meses após a desnervação renal para avaliar o estado das artérias renais; se pertencer ao grupo que realizou OCT, esta técnica será repetida nessa altura.

Descrição dos Procedimento (resumo):

Serão realizados os seguintes procedimentos/exames não invasivos:

- MAPA previamente ao procedimento e aos 6 meses
- Holter das 24 horas previamente ao procedimento e aos 6 meses
- Electrocardiograma previamente ao procedimento (durante o internamento) e aos 6 meses
- Ecocardiograma transtorácico previamente ao procedimento (durante o internamento) e aos 6 meses
- Estudo do sono previamente ao procedimento (durante o internamento) e aos 6 meses
- Análises ao sangue previamente ao procedimento, após 24h, ao 1° mês e após 6 meses. Estas análises incluirão a realização de um estudo ao seu sistema imunitário. Será guardada uma amostra de sangue (para o caso de haver necessidade de esclarecer pormenores) durante o tempo de duração do estudo.
- Análises à urina previamente ao procedimento (durante o internamento) e aos 6 meses.
- Cintigrafia cardíaca com I123MIBG (subgrupo de doentes selecionados aleatoriamente) previamente ao procedimento e após 6 meses.

Serão realizados os seguintes procedimentos/exames invasivos:

- Angiografia renal: administração de contraste nas artérias renais para avaliar a anatomia das mesmas, previamente à desnervação e após 6 meses.
- Desnervação renal
- OCT (subgrupo de doentes selecionados aleatoriamente), previamente ao procedimento, logo após a desnervação e aos 6 meses.

2.2. Calendário das visitas/ Duração

Será realizada uma primeira consulta para avaliar se cumpre os critérios para a realização da desnervação renal. Após o procedimento serão realizadas consultas ao 1°, 3° e 6° meses. Terão a duração de cerca de 20 minutos.

2.3. Tratamento de dados/ Randomização

Serão incluídos neste estudo todos os doentes submetidos a desnervação renal por HTA resistente, que cumpram os critérios de inclusão e assinem o Consentimento Informado. O tratamento de dados ficará a cargo da Investigadora Principal.

3. RISCOS E POTENCIAIS INCONVENIENTES PARA O DOENTE

As complicações da desnervação renal são muito raras mas podem ocorrer. Pode observar uma equimose na região da virilha, perna ou abdómen, ou no braço (depende do local da punção). Podem também ocorrer lesões das artérias renais, aorta ou das artérias da região da virilha/braço. Poderá ocorrer lesão renal que geralmente é transitória, devido à administração de contraste.

4. POTENCIAIS BENEFÍCIOS

Este estudo tem a vantagem de estudar a sua doença e permitir um melhor conhecimento da desnervação renal, como tratamento para a mesma. Actualmente não se sabe a razão pela qual só alguns doentes respondem ao tratamento e este estudo tem como objectivo esclarecer esse aspecto. Além disso, a informação que será recolhida irá contribuir para uma melhor informação dos médicos de forma a melhorar os cuidados clínicos a prestar aos doentes com situações idênticas à sua.

Este tratamento não é curativo, ou seja, tem que continuar a tomar todos os medicamentos que estava a tomar antes da desnervação, devendo seguir as intruções do seu cardiologista. Este procedimento vai ajudá-lo a alcançar um valor de tensão arterial normal, juntamente com os medicamentos (que provavelmente irão acompanhá-lo durante toda a vida), e desta forma diminuir o risco complicações (acidente vascular cerebral, insuficiência renal, etc.)

5. NOVAS INFORMAÇÕES

Ser-lhe-á dado conhecimento de qualquer nova informação que possa ser relevante para a sua condição ou que possa influenciar a sua vontade de continuar a participar no estudo.

6. TRATAMENTOS ALTERNATIVOS

Em doentes com hipertensão arterial resistente, não existem tratamentos alternativos à desnervação renal.

7. PARTICIPAÇÃO/ ABANDONO VOLUNTÁRIO

É inteiramente livre de aceitar ou recusar participar neste estudo. Pode retirar o seu consentimento em qualquer altura sem qualquer consequência para si, sem precisar de explicar as razões, sem qualquer penalidade ou perda de benefícios e sem comprometer a sua relação com o Investigador que lhe propõe a participação neste estudo. Ser-lhe-á pedido para informar o Investigador se decidir retirar o seu consentimento.

O Investigador do estudo pode decidir terminar a sua participação neste estudo se entender que não é do melhor interesse para a sua saúde continuar nele. A sua participação pode ser também terminada se não estiver a seguir o plano do estudo, por decisão administrativa ou decisão da Comissão de Ética. O médico do estudo notificá-lo-á se surgir uma dessas circunstâncias, e falará consigo a respeito da mesma.

8. CONFIDENCIALIDADE

Sem violar as normas de confidencialidade, serão atribuídos a auditores e autoridades reguladoras acesso aos registos médicos para verificação dos procedimentos realizados e informação obtida no estudo, de acordo com as leis e regulamentos aplicáveis. Os seus registos manter-se-ão confidenciais e anonimizados de acordo com os regulamentos e leis aplicáveis. Se os resultados deste estudo forem publicados a sua identidade manter-se-á confidencial.
Ao assinar este Consentimento Informado autoriza este acesso condicionado e restrito.

Pode ainda em qualquer altura exercer o seu direito de acesso à informação. Pode ter também acesso à sua informação médica directamente ou através do seu médico neste estudo. Tem também o direito de se opor à transmissão de dados que sejam cobertos pela confidencialidade profissional.

Os registos médicos que o identificarem e o formulário de consentimento informado que assinar serão verificados para fins do estudo pelo promotor e/ou por representantes do promotor, e para fins regulamentares pelo promotor e/ou pelos representantes do promotor e agências reguladoras noutros países. A Comissão de Ética responsável pelo estudo pode solicitar o acesso aos seus registos médicos para assegurar-se que o estudo está a ser realizado de acordo com o protocolo. Não pode ser garantida confidencialidade absoluta devido à necessidade de passar a informação a essas partes.

Ao assinar este termo de consentimento informado, permite que as suas informações médicas neste estudo sejam verificadas, processadas e relatadas conforme for necessário para finalidades científicas legítimas.

Confidencialidade e tratamento de dados pessoais

Os dados pessoais dos participantes no estudo, incluindo a informação médica ou de saúde recolhida ou criada como parte do estudo (tais como registos médicos ou resultados de testes), serão utilizados para condução do estudo, designadamente para fins de investigação científica relacionados com a patologia em estudo.

Ao dar o seu consentimento à participação no estudo, a informação a si respeitante, designadamente a informação clínica, será utilizada da seguinte forma:

1. O promotor, os investigadores e as outras pessoas envolvidas no estudo recolherão e utilizarão os seus dados pessoais para as finalidades acima descritas.
2. Os dados do estudo, associados às suas iniciais ou a outro código que não o (a) identifica directamente (e não ao seu nome) serão comunicados pelos investigadores e outras pessoas envolvidas no estudo ao promotor do estudo, que os utilizará para as finalidades acima descritas.
3. Os dados do estudo, associados às suas iniciais ou a outro código que não permita identificá-lo(a) directamente, poderão ser comunicados a autoridades de saúde nacionais e internacionais.
4. A sua identidade não será revelada em quaisquer relatórios ou publicações resultantes deste estudo.
5. Todas as pessoas ou entidades com acesso aos seus dados pessoais estão sujeitas a sigilo profissional.
6. Ao dar o seu consentimento para participar no estudo autoriza o promotor e seus colaboradores e/ou autoridades de saúde, a aceder aos dados constantes do seu processo clínico, para conferir a informação recolhida e registada pelos investigadores, designadamente para assegurar o rigor dos dados que lhe dizem respeito e para garantir que o estudo se encontra a ser desenvolvido correctamente e que os dados obtidos são fiáveis.

7. Nos termos da lei, tem o direito de, através de um dos médicos envolvidos no estudo, solicitar o acesso aos dados que lhe digam respeito, bem como de solicitar a rectificação dos seus dados de identificação.
8. Tem ainda o direito de retirar este consentimento em qualquer altura através da notificação ao investigador, o que implicará que deixe de participar no estudo. No entanto, os dados recolhidos ou criados como parte do estudo até essa altura que não o(a) identifiquem poderão continuar a ser utilizados para o propósito de estudo, nomeadamente para manter a integridade científica do estudo, e a sua informação médica não será removida do arquivo do estudo.
9. Se não der o seu consentimento, assinando este documento, não poderá participar neste estudo. Se o consentimento agora prestado não for retirado e até que o faça, este será válido e manter-se-á em vigor.

9. COMPENSAÇÃO

Este estudo é da iniciativa do investigador e, por isso, se solicita a sua participação sem uma compensação financeira para a sua execução, tal como também acontece com os investigadores e o Centro de Estudo.

10. CONTACTOS

Se tiver perguntas relativas aos seus direitos como participante deste estudo, deve contactar:
Presidente da Comissão de Ética da FMUC,
Azinhaga de Santa Comba, Celas – 3000-548 Coimbra
Telefone: 239 857 707
e-mail: comissaoetica@fmed.uc.pt
Se tiver questões sobre este estudo deve contactar:
(Joana Delgado Silva, 239 800093)

NÃO ASSINE ESTE FORMULÁRIO DE CONSENTIMENTO INFORMADO A MENOS QUE TENHA TIDO A OPORTUNIDADE DE PERGUNTAR E TER RECEBIDO RESPOSTAS SATISFATÓRIAS A TODAS AS SUAS PERGUNTAS.

CHUC

CARDIOLOGIA
CHUC-HG

CONSENTIMENTO INFORMADO

De acordo com a Declaração de Helsínquia da Associação Médica Mundial e suas actualizações:

1. Declaro ter lido este formulário e aceito de forma voluntária participar neste estudo.
2. Fui devidamente informado(a) da natureza, objectivos, riscos, duração provável do estudo, bem como do que é esperado da minha parte.
3. Tive a oportunidade de fazer perguntas sobre o estudo e percebi as respostas e as informações que me foram dadas.

   A qualquer momento posso fazer mais perguntas ao médico responsável do estudo. Durante o estudo e sempre que quiser, posso receber informação sobre o seu desenvolvimento. O médico responsável dará toda a informação importante que surja durante o estudo que possa alterar a minha vontade de continuar a participar.
4. Aceito que utilizem a informação relativa à minha história clínica e os meus tratamentos no estrito respeito do segredo médico e anonimato. Os meus dados serão mantidos estritamente confidenciais. Autorizo a consulta dos meus dados apenas por pessoas designadas pelo promotor e por representantes das autoridades reguladoras.
5. Concordo em que seja guardada uma amostra do meu sangue transitoriamente (pelo período de duração deste estudo) desde que os meus dados sejam estritamente confidenciais.
6. Aceito seguir todas as instruções que me forem dadas durante o estudo. Aceito em colaborar com o médico e informá-lo(a) imediatamente das alterações do meu estado de saúde e bem-estar e de todos os sintomas inesperados e não usuais que ocorram.
7. Autorizo o uso dos resultados do estudo para fins exclusivamente científicos e, em particular, aceito que esses resultados sejam divulgados às autoridades sanitárias competentes.
8. Aceito que os dados gerados durante o estudo sejam informatizados pelo promotor ou outrem por si designado.

   Eu posso exercer o meu direito de rectificação e/ ou oposição.

QUINTA DO VALES - S. MARTINHO
DO BISPO • 3040 COIMBRA
Tel.: 239-800093 • Fax: 239-445737

9. Tenho conhecimento que sou livre de desistir do estudo a qualquer momento, sem ter de justificar a minha decisão e sem comprometer a qualidade dos meus cuidados médicos. Eu tenho conhecimento que o médico tem o direito de decidir sobre a minha saída prematura do estudo e que me informará da causa da mesma.
10. Fui informado que o estudo pode ser interrompido por decisão do investigador, do promotor ou das autoridades reguladoras.

Nome do Participante_____

Assinatura : _____ ***Data:***_____/____/___

Nome de Testemunha / Representante Legal:_____

Assinatura: _____ ***Data:***_____/____/___

Confirmo que expliquei ao participante acima mencionado a natureza, os objectivos e os potenciais riscos do Estudo acima mencionado.

Nome do Investigador:_____

Assinatura: _____ ***Data:***_____/____/___

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Tel.: 239-800093 • Fax: 239-445737

Supplement Number 5

Informed Consent: First Version and Informative Brochure

ANEXO I

Declaração de Consentimento Informado

Designação do projeto: Segurança e eficácia da desnervação renal no tratamento da hipertensão resistente: avaliação clínica, imagiológica e imunológica.

Investigador responsável: Joana Delgado Silva

Local onde decorre o estudo: Serviço de Cardiologia, Centro Hospitalar e Universitário de Coimbra, Hospital Geral.

Data do parecer favorável pela respetiva Comissão de Ética: ____/____/_____

Eu, _____, _____ anos, portador do BI nº_____, compreendi a explicação que me foi fornecida acerca do meu caso clínico e da investigação que se tenciona realizar, bem como do estudo em que serei incluído(a). Foi-me dada a oportunidade de fazer as perguntas que julguei necessárias e, de todas, obtive resposta satisfatória.

Tomei conhecimento de que, de acordo com as recomendações da Declaração de Helsínquia, a informação e a explicação que me foram prestadas versou os objetivos, os métodos, os benefícios previstos, os riscos potenciais e o eventual desconforto. Além disso, foi-me afirmado que tenho o direito de recusar a qualquer momento a minha participação no estudo, sem que isso possa ter como efeito qualquer prejuízo na assistência que me é prestada. Os registos dos resultados poderão ser consultados pelos responsáveis científicos e ser objeto de publicação, mas os elementos da identidade pessoal serão sempre tratados de modo estritamente confidencial. Concordo em que seja guardada uma amostra do meu sangue transitoriamente (pelo período de duração deste estudo) desde que só o investigador principal tenha acesso à minha identidade.

Por isso, concordo em participar neste estudo e realizar o tratamento e exames complementares de diagnóstico propostos pela equipa.

Coimbra, ____ de _____ de 20____

_____

Assinatura do(a) doente

_____

Nome e Assinatura da Médica

ANEXO II

FOLHETO INFORMATIVO

(Esta informação é complementar a que já lhe foi fornecida pelo seu Médico cardiologista, sendo este a pessoa mais indicada para responder a todas as suas questões).

A tensão arterial alta (hipertensão arterial) é um estado de saúde muito perigoso que afecta milhões de pessoas em todo o mundo. Significa que o coração tem que trabalhar mais arduamente que o normal para bombear o sangue para o resto do corpo e este esforço extra exerce uma pressão adicional nos vasos sanguíneos e em vários orgãos importantes, como os rins. Se não for tratada pode estar associada a várias patologias como os acidentes vasculares cerebrais e insuficiência cardíaca.

No seu caso, a hipertensão é considerada resistente, ou seja, não responde à terapêutica receitada pelo seu médico ou às mudanças que já efectuou no seu estilo de vida (perda de peso, regime alimentar cuidado, exercício físico, redução do consumo de álcool, deixar de fumar).

Qual o tratamento a que me vou submeter?

Esta nova opção no tratamento da hipertensão arterial chama-se desnervação renal. Os nervos que existem nos rins (nervos simpáticos renais) contribuem para a tensão arterial alta. Os estudos demonstram que a interrupção destes sinais nervosos reduz a tensão arterial. A desnervação renal é um procedimento que interrompe de modo eficaz os nervos renais. Realiza-se numa sala de hemodinâmica, por um médico especializado em procedimentos cardiovasculares. Antes do tratamento irão ser-lhe administrados sedativos para o ajudar a manter-se relaxado e analgésicos a fim de aliviar qualquer desconforto que possa sentir. O tubo para a desnervação será introduzido pela virilha (zona que será anestesiada). Depois, com o auxílio de RX, o catéter será introduzido na artéria renal onde serão aplicados impulsos de energia aos nervos. O tratamento será repetido na outra artéria renal. Depois de concluída a desnervação, o médico removerá cuidosamente todo o material da sua virilha e encerrará o ponto de inserção com um tampão cirúrgico. Deverá permanecer deitado até ao dia seguinte para evitar complicações como hemorragias; poder-se-á levantar de acordo com as indicaçoes das equipas médica e de enfermagem. A maioria dos doentes terá alta no dia seguinte ao procedimento, podendo regressar à sua vida normal em poucos dias.

O tratamento é seguro?

As complicações são muito raras mas podem ocorrer. Pode observar uma equimose na região da virilha, perna ou abdómen. Podem também ocorrer lesões das artérias renais ou das artérias da região da virilha.

ANEXO II

Vou ficar curado?

Tem que continuar a tomar todos os medicamentos que estava a tomar antes do procedimento, devendo seguir as intruções do seu cardiologista. Este procedimento vai ajudá-lo a alcançar um valor de tensão arterial normal, juntamente com os medicamentos (que provavelmente irão acompanhá-lo durante toda a vida).

Terei que realizar outros exames?

Sim. Para garantirmos que o seu caso é adequado, que pode beneficiar deste tratamento, que é realizado sem complicações e de forma a monitorizar a resposta do seu corpo, vai necessitar de realizar análises ao sangue e urina, Holter das 24h, MAPA (medição da tensão arterial durante 24h), electrocardiograma, ecocardiograma e estudo do sono. Vai necessitar de consultas de seguimento e de repetir estes exames aos 6 meses.

Poderá ter que realizar uma cintigrafia cardíaca (antes da desnervação e após 6 meses) para avaliar os nervos do coração; ser-lhe-á administrado um marcador inócuo e as imagens serão obtidas através de RX (é um exame não invasivo). Terá uma duração de 4-5 horas.

Será ainda realizado um estudo do seu sistema imunitário (que implica apenas a colheita de amostras de sangue antes do procedimento, após 24h e nas consultas de seguimento do 1º e 6º meses). Será guardada uma amostra do mesmo (para o caso de haver necessidade de esclarecer pormenores) durante o tempo de duração do estudo.

Supplement Number 6

Request for statistical support from the Portuguese Society of Cardiology

Ao Exmº Presidente

Do Centro Nacional de Coleção de Dados em Cardiologia (CNCDC),

Da Sociedade Portuguesa de Cardiologia,

Dr. Jorge Mimoso

O meu nome é Joana Silvia Delgado Silva e sou Assistente Hospitalar de Cardiologia no S. de Cardiologia B do Centro Hospitalar e Universitário de Coimbra, Hospital Geral (CHUC-HG). Estou atualmente a frequentar o Programa de Doutoramento em Ciências da Saúde da Faculdade de Medicina da Universidade de Coimbra (FMUC) e encontro-me a desenvolver um projeto de investigação translacional intitulado 'Segurança e eficácia da desnervação renal no tratamento da hipertensão resistente: avaliação clínica, imagiológica, imunológica e histológica'. Este trabalho tem como orientador o Prof. Lino Gonçalves, atual Director do S. de Cardiologia B do CHUC-HG, e como tutor o Prof. Manuel Santos Rosa (Departamento de Imunologia, FMUC).

Neste sentido, gostaria de solicitar à Sociedade Portuguesa de Cardiologia o apoio estatístico necessário, para as questões de amostragem e a análise estatística dos dados, de forma que os trabalhos tenham a metodologia e qualidade esperados.

Coimbra, 28 de Outubro de 2014

Com os melhores cumprimentos,

_____

Joana Delgado Silva
(Assistente Hospitalar de Cardiologia/estudante de Doutoramento)

_____

Prof. Lino Gonçalves
(Director do S. de Cardiologia B, CHUC-HG)

Supplement Number 7

Thesis related Presentations and Informative Session

1. **Presentations to General Practicioners in Local Health Centers – *Resistant Hypertension and the Role of Renal Denervation***

   - *Centro de Saúde Norton de Matos* – March 2014
   - *Centro de Saúde de Eiras* – April 2014
   - *Centro de Saúde da Anadia* – June 2014
   - *Centro de Saúde da Mealhada* – September 2014

2. **Presentations to Cardiologists – *Renal Denervation: final step in the treatment of Resistant Hypertension***

   - Department of Cardiology of *Centro Hospitalar e Universitário de Coimbra, Hospital Geral* – October 2014

   - Department of Cardiology of *Hospital de Santo André, Leiria* – November 2014

   - Department of Cardiology of *Centro Hospitalar Baixo Vouga, Aveiro* – March 2015

3. **Presentations in National Congresses/Reunions**

   - 'Renal Denervation in the Treatment of Arterial Hypertension: update' – *21º Congresso Português de Cardiopneumologia*, Curia, March 2016

   - 'The Patient with Resistant Hypertension: day to day problems' – *Jornadas de Cardiologia do Centro*, Guarda, October de 2016

   - 'Resistant Arterial Hypertension' – *Encontro Renal*, Vilamoura, March 2018

- 'Device-based Treatment in Uncontrolled Hypertension: state of the art' – *Curso de Hypertensão Resistente*, Coimbra, June 2018

- 'Renal Denervation – Bench to Bedside. Investigation Corner' – 2VRT – Vascular and Valvular Restorative Therapy, Lisbon, May 2019

Supplement Number 8

Case Report: Reinnervation After Denervation – A Myth?

Original Article

Title: Reinnervation After Denervation – A Myth?

Submitted to *Arquivos Brasileiros de Cardiologia*

Eric Monteiro [a*], Gonçalo Costa [a], Joana Delgado-Silva [a,b], Lino Gonçalves[a,b]

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Abstract

We present two cases of idiopathic resistant hypertension treated with catheter-based renal denervation with an optimal initial response. Nevertheless, both patients had their blood pressure back to baseline values at 24 and 18-month follow-up, respectively. Secondary causes of hypertension were once again excluded. Given the unacceptable high levels of blood pressure with a poor response to optimal antihypertensive treatment, a new renal denervation was scheduled. After the second procedure the blood pressure was substantially lowered in both patients, lasting until the present day (6 months follow-up for patient one and more than 3 years for patient two). There were no procedural related complications. We intend to raise concern about the possibility of functional re-innervation and development of supersensitivity to norepinephrine after renal denervation in humans. It is crucial to know whether re-innervation occurs, if it influences the long-term results of the intervention and in which subset of patients this phenomenon is more likely to occur.

Keywords: resistant hypertension; renal denervation; renal re-innervation

Introduction

Hypertension is a leading risk factor influencing the global burden of cardiovascular disease (1). In spite of the fact that measures such as lifestyle changes and pharmacological treatment reduce blood pressure (BP) and cardiovascular complications in hypertensive patients, worldwide, the treatment of hypertension remains suboptimal with consequent inadequately controlled BP in many patients (2). The true prevalence of the so-called 'resistant hypertension' is still unknown, but apparently revolves around 12% among all hypertensive patients (3). According to the current guidelines of European Society of Hypertension, it is defined when target BP values are not reached despite prescription of triple therapy, including a diuretic at maximum tolerated dose (4). Catheter-based renal denervation (RDN) is one of the most frequently used invasive methods for the treatment of resistant arterial hypertension (5). Nevertheless, its role in clinical practice is controversial and there is scarce information about the different responses to this procedure (4). We report two cases of idiopathic resistant hypertension treated with RDN. Both patients had a profound initial response to the procedure. Nevertheless, both patients had their BP back to baseline values at 24 and 18-month follow-up, respectively. An investigation to detect secondary causes was performed with no findings that justified the BP changes. Therefore a new RDN procedure was performed, with good results, lasting until the present day (6 months follow-up for patient one and more than 3 years for patient two). This is a report about the heterogeneous response to RDN, the possible role of functional re-innervation and the potential development of supersensitivity to norepinephrine after RDN. These mechanisms could be responsible for rising the BP back to baseline values after an optimal initial response.

Case reports

Case 1

A 49-year-old man with a history of arterial hypertension, presented with episodes of dizziness and chest pain associated with hypertensive peaks. The patient had a medical history of type 2 diabetes, dyslipidemia, gout and obesity. He was on five antihypertensive drugs: amlodipine 80 mg/valsartan 5 mg bid, spironolactone 100 mg od, nebivolol 5 mg od and chlortalidone 50 mg od. He was an active smoker (5 pack-units-year) and had no history of alcohol or caffeine excess. On initial examination his office BP was 195/125mmHg, with no inter-arm disparity. The remaining physical examination was normal. There was evidence of hypertensive end-organ damage (left ventricular hypertrophy criteria on ECG and moderate concentric hypertrophy of left ventricle on transthoracic echocardiography). The patient had done a previous CT coronary angiogram that revealed no coronary disease. Secondary causes of hypertension were excluded (screening with full biochemistry and haematology profile, imaging assessment and polysomnography) and idiopathic resistant hypertension was confirmed by ambulatory blood pressure monitoring (ABPM). We proposed the patient to be submitted to RDN which was performed with the multielectrode Spyral catheter, without complications. At the 6 months follow-up, the patient was asymptomatic, on four antihypertensive drugs and systolic and diastolic BP in ABPM had dropped 15 and 10 mmHg, respectively (24h average BP 144/96 mmHg). Nevertheless, at 24 month follow-up the patient was back on five hypertensive drugs and had a 24h average BP of 181/120 mmHg in ABPM.

Case 2

A 74-year-old woman presented with episodes of headache associated with hypertensive peaks and excessive daytime sleepiness. The patient had a medical history of arterial hypertension and dyslipidemia. She was medicated with four antihypertensive drugs: nifedipine 60 mg in the morning and 30 mg at dinner, perindopril 5 md bid, carvedilol 12.5 mg bid and chlortalidone 50 mg od. The patient had no history of smoking, alcohol or caffeine excess. At examination, her

office BP was 200/90 mmHg, with no inter-arm disparity and no other abnormal findings in the physical examination. There was no evidence of hypertensive end-organ damage. A previous CT renal angiogram revealed atheromatous plaques in both renal arteries ostium, but without hemodynamically significant stenosis. Secondary causes of hypertension were assessed, which revealed a mild obstructive sleep apnea. Nevertheless, the ABPM values did not improve with continuous positive airway pressure (CPAP), despite confirmed compliance. RDN was proposed and performed with the multielectrode Spyral catheter, with no complications. At 6 months follow-up, the patient had no cardiovascular complaints. She was still on four antihypertensive drugs, but the ABPM presented a 24h average BP of 110/60 mmHg (systolic and diastolic reduction of 48 and 19 mmHg, respectively). However, at 18 months follow-up, the patient presented with new hypertensive crisis with a BP of 190/85 mmHg. A new ABPM was performed and revealed a 24h average BP of 146/70 mmHg.

Investigations and treatment

The patients were reassessed for secondary causes of hypertension, but none was found. Since no justifiable cause for the rising of BP was found in both patients, we proposed a new RDN procedure which they accepted. Both procedures were performed through the femoral artery, using the multielectrode Spyral catheter, with no procedural related complications.

Outcome and follow-up

Case 1

At 6-month follow-up of the second procedure, average 24h BP registered by ABPM was 159/103mmHg (systolic and diastolic BP drop of 22 and 17 mmHg, respectively). The patient was asymptomatic and remained with the same antihypertensive medication.

Case 2

At 6-month follow-up of the second procedure, average 24h BP registered by ABPM was 127/68mmHg (systolic and diastolic BP drop of 19 and 2 mmHg, respectively). The BP remained stable at 1-year, 2-year and 3-year follow-up. During this period the patient's antihypertensive medication was progressively reduced due to hypotensive episodes. Overall, the patient general condition was improved, with no record of hypertensive symptoms or signs until the present day.

Discussion

The limitations of currently available pharmacological strategies to control the BP in some patients, defining the so-called resistant hypertension, is thought to reflect the complexity and multitude of potential mechanisms responsible for the genesis and maintenance of elevated BP. This led to a renewed interest in invasive strategies to control BP (6, 7). Renal sympathetic nerves contribute to development and perpetuation of hypertension, and sympathetic outflow to the kidneys is activated in patients with essential hypertension (8). The chronic activation of the sympathetic nervous system constitutes a central mechanism in resistant hypertension and has been a target of percutaneous RDN (7).

There is robust evidence derived from well designed and rigorously conducted sham-controlled studies (SPYRAL HTN-OFF MED, SPYRAL HTN-ON MED, and RADIANCE-HTN SOLO) supporting the efficacy and safety of RDN (9-11). Nevertheless the available results are only short term, and long-term efficacy is still lacking (12). There is still little information regarding the extent of re-innervation following catheter-based RDN, but studies in animal models show evidence of functional and anatomical renal nerve re-innervation, along with denervation related supersensitivity to norepinephrine. A study conducted in sheep assessed the effectiveness of renal nerve denervation with the Symplicity Flex catheter and the functional and anatomical re-innervation at 5.5- and 11-months post-denervation. It was found that the procedure effectively

denervated the afferent and efferent renal nerves, but by 11 months post-RDN there was functional and anatomical evidence of afferent and efferent renal nerve re-innervation (13). Similarly, a study conducted in rats indicates that following RDN, functional re-innervation of the renal vasculature begins to occur between 14 and 24 days after the procedure, and that complete return of function may occur by 8 weeks. The study also suggested that the response to renal nerve stimulation during re-innervation could be due to a combination of regeneration of the nerve fibres and denervation related supersensivity to norepinephrine (14). Although the final 3-year results of the Symplicity HTN-1 study (15) suggest that no re-innervation or any counter-regulatory mechanisms develop over time that could lessen the efficacy of the procedure, these two cases that we report, along with the evidence available on animal models, seem to indicate that this may not be universally true. We considered the possibility of incomplete denervation on the initial procedure. Unfortunately, in clinical practice, methods such as renal norepinephrine spillover are not readily available to objectively measure the efficacy of the RDN procedure. Nevertheless, we firmly believe that quality procedures were performed in both cases, since care was taken to achieve an adequate number of ablations, throughout the ostium, main renal artery and branches. In our opinion, the fact that in both the described cases there was a marked BP response to the first RDN procedure, followed by re-elevation of the BP to baseline values at follow up, could indicate that re-innervation plays a clinically significant role in the long-term efficacy of the procedure. Additionally, both patients responded to a repeat procedure, fact that seems to further validate our hypothesis.

Taking these aspects together, our goal is to raise concern about the possibility of re-innervation and development of super-sensitivity to norepinephrine after RDN in humans. It is crucial to know whether re-innervation occurs, if it influences the long- term results of the intervention and in which subset of patients this phenomenon is more likely to occur.

Conclusions

- Hypertension is a leading risk factor influencing the global burden of cardiovascular disease. Many patients are not able to reach target blood pressure values despite lifestyle changes and pharmacological treatment.

- Catheter-based renal denervation presents as a safe and effective alternative for this subset of patients with resistant hypertension.

- Although the final 3-year results of the Symplicity HTN-1 study suggest that no functional re-innervation or any counter-regulatory mechanisms develop over time that could lessen the efficacy of the procedure, these two cases that we report, along with the evidence available on animal models, seem to indicate that this may not be universally true.

- It is therefore crucial to know whether re-innervation occurs, if it influences the long-term results of the intervention and in which subset of patients this phenomenon is more likely to occur.

Figure 1: Panels A-D – case 1 renal arteries pre 1st renal denervation (RDN), immediately post 1st RDN, 6 months follow-up after 1st RDN and immediately post 2nd RDN, respectively; Panels E-H – case 2 renal arteries pre 1st RDN, immediately post 1st RDN, 6 months follow-up after 1st RDN and immediately post 2nd RDN, respectively. Only the left renal artery of each patient is shown. The contralateral renal artery was in similar conditions.

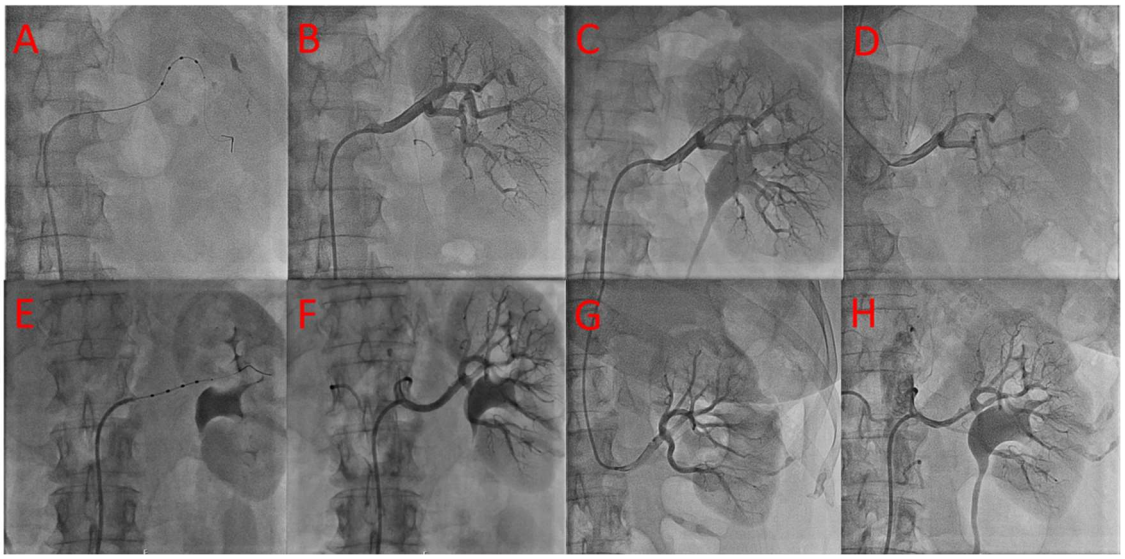


Figure 2: Case 1 BP evolution registered by ABPM.

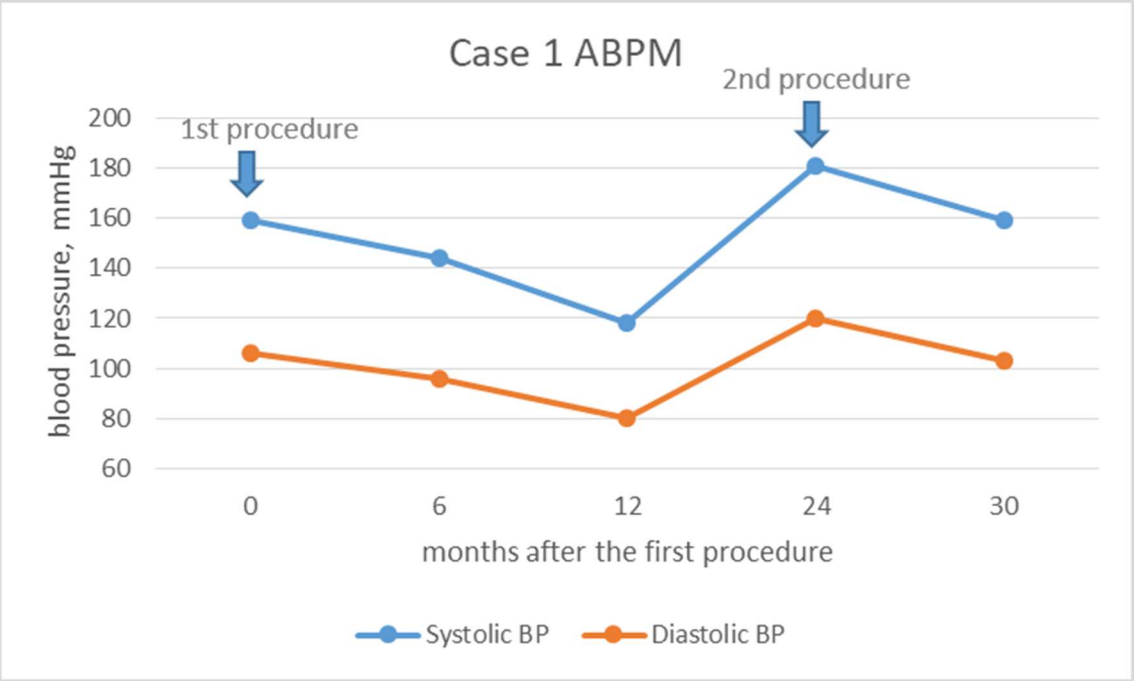
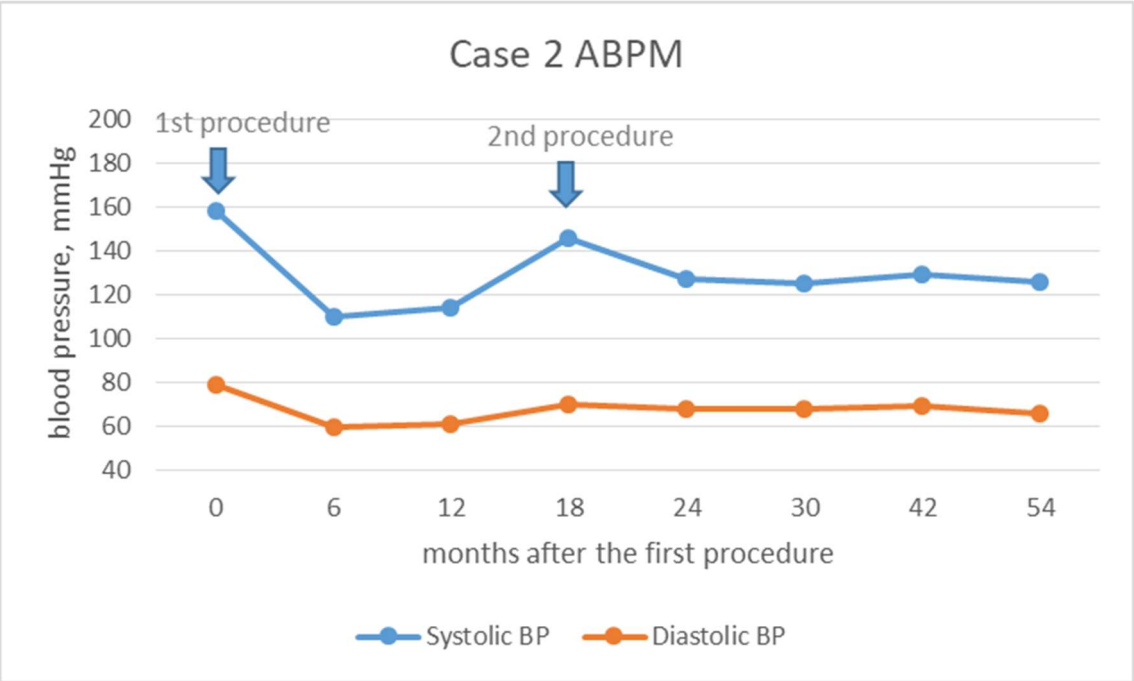


Figure 3: Case 2 BP evolution registered by ABPM.



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