BRIEF REPORT

Expression of nicotinic acetylcholine receptor subunits alpha 4, alpha 7 and beta 2 in human internal mammary arteries of non-smokers and smokers

Sandy von Salisch[1,*], Maja-Theresa Dieterlen[1,*], Jan Bucerius[2,3,4], Felix M. Mottaghy[2,4], Dirk Neubert[1], Friedrich W. Mohr[1,#]

[1]*Heart Center Leipzig, University Hospital, HELIOS Clinic, Department of Cardiac Surgery, Leipzig, 04289, Germany*
[2]*Department of Nuclear Medicine, Maastricht University Medical Center, Maastricht, 6200, The Netherlands*
[3]*Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Center, Maastricht, 6200, The Netherlands*
[4]*Department of Nuclear Medicine, University hospital RWTH Aachen, Aachen, 52062, Germany*

[*]These authors contributed equally to this work.
[#]Correspondence: Maja-Theresa Dieterlen
E-mail: Maja-Theresa.Dieterlen@helios-kliniken.de
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Nicotinic acetylcholine receptors (nAChR) are widely expressed in non-neuronal tissue, but data about their expression in vascular tissue are rare. To study the expression of nAChR α4, α7 and β2 in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in human arteries of smokers and non-smokers, arteriae thoracicae internae dissected for coronary artery bypass grafting were analysed immunhistochemically and by polymerase chain reaction (PCR). ECs of the tunica intima and the vasa vasorum as well as the VSMCs of the tunica media showed clear staining for nAChR α4, α7 and β2, without significant differences between non-smokers and smokers in all vascular layers. Further verification of nAChR α4, α7 and β2 expression of whole-tissue homogenates using PCR analysis showed no differences in the subtype expression between non-smokers and smokers. This provides an important basis for further investigations using positron electron tomography (PET) tracers for imaging of nAChRs in vascular health and disease.

Keywords: nicotinic acetylcholine receptor; endothelial cells; vascular smooth muscle cells; smoker

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Introduction

Nicotinic acetylcholine receptors (nAChR) are a family of ligand-gated ion channels, which exhibit Na^+, K^+ and Ca^{2+} permeability and consist of 17 distinct isoforms of five subunits (α1- α10, β1- β4, γ, δ and ε). The subunits can assemble as homogeneous or heterogeneous pentamers in many possible combinations. Beside their well-known expression in the nervous system and at motor endplates, nAChRs are widely distributed in non-neuronal mammalian cells including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), bronchial epithelial cells, keratinocytes, immune cells and cancer cells [1-4].

Table 1. Patients characteristics of the study cohort (n=27)

	Non-smokers (n=14)	Smokers (n=13)
Age	68.6yrs ± 2.3yrs	61.3yrs ± 2.4yrs
% female (n)	42.9 (6)	0 (0)*
Body mass index (BMI)	28.3 ± 0.7	26.8 ± 0.9
% diabetes mellitus (n)	57.1 (8)	23.1 (3)
% hypertension (n)	92.9 (13)	92.3 (12)
% heart failure (n)	28.6 (4)	30.8 (4)

Data are represented as mean ± S.E.M. Footnote: * p=0.01

A number of nAChR subunits are expressed in ECs (α3, α5, α7, α10, β2, β and β4) and VSCMs (α2, α3, α4, α5, α7, α10) of blood vessels, whereas the most abundant receptor subunit is α7 [5]. α7-nAChRs on the plasma membrane of VSMCs and ECs are responsible for the nicotine response of these cells, which is characterized by stimulated proliferation and migration of ECs and increased release of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) as well as for pro-angiogenic processes [6]. NAChRs in VSMCs have been shown to be involved in pathological processes [7-9]. They mediate the nicotine-induced migration, inhibit apoptosis and stimulate proliferation of human aortic smooth muscle cells, at least via an increased expression of the platelet-derived growth factor (PDGF). It was speculated that these processes contribute to the development and progression of atherosclerosis [8]. All these findings emphasize the pivotal role of vascular nAChRs in normal intercellular communication, proliferation, invasion and angiogenesis.

However, systematic and detailed information about subunits of nAChRs, along with their incidence and distribution in the human vascular system is lacking. Therefore, the aim of this study was to investigate and quantify the abundance of nAChR subunits in human arteries, especially on ECs and VSMCs. Furthermore, we investigated whether there is a difference in the expression in the arteries of non-smokers and smokers.

Materials and methods

Study population und sample collection

The study was approved by the local ethical board and performed according to the declaration of Helsinki. All patients gave written informed consent. Twenty-seven patients undergoing elective cardiac surgery for coronary artery bypass grafting were included in this study. Samples of arteriae thoracicae internae were collected from patients undergoing bypass surgery and either paraffin-embedded (n=17) or snap-frozen for ribonucleic acid (RNA) extraction (n=10).

RNA extraction and reverse transcriptase PCR analysis

Snap frozen tissue specimen was homogenized in TRIzol® (Life Technologies GmbH, Darmstadt, Germany). RNA was extracted following the manufacturers protocol. 2 µg RNA were reverse transcribed using 0.5 µg Oligo(dT) primer (Promega, Mannheim, Germany), 1 mM dNTPs, 10x reaction buffer, 4 U Omniscript® reverse transcriptase (Qiagen, Hilden, Deutschland) and 10 U RiboLock RNase Inhibitor (Thermo Scientific, Schwerte, Germany). 2 µl of cDNA was amplified by adding 1 U Platinum® Taq Polymerase (Life Technologies GmbH), 10x reaction buffer, 1.5 mM magnesium chloride, 0.2 mM desoxy-nucleotides (dNTPs) and 0.2 µM specific forward (fw) and reverse (rev) primer (nAChRα4: fw 5'-cgaccagcagaactgcacca-3', rev 5'-agcaggaagacggtgagcga-3'; nAChRα7: fw 5'-tgtggccgcatggcctgctc-3', rev 5'-ctttggacacggcctccacg-3'; nAChRβ2: fw 5'-ggcgagaagatgacgttgtg-3', rev 5'-gttgacgaagcacgtgcagg-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): fw 5'-tcgtggaaggactcatgacc-3', rev 5'-ccttgcccacagccttggca-3'). PCR products were separated on a 1% agarose gel and visualized using ethidium bromide.

Immunohistochemistry

Tissue specimen were fixed with 4% formalin and embedded in paraffin. Paraffin sections were stained using the following primary antibodies: nAchR subunit α4, α7 or β2 (1:50, respectively, all Santa Cruz Biotechnolgies, Santa Cruz, CA). After washing, the signal was amplified and visualized using the EnVisionTM FLEX System and 3,3′-diaminobenzidine (DAB) staining according to the manufacturer's instructions (Dako). A counterstaining with haemalaun was also performed. Sections without primary antibody served as negative, sections of neonatal rat brain as positive controls. Analysis was performed using a Zeiss microscope and AxioVision software (Carl Zeiss Microscopy GmbH, Jena, Germany). DAB-stained areas were analysed using ImageJ software.

Statistics

Data are shown as mean ± standard error of the mean (S.E.M). Statistical analysis was performed with Student´s t-test using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). A p-value ≤0.05 was considered to be significant.

Results

The study cohort did not show significant differences regarding age, body mass index and for the frequencies of diabetes mellitus, hypertension and heart failure in the two

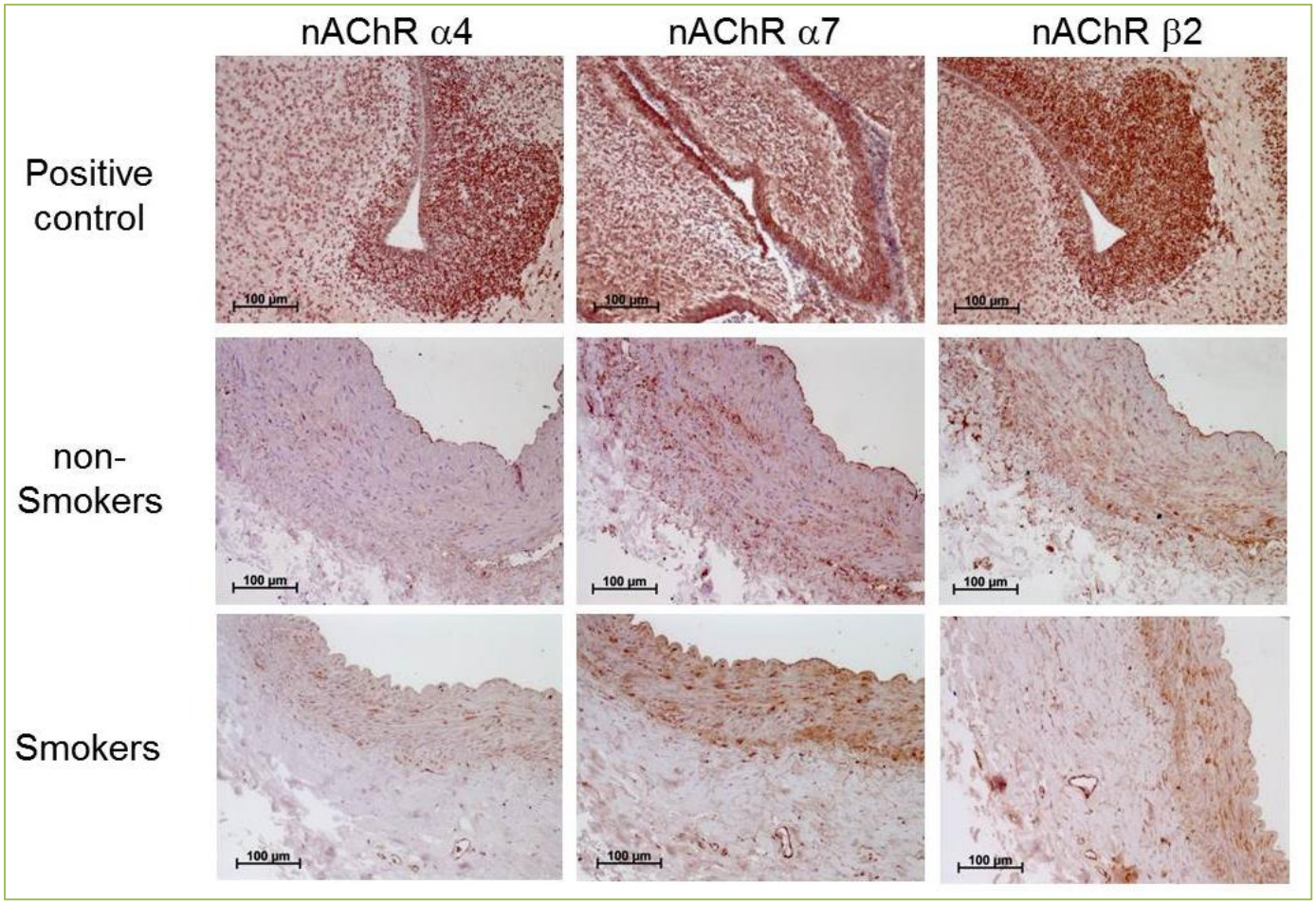

Figure 1. Immunohistochemistry of nicotinic acetylcholine receptor (nAChR) subunits α4, α7 and β2 in the internal mammary artery of smokers and non-smokers. Diaminobenzidine (DAB) staining of α4, α7 and β2 subunits of nAChRs in arteriae thoracicae internae of non-smokers and smokers. Staining of all three subunits on neonatal rat neocortex of the ventricle (α4, β2) and of the cerebellum (α7) served as positive controls. Magnification: ×100.

study groups 'smokers' and 'non-smokers' (Table 1). The distribution of gender was significantly different (p=0.01) between both study groups: the group 'smokers' consisted solely of male patients.

Immunohistochemical analysis of nAChR α4, α7 and β2 subunits was analysed in the different vascular layers (endothelium, VSMCs in the tunica media, in adventitial cells and ECs of the vasa vasorum) and did not show apparent differences regarding the staining intensity between the internal mammary arteries of non-smokers and smokers (Figure 1). Analysis of the endothelium showed that the nAChRs containing the α4-subunit were expressed as a lower percentage (non-smokers: 68.3% ± 7.4%, smokers: 51.0% ± 12.6%) compared to nAChRs containing α7 (non-smokers: 85.2% ± 3.8%, smokers: 79.0% ± 8.0%) or β2 (non-smokers: 77.4% ± 9.4%, smokers: 67.6% ± 13.3%) (Figure 2A). The relative stained area of VSMCs exhibited a similar expression of nAChR subunits. The α4-subunit (non-smokers: 3.8% ± 0.9%, smokers: 2.5% ± 0.6%) was expressed at a lower level than α7-subunit (non-smokers: 12.6% ± 2.7%, smokers: 12.1% ± 3.4%) or 2 (non-smokers: 13.0% ± 2.3%, smokers: 14.2% ± 2.9%) (Figure 2B). However, no significant differences could be detected in the average percentages of nAChR α4, α7 and β2 immunostained endothelium as well as in stained areas of VSMCs of the tunica media. In accordance with the immunohistochemical results, reverse transcriptase PCR (RT-PCR) analysis revealed that the mRNA-expression of nAChR subunits α4, α7 and β2 of the arteriae thoracicae internae did not differ between smokers and non-smokers (Figure 3).

Discussion

The results of this study demonstrated an abundance of nAChR α4, α7 and β2 subunits in the endothelium and in VSMCs of the tunica media in human arteriae thoracicae internae.

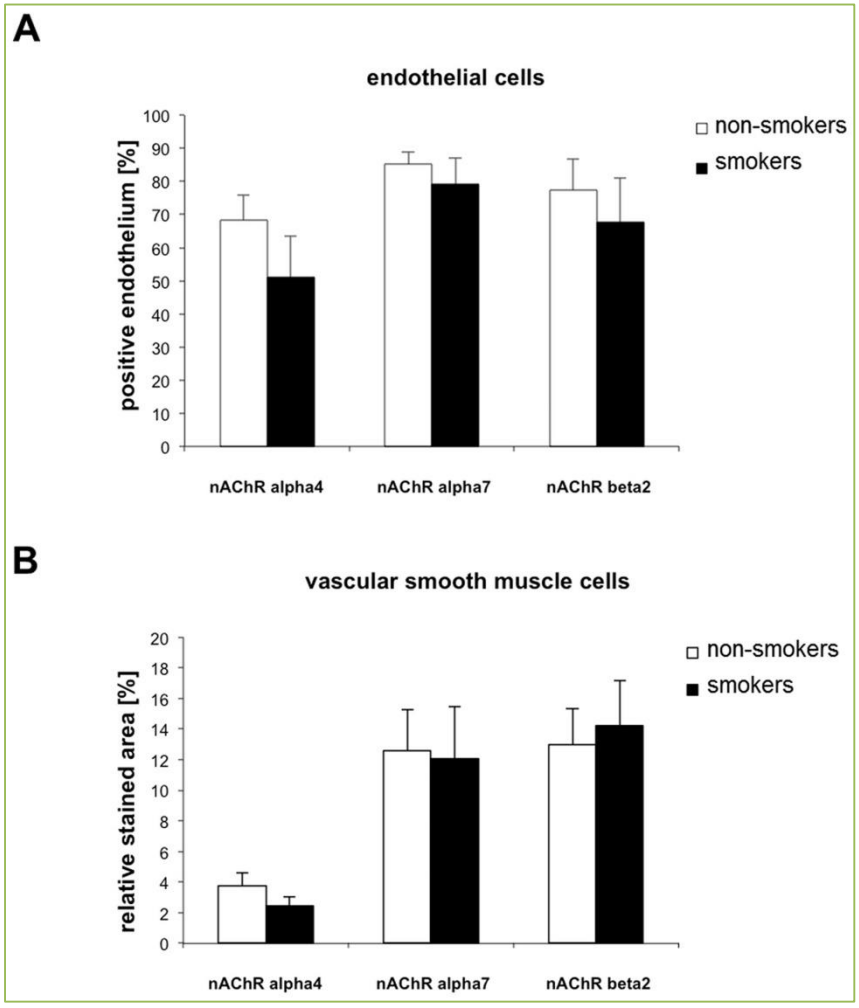

Figure 2. Immunohistochemical quantification of nicotinic acetylcholine receptor (nAChR) α4, α7 and β2 expression in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in the internal mammary artery of smokers and non-smokers. (A) Positive nAChR α4, α7 and β2 staining in the endothelium of the arteriae thoracicae internae of non-smokers and smokers. **(B)** Positive nAChR α4, α7 and β2 staining of the tunica media vascular smooth muscle cells in non-smokers (n=9) and smokers (n=8). Data are shown as mean ± S.E.M.

The expression of nAChRs in non-neuronal cells such as ECs and VSMCs was reported in previous in vitro studies [10, 11]. It has been reported that the nAChR α4 subunit is expressed in human umbilical vein ECs and in human microvascular ECs in vitro [12, 13]. Duerrschmidt and colleagues [12] investigated the intercellular communication of umbilical vein ECs and stated that nAChRs play a role in the impairment of intercellular communication. According to their results, this process is mediated through α4β2 and α3β2 receptors but not α7 nAChRs. A further study showed that α7-containing nAChRs play a role in the cardiovascular system and act as an important protector in myocardial ischemia, reperfusion injury, stroke, atherosclerosis and hypertension [14].

Our study is one of the few reports that confirm the *in vivo* expression of nAChRs on human vascular tissue. Our study showed that α4 and α7 is present in ECs and VSMCs of smaller human arteries such as arteriae thoracicae internae. Furthermore, the expression was quantified and the results demonstrated that the α4 subunit was expressed to a lower extend in ECs as well as in VSMCs compared to α7 or β2 subunit. Scientific reports indicate reverse results on expression of nAChR subunits in vascular tissue. Regarding the α4, α7 and β2 subunits, a recent study proved the expression of α4 and α7 on VSMCs and of α7 and β2 for aortic ECs in humans [15]. Our data are in accordance with *in vitro* results of human primary cultures of ECs by Wang *et al*., who reported the expression of almost all nAChR subunits in ECs [5]. Furthermore, they report that the most abundant receptor subunit is α7 nAChRs.

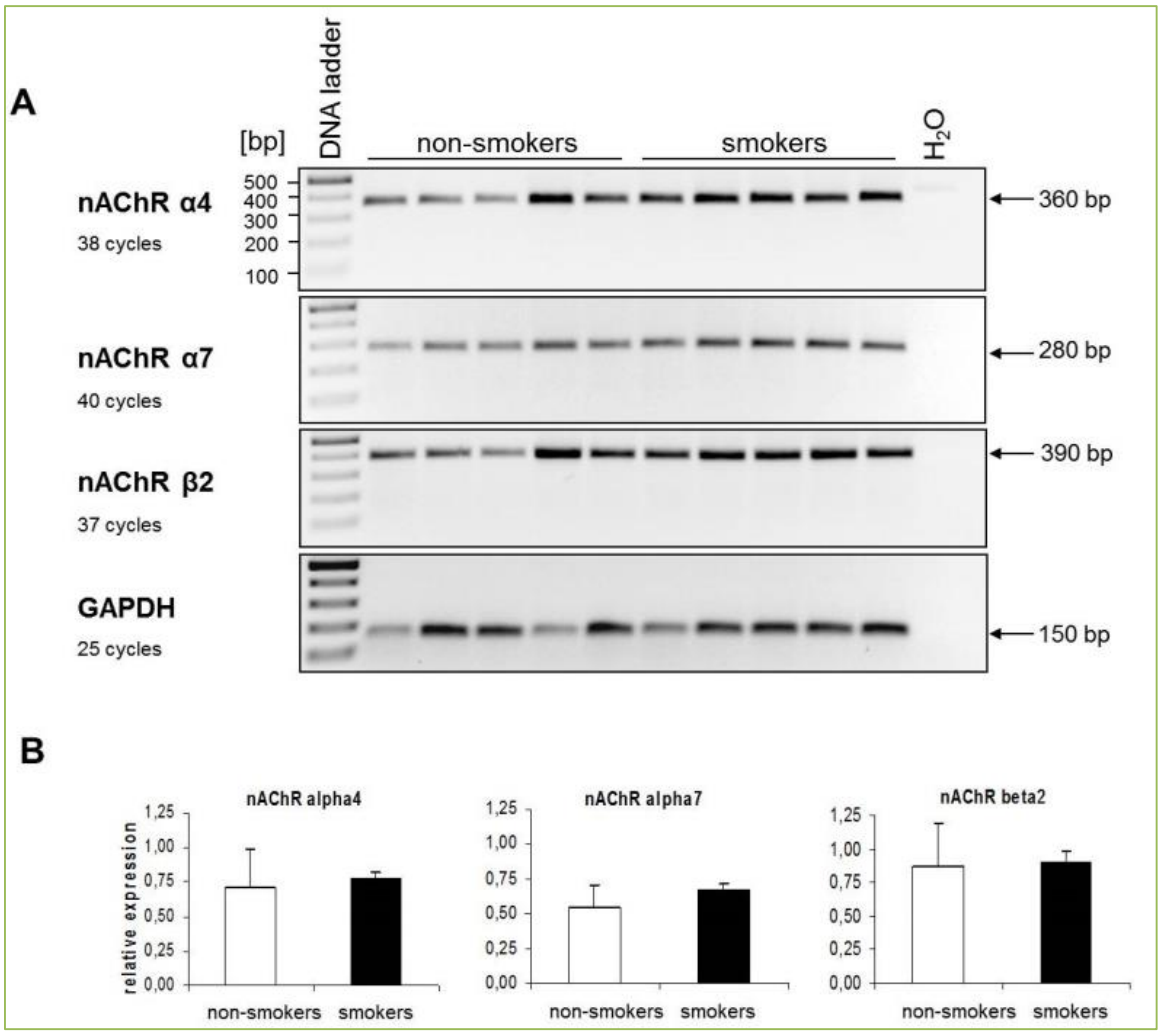

Figure 3. PCR analysis of nicotinic acetylcholine receptor (nAChR) α4, α7 and β2 in the internal mammary artery of non-smokers und smokers. (A) Agarose gel electrophoresis of PCR products of nAChR subunits α4 (360bp), α7 (280bp), β2 (390bp) and GAPDH (150bp). The housekeeping gene GAPDH was used as internal control. **(B)** Relative expression of the nAChR α4, α7 and β2 subunits in internal mammary arteries of non-smokers (n=5) and smokers (n=5). Data are shown as mean ± SEM.

Presumably, differences exist between *in vitro*-analyses (e.g. primary cells, cell lines) and *in vivo*-studies of tissue samples as well as of the different vessel types (e.g. aorta, arteriae) and vessel layers (tunica intima, media and adventitia). Therefore, it is reasonable that a clear separation of *in vitro* and *in vivo* results as well as those obtained in different species and vessel types are necessary to generate an expression map of nAChRs in the vascular system.

Furthermore, this study revealed, that there is no significant difference in the expression of the three investigated nAChR subunits in non-smokers and smokers, neither in ECs, nor in the VSMCs of the tunica media. In general, the effect of nicotine as a major component of cigarette smoke on the cells of the vascular system is very complex. For example, *in vitro* studies with cigarette smoke extract causes apoptosis of aortic VSCMs and aortic ECs in humans and rodent animals [6]. In contrast, it has been reported that nicotine inhibits apoptosis of aortic VSMCs and ECs through nAChRs [7]. Several studies have shown that nicotine stimulates the proliferation and migration of ECs, increase VEGF and FGF release by vascular VSMCs and ECs and promote angiogenesis. In turn, angiogenesis is regulated by VEGF receptor- and α7 nAChR-mediated pathways [6]. Although nicotine as exogenous agonist of nicotinic acetylcholine receptors in the vasculature is studied very well, clinical data on receptor expression under chronic nicotine exposure are rare. In addition, it has to be stated that nicotine is one of the major and well-studied components of cigarette smoke, but it is one of 4000 other ingredients [16].

The actual correlations between chronic nicotine exposure and the distribution and changes of nAChRs in the vascular system *in vivo* have to be clarified in further studies. A very

promising starting point for studying nAChRs *in vivo* is the usage of nAChR analogs in imaging systems. Bucerius *et al*. [17] evaluated a radiolabeled nAChR ligand for *in vivo* positron emission tomography (PET) imaging in humans, and quantified the uptake in the ascending and descending aorta, the aortic arch and the carotids. Although, these studies are designed to detect arterial disease and pathological changes in the vascular system, important insights can be generated about the distribution of nAChRs from these *in vivo* studies.

Conflicting interests

The authors have declared that no competing interests exist.

Abbreviations

DAB: 3,3′-diaminobenzidine; dNTPs: desoxy-nucleotides; ECs: endothelial cells; FGF: fibroblast growth factor; fw: forward; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; nAChR: nicotinic acetylcholine receptors; PCR: polymerase chain reaction; PET: positron electron tomography; PDGF: platelet-derived growth factor; rev: reverse; RNA: ribonucleic acid; RT-PCR: reverse transcriptase polymerase chain reaction; S.E.M: standard error of the mean; VEGF: vascular endothelial growth factor; VSMCs: vascular smooth muscle cells.

Author contributions

J.B. and F.M.M. conceived and designed the experiments. D.N. and S.v.S. performed the experiments and analysed the data. S.v.S. and M.T.D. wrote the paper. F.W.M. has given final approval of the manuscript.

References

1. Gotti C, Clementi F. Neuronal nicotinic receptors: From structure to pathology. Progress in neurobiology 2004; 74:363-396.
2. Lindstrom JM. Nicotinic acetylcholine receptors of muscles and nerves: Comparison of their structures, functional roles, and vulnerability to pathology. Annals of the New York Academy of Sciences 2003; 998:41-52.
3. Schuller HM. Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nature reviews. Cancer 2009; 9:195-205.
4. Schuller HM. Regulatory role of the alpha7nachr in cancer. Current drug targets 2012; 13:680-687.
5. Wang Y, Pereira EF, Maus AD, Ostlie NS, Navaneetham D, Lei S, *et al*. Human bronchial epithelial and endothelial cells express alpha7 nicotinic acetylcholine receptors. Molecular pharmacology 2001; 60:1201-1209.
6. Li ZZ, Dai QY. Pathogenesis of abdominal aortic aneurysms: role of nicotine and nicotinic acetylcholine receptors. Mediator Inflamm 2012; 2012:103120.
7. Cucina A, Fuso A, Coluccia P, Cavallaro A. Nicotine inhibits apoptosis and stimulates proliferation in aortic smooth muscle cells through a functional nicotinic acetylcholine receptor. The Journal of surgical research 2008; 150:227-235.
8. Li S, Zhao T, Xin H, Ye LH, Zhang X, Tanaka H, *et al*. Nicotinic acetylcholine receptor alpha7 subunit mediates migration of vascular smooth muscle cells toward nicotine. Journal of pharmacological sciences 2004; 94:334-338.
9. Pestana IA, Vazquez-Padron RI, Aitouche A, Pham SM. Nicotinic and pdgf-receptor function are essential for nicotine-stimulated mitogenesis in human vascular smooth muscle cells. Journal of cellular biochemistry 2005; 96:986-995.
10. Macklin KD, Maus AD, Pereira EF, Albuquerque EX, Conti-Fine BM. Human vascular endothelial cells express functional nicotinic acetylcholine receptors. The Journal of pharmacology and experimental therapeutics 1998; 287:435-439.
11. Gu Z, Fonseca V, Hai CM. Nicotinic acetylcholine receptor mediates nicotine-induced actin cytoskeletal remodeling and extracellular matrix degradation by vascular smooth muscle cells. Vascul Pharmacol. 2013; 58:87-97.
12. Duerrschmidt N, Hagen A, Gaertner C, Wermke A, Nowicki M, Spanel-Borowski K, *et al*. Nicotine effects on human endothelial intercellular communication via alpha4beta2 and alpha3beta2 nicotinic acetylcholine receptor subtypes. Naunyn-Schmiedeberg's archives of pharmacology 2012; 385:621-632.
13. Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. The Journal of clinical investigation 2002; 110:527-536.
14. Li DJ, Zhao T, Xin RJ, Wang YY, Fei YB, Shen FM. Activation of α7 nicotinic acetylcholine receptor protects against oxidant stress damage through reducing vascular peroxidase-1 in a JNK signaling-dependent manner in endothelial cells. Cell Physiol Biochem. 2014; 33:468-478.
15. Santanam N, Thornhill BA, Lau JK, Crabtree CM, Cook CR, Brown KC, *et al*. Nicotinic acetylcholine receptor signaling in atherogenesis. Atherosclerosis. 2012; 225:264-273.
16. Haussig S, Schubert A, Mohr FW, Dhein S. Sub-chronic nicotine exposure induces intercellular communication failure and differential down-regulation of connexins in cultured human endothelial cells. Atherosclerosis 2008; 196:210-218.
17. Bucerius J, Manka C, Schmaljohann J, Mani V, Gundisch D, Rudd JH, *et al*. Feasibility of [18f]-2-fluoro-a85380-pet imaging of human vascular nicotinic acetylcholine receptors in vivo. JACC. Cardiovascular imaging 2012; 5:528-536.