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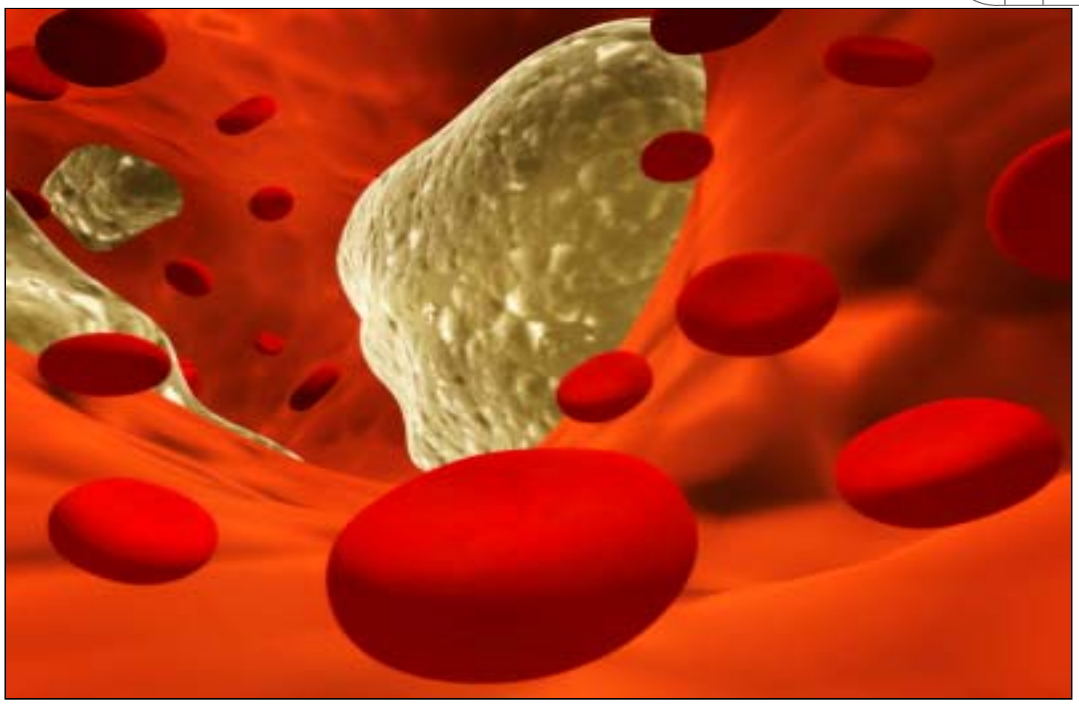

Picture: Obstructed artery and erythrocytes, the grayish mass in the middle depicts atherosclerotic plaque narrowing the lumen

Investigation of expression and function of alpha-7 nicotinic receptor as an anti-inflammatory target in atherosclerosis

-An approach to attenuate inflammation using alpha-7 nicotinic receptor as a primary tool

Master of Science thesis - Biotechnology

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Investigation of expression and function of alpha-7 nicotinic receptor as an anti-inflammatory target in atherosclerosis

UNIVERSITY OF GOTHENBURG

CHALMERS

This master thesis work is carried at the Sahlgrenska academy, Gothenburg University at the dept. of Physiology under the supervision of Dr. Maria Johansson

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“Live as if you were to die tomorrow. Learn as if you were live forever.”

— Mahatma Gandhi

Abstract

Atherosclerosis is a cardiovascular disease, the leading cause of mortality with an astonishing increase in death rate worldwide according to WHO. This is twice as many death cases caused by cancer. Atherosclerosis is a chronic inflammatory disease resulting in intense complications such as either a myocardial infarction or a stroke. It is deemed as inflammation process triggered by innate immune cells with response to pathogen invasion, tissue injury etc. The cholinergic anti-inflammatory pathway is a key regulator of inflammation by suppressing cytokine activity. It functions as a neural circuit aiding in immunomodulation process, reacting to and tuning inflammation to optimal rate. The pathway comprises two main components, the vagus nerve and neurotransmitter acetylcholine. Stimulation of efferent vagus nerve releases acetylcholine which later interacts with alpha-7 nicotinic receptor (α7nAChR) on macrophages. This binding leads to activation of intracellular signal transduction later inhibiting pro-inflammatory cytokine release.

Not much is known about function of α7nAChR in atherosclerosis. However few researches suggested their importance in anti-inflammation role. Thus the overall aim of this thesis was to detect and evaluate expression and function of α7nAChR using RT-PCR technique in healthy and atherosclerosis prone mouse aortas. Receptor stimulation in-vitro is carried out using nicotine, a potent ligand for α7nAChR. LPS stimulation was performed with or without combining effect of nicotine to evaluate receptor function in attenuating cytokine release.

The result indicated the expression of α7nAChR in normal and atherosclerotic prone samples with absence in α7R knockout mice samples suggesting presence of receptor for further evaluation on its function. The in-vitro stimulation failed to explain the function of receptor the in reducing released cytokines. Thus the report provides evidence implicating expression pattern of receptor in healthy and atherosclerotic samples. Further experiments are required to elucidate function of receptor on inflammatory profile of atherosclerotic plaque.

Keywords: Atherosclerosis, alpha-7 nicotinic receptor, Cytokine, Cholinergic anti-inflammatory pathway

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I turn my attention to prime part of this journey in saying ‘tack så mycket’ to everybody involved. Sure it’s been a wonderful six months of intense learning.

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Finally I’m ever grateful to my parents for trusting and providing me with mental and financial support to pursue my master degree and conclude with a project of interest.

Abbreviations

CVD	Cardiovascular disease
nAChRα7	Nicotinic alpha-7 acetylcholine receptor
LDL	Low density lipoprotein
HDL	High density lipoprotein
Ox-LDL	Oxidized low density lipoprotein
TNFα	Tumor necrosis factor-α
CNS	Central nervous system
ANS	Autonomic nervous system
NF-κB	Nuclear factor kappa B
JAK-STAT	Janus Kinase – Signal Transducer and Activation of Transcription
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-18	Interleukin-18
ApoE-/-	Apolipoprotein E knockout
α 7-/-	Alpha 7 receptor knockout
LPS	Lipopolysaccharide
VCAM	Vascular cell adhesion molecule
M-CSF	Macrophage colony stimulating factor
RQI	RNA quality indicator

YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta Polypeptide
HPRT	Hypoxanthine phosphoribosyl transferase
TBP	TATA box binding protein
WHO	World Health Organization
HFD	High Fat Diet
SD	Standard Diet

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1. Introduction

Cardiovascular disease (CVD) is considered as one of the prominent diseases in the world accounting to largest possible single cause of death, summing up to 17.3 million deaths in 2008 according to WHO. It is estimated around 23.6 million people will die from CVD by year 2030. On an average, in every 40seconds, someone in United States has a stroke [1]. The Coronary artery disease (CAD) and cerebrovascular disease are most communal form of CVD. Their underlying prime cause is atherosclerosis, an inflammatory process affecting the vessel wall [2-4]. Atherosclerosis can be defined as disease of artery, characterized by chronic inflammatory response with respect to accumulation of fatty material in the walls of the vessel [5, 6]. This leads to thickened, hardened and narrowed arteries. The inflammation mostly emerges at specific areas such as bifurcations, aortic arch etc, where a disturbed blood flow pattern exists. The inception of atherosclerosis occurs with a complex interactions of circulating factors and immune cells such as lymphocytes, monocytes and smooth muscle cells [7]. The leukocyte infiltration into the intima and production of pro-inflammatory cytokines marks initial plaque development process [8]. Atherosclerosis is a slow, progressive and complex disease initiated during childhood and progress as people grow old. This fatal process is preventable and treatable by adapting healthy lifestyle. Therefore new insight of the role of inflammation in atherosclerosis provides a perception in understanding clinical benefits in immune-suppression therapies. The quest for triggers of inflammation and their respective inflammatory pathways in pathogenesis of atherosclerosis can eventually help in prevention and therapy of the disease.

The greatest enigma for the disease being, absence of clinical symptoms for inflammation. Thus clinical manifestation for atherosclerosis fails to show up until an occlusion of blood flow occurs, resulting in an ischemic tissue.

The risk factors for atherosclerosis include both genetic and environmental factors, most significant being: Physical inactivity, diabetes, hypertension, hyperlipidemia, obesity and tobacco smoking, enhancing the risk of endothelial dysfunction [9, 10]

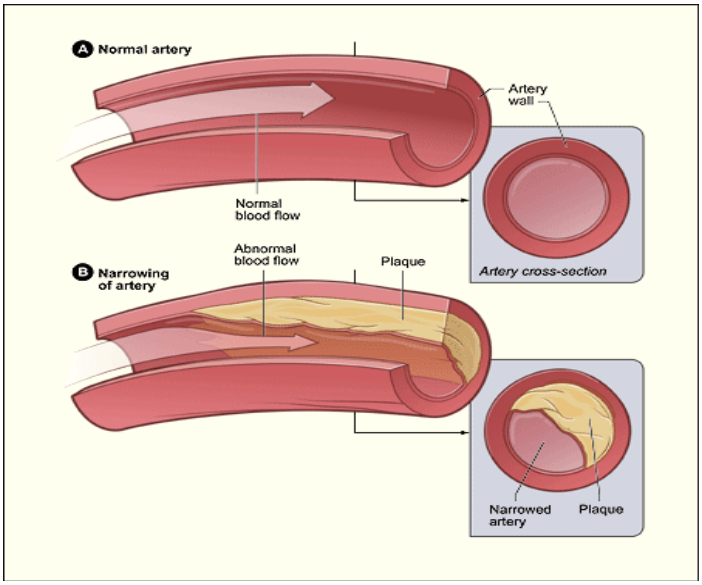


Fig 1: A) A normal artery with smooth blood flow B) Diseased artery with plaque buildup having abnormal blood flow. Picture adapted from: http://www.nhlbi.nih.gov/health/health-topics/topics/atherosclerosis/

1.1 *Atherosclerosis - An inflammatory disease*

Inflammation is a physiological response to pathological invasion [11]. The magnitude of inflammatory response plays a key role in maintaining homeostasis [12]. Homeostasis is renewed upon activation of anti-inflammatory response. In atherosclerosis the homeostasis is altered when the normal function of endothelium layer is modified, advancing inflammation. Years before, atherosclerosis was deemed to be a degenerative disorder of arteries that was inevitable with passive accumulation of cholesterol in vessel wall, but current scenario is complex, with disease being reckoned as a chronic inflammatory process mediating all phases of disease and having an active contribution to plaque rupture [2, 13, 14]. The immunohistochemical studies and test with inflammatory marker CRP manifested inflammatory activity in atherosclerotic lesions [15, 16].

1.2 *Anatomy of the artery*

In order to understand the process of atherosclerosis, it is necessary to know the structure and composition of an artery.

An artery wall consists of three layers as shown in fig 2 with each having a unique role and composition [17]

- *Tunica adventitia/externa*: Strong outer cover of artery composed of connective tissue with elastic and collagen fibers
- *Tunica media*: The middle layer, primarily consisting of smooth muscle cells with elastic fibers
- *Tunica intima/interna*: Innermost layer having a direct contact with blood. The layer is smooth with endothelial cells and consists of internal elastic lamina membrane which separates the intima from media. The fatty buildup takes place in tunica intima.

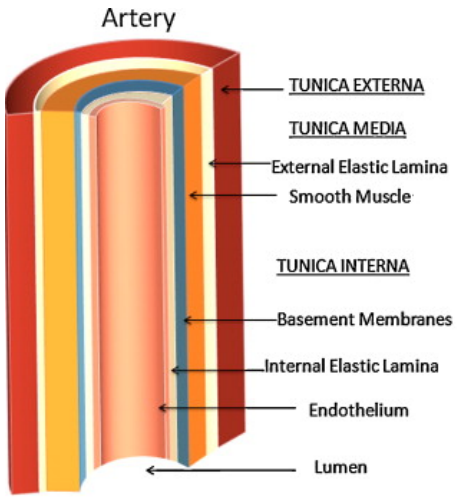


Fig 2 Layers and composition of artery. Picture modified from [17]

1.3 *Stages and mechanism in Atherosclerosis*

Initiation of atherosclerosis is complex [18] and thus there are principal stages contributing to the progression of disease.

Fatty streak & plaque formation

The initial stage present virtually in every person's early life where lipids, foam cells (macrophages) loaded with cholesterol and T cells gets deposited in the wall (in the sub-endothelial space) [19]. Cholesterol, a key player is responsible for the initiation of atherosclerosis through transportation by Low density lipoprotein (LDL) [20] which comprises infiltration of LDL's from blood to endothelial monolayer layer (tunica intima). The monocyte derived macrophage is the hallmark of fatty streak production and constitute a large proportion [20]. Fatty streak may either disappear or can transform into advanced lesion. This stage doesn't have an clinical symptoms initially, but later contributes for plaque formation [20]

The plaque formation is a result of complex cellular interaction between smooth muscle cells (SMC), endothelial cells and immune cells (leukocytes). According to the hypothesis of 'Response to retention' theory; the LDL particles penetrate endothelial layer and settles in the internal lamina by binding to proteoglycans. Later these molecules undergo extensive oxidative modifications by enzymes and oxygen radicals in intima layer, transforming into so called '*oxidized low density lipoprotein*' (ox-LDL) [18]. Upon transformation, they act as an pro-inflammatory agent, subsequently stimulating vascular cell adhesion molecule (VCAM) and also producing chemokine and pro-inflammatory cytokines [18]

The subsequent stage induces the migration of monocytes and T cells into intima soon after VCAM expression and is therefore a significant step in lesion development as shown in fig 3 (A). Under the influence of cytokines and induction of macrophage colony stimulating factor (M-CSF) secreted by endothelial cells and smooth muscle cells, the invaded monocytes transforms into tissue macrophages within sub endothelial space [5]. The resulting cells has a high ligand specificity for Ox-LDL and therefore phagocytose Ox-LDL via scavenger receptors transforming into *foam cells* [21]. Macrophages further secrete cytokines which helps recruiting more monocytes and T-cells, eventually amplifying inflammation [22]. This continued recruitment of T-cells and macrophages marks lesion initiation and progression. At later stages, the foam cell dies and constitutes the growth of plaque together with other extracellular matrix components [22]. Therefore a fully developed plaque contains high proportion of inflammatory cells

Later T lymphocytes, smooth muscle cells and platelets combined with foam cells broadens the plaque area [22]. Activation of smooth muscle cells triggered by inflammatory process due to deposition of fatty debris and macrophages leads to production of collagen. This eventually turns fatty streak to a fibrous plaque [20]. Thus thickening of vessel wall occurs as intense LDLs infiltration and foam cell formation occurs, where the real progression of the disease tunes in [20]

Plaque rupture & Thrombosis

The blood flow pattern and lipids are the driving force in this process. Inflammation also appeared to be associated with plaque rupture containing intense inflammation where inflammatory cells release various inflammatory products [23]. The macrophages are capable of releasing proteolytic enzymes, inflammatory cytokine TNF-α and larger amount of thrombosis initiator tissue factor. They also increase number of interleukin 2 receptor on T-cells which serves as a marker for T-cell activation in unstable lesions [23].

Plaques can be divided into clinically stable or vulnerable, depending upon their composition [23]. Stable plaques are characterized by high fibrous tissue and small amount of lipids, whereas on the other hand vulnerable plaques are made up of high lipid and absence of fibrous cap. The fibrous cap helps in structural integrity of plaque, however an intense build of plaque covered by a fibrous cap, an extracellular matrix rich in collagen can weaken and fissure with respect to secretion of matrix metalloproteinase's by inflammatory mediators, assisting in degradation of collagen and other matrix components [23]. Also high arterial blood pressure plays vital role in disintegration of plaque. Thus released plaque components and tissue factors, an pro-coagulant stimulating platelet aggression and thrombosis enters lumen eventually forming blood clots in lesion site which likely results in ischemic tissue as shown in fig 3(D) [2, 24]

A myocardial infarction occurs if a blockage occurs in one of the artery carrying blood to the heart, wherein a stroke can result if a carotid or cerebral artery gets obstructed through an embolus, showing the severity of disease [24]

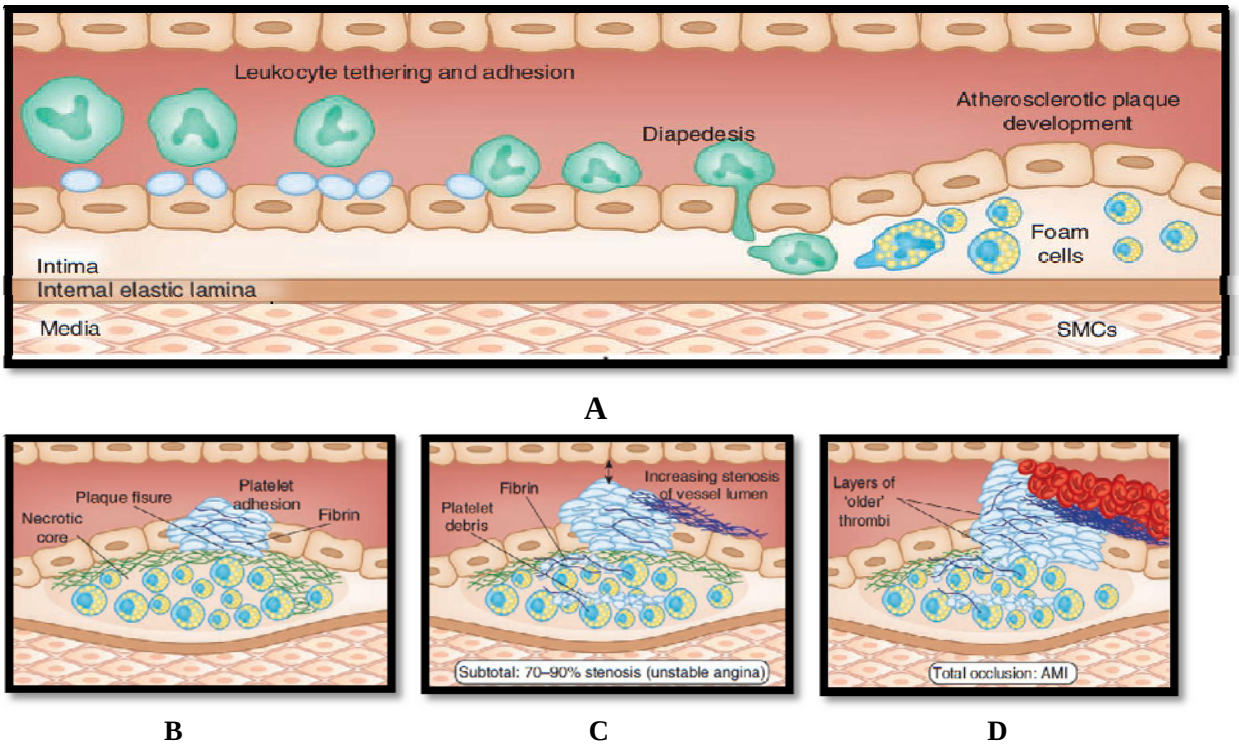


Fig 3: Depicting important events in plaque formation and rupture A) shows the migration of leukocyte from lumen to intima through endothelial layer with help of adhesion molecules. B&C) Lesion progression & vessel stenosis assisted by blood clotting factors D) Thrombosis. Picture adapted from [25]

1.4 *Macrophage - their role and significance*

Macrophages are important mediators of inflammation, assisting in cytokine release. They also widely contribute to lesion remodeling and plaque rupture through secretion of matrix metalloproteinase's [26]. The macrophages are potent cells involved in immune regulation, capable of producing pro-inflammatory cytokines upon specific stimuli [26]. Therefore the project intends in discussing macrophages over other immune cells since they play a diverse role in pathogenesis of atherosclerosis.

1.5 *The Neural-immune connection*

The inflammatory response is modulated through bi-directional communication between brain and immune system as shown in fig 4. This constitutes the neuronal way by which brain regulates immune system and in turn immune system modulating brain through cytokines [27]. This bi-directional communication forms a negative loop in healthy individuals thus retaining the balance. Changes in regulatory cycle can lead to either hyper immune response resulting in inflammatory disease or over suppression leading to infectious disease [28].

Thus in general, the immune system modulates inflammation through neural pathways; therefore a general understanding of nervous system is required to further elucidate the connective mechanism of these systems.

Central nervous system (CNS) & Peripheral nervous system (PNS)

The CNS is the processing centre of the nervous system, responsible for integrating sensory information from other parts of the body and responds accordingly. The CNS consists of the brain and the spinal cord and therefore serving as central unit. Whereas PNS makes up sensory nerves outside brain and spinal cord functioning in connecting and sending information to brain. PNS is subdivided into somatic and autonomic nervous system [29]

The autonomic nervous system (ANS)

The autonomic nervous system (ANS) consists of sensory neurons acting as a control system which functions under subconscious level. The action of ANS is highly involuntary, controlling various visceral organs such as heart rate, digestion and respiration etc. [12]

ANS is subdivided into Parasympathetic and Sympathetic nervous system [12]

The parasympathetic stimulates activities associated with 'Rest and digest' while the sympathetic is for 'Fight or flight response' [12]

The CNS plays a distinct role in modulating inflammation through activation of sympathetic and parasympathetic division of ANS. These nerves innervate various major organs.

Stimulation of these nerves by brain releases neurotransmitter at a particular site of inflammation. Thus there is a bridge between immune and nervous system [12].

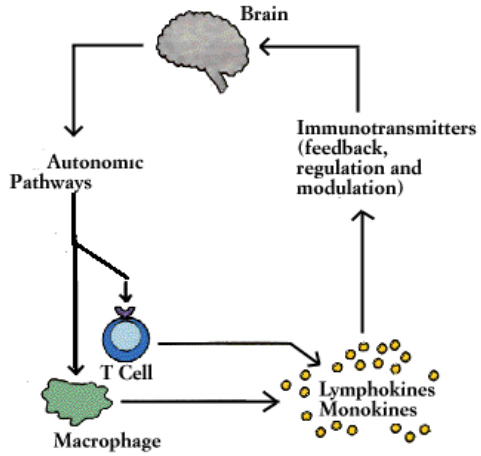


Fig 4 A bi-directional communication between brain and neural system. Picture modified from http://thyroid.about.com/library/immune/blimm28.htm

1.6 *Cholinergic anti-inflammatory pathway*

Though there are various notable mechanisms and pathways involved in regulating inflammation, the 'Cholinergic anti-inflammatory pathway' is established as an advanced neural mechanism having an substantial role in attenuating release of pro-inflammatory cytokines through inactivation of macrophages [11, 30]

The mechanism involves two important components, the *vagus nerve* and principal neurotransmitter *acetylcholine* (ACh) [11]. The vagus nerve is an important nerve of parasympathetic division, originating from brain innervating important organs of the body, responsible for maintaining homeostasis [31]. Connectivity between brain and immune system is modulated through nicotinic acetylcholine receptor α7sub unit (α7nAChR) [11], a homo-pentameric ion channel cholinergic receptor [32] found on macrophages, highly sensitive to cholinergic agonist's acetylcholine and nicotine.

Inflammation at a localized site sends signal to the brain through afferent part of vagus nerve and the counter anti-inflammatory output then reaches the specific site [12]. Thus combining effect of afferent and efferent vagus nerve is highly crucial for receiving and sending information concerning inflammation from periphery to brain, and to counteract by release of anti-inflammatory signal, a mechanism so called 'inflammatory reflex' [33, 34] illustrated in fig 5.

The mechanism is initiated, once information about inflammation at a specific site is conveyed to brain by afferent vagus nerve [27]. This activates the parasympathetic nervous system resulting in stimulation of cholinergic nerve fibers of innervated efferent vagus immediately acting and releasing acetylcholine at the site [28]. The neurotransmitter then interacts with α7nAchR located on surface of macrophages and other immune cells [35]. This mechanism attenuates pro-inflammatory cytokines TNF, IL-1, IL-6 and IL-18 etc, with no effect on anti-inflammatory cytokines IL-10 [35]

Attenuation of pro-inflammatory cytokines via α7nAchR is mediated through two important pathways: The NF-κB (pro-inflammatory) and Jak-STAT pathway (anti or pro inflammatory) [11, 31, 36]. Studies have shown, α7nAchR receptor attenuates the pro-inflammatory TNF release and other important inflammatory cytokines in human macrophages through nicotine [11] .Yoshikawa *et al*., [36] demonstrated the expression and function of α7nAchR in LPS stimulated human monocyte by examining the attenuation of pro-inflammatory cytokine release, inhibited by nicotine through α7nAchR. In addition, Wang *et al*., [37] showed the comparison of functional outcome of α7nAchR in α7nAchR knockout and wild type mice. The study gave an insight of serum level of TNF in α7nAchR knockout mice which was much higher compared with wild type endotoxaemic mice. Thus in conclusion, knocking out α7nAchR fails to reduce inflammation through vagus nerve, indicating significance of the protein in anti-inflammation therapy [11, 37]

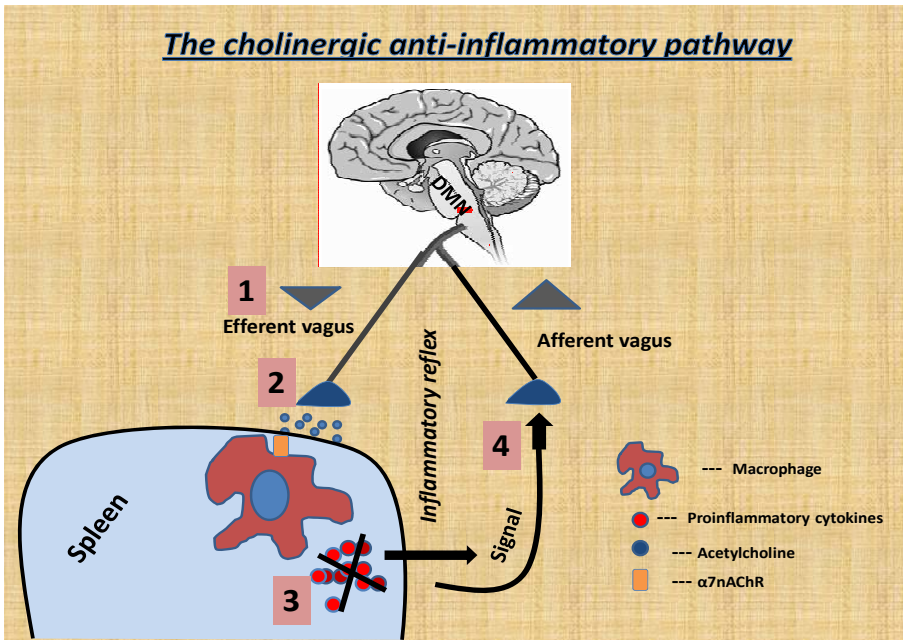


Fig 5: Shows the mechanism behind the pathway 1) Signal from the brain passes through the efferent vagus nerve 2) Release of neurotransmitter acetylcholine at the site of inflammation where it then binds to α7nAchR present on surface of macrophage 3) Attenuation of pro-inflammatory cytokines due to suppression of NF-κB pathway 4) The output information concerning pro-inflammatory cytokine release is then conveyed to the brain through afferent vagus nerve, thus maintaining homeostasis.

1.7 *Nicotine alpha 7 acetylcholine receptor structure, expression and role*

Nicotinic acetylcholine receptors belongs to super family of five trans membrane domain of neurotransmitter-gated ion channels formed by combination of subunits α and β, forming either homo or heteropentameric receptors [32]. They play a prominent role in synaptic communication and intracellular signaling [38]. The α7nAChR is a homo-pentameric ion channel receptor and the only α-bungarotoxin (antagonist) sensitive receptor found on

Macrophages [32]. Subunits of receptor collectively form a homo-pentameric structure forming a principal ion pore at the centre, making way to calcium and sodium permeability. Binding of acetylcholine and other agonists leads to change in receptor conformation resulting in channel opening by which ions pass through [38].

The receptor is suggested to be an important target for opening up the possibilities of treating inflammatory disease. Pathway transition using receptor is mediated through vagus nerve, with neurotransmitter acetylcholine acting on it [38]. Studies have proven that vagus nerve stimulation has failed to attenuate pro-inflammatory cytokines in alpha 7 knockout mice [11]. Nicotine is agonists for the receptor. Further, studies have proven the efficiency of nicotine in assisting receptor for attenuating pro-inflammatory cytokine release [37].

*2. **Hypothesis & Aim***

Not much known about function of α7nAChR in atherosclerosis but research suggests their importance in regulating inflammation. The speculation being, that stimulation of α7nAchR present on macrophage and subsequent activation of cholinergic anti-inflammatory pathway can reduce cytokine release. Previous studies [38, 39] have shown a promising effect of receptor in various inflammatory diseases [36]. Thus dose dependent treatment of nicotine for α7nAchR can lower release of pro-inflammatory cytokines. So we presume that α7nAchR can have a therapeutic effect for atherosclerosis through anti-inflammatory activity.

Experimental studies imply that intervention with immune mechanism could be used to hinder progression of atherosclerosis [40], but however existing knowledge is insufficient for expanding successful therapeutic strategies. If in-depth research is centered on investigating immune mechanism and vascular inflammation, a key to the lock can be established.

Thus the overall aim of this project is to evaluate expression and also function of α7nAChR in attenuating pro-inflammatory cytokine (TNF-α) release from macrophages, thereby serving as an anti-inflammatory target in atherosclerosis. Special emphasis was placed on finding expression pattern of receptor in healthy and atherosclerotic prone mouse aortas by real time PCR. Receptor stimulation in-vitro was carried using nicotine, a potent ligand for α7nAChR. LPS stimulation was performed with or without combining effect of nicotine to evaluate receptor function in attenuating cytokine release.

*3. **Methods***

3.1 *Animals*

Mouse models

Male apolipoproteinE knockout (ApoE-/-) mice was used in this study, since they are prone to develop atherosclerosis [41] and α7 receptor knockout (α7-/-) mice, were gene for α7nAchR is knocked out is used as negative control for analyzing receptor expression. Thus in total 20 ApoE-/-, 16 C57BL/6 and 9 α7R-/- were employed for the study.

The ApoE-/- mice (Taconic Trangenic Models™ strain B6.129P2- Apoe^{tm1Unc}/N11 Taconic, Denmark), α7-/- mice (Jackson Laboratories, Maine, USA) and C57BL/6 mice (Taconic,

Denmark) were all housed at 21-24ºC room with 12h light/12 h dark cycle. All animals had free accesses to food and water. The study was approved by Regional animal ethics committee at Gothenburg University, Gothenburg Sweden in accordance with European Communities council Directives of 24 November 1986 (86/609/ECC)

3.2 *Experimental design*

A subgroup of the ApoE-/- mice (n=10) were fed a high fat diet, ie Western diet, with a composition: 21% fat and 0.15% cholesterol, R638 Lantmännen, Sweden for 6 weeks. The rest of the mice's used were fed with normal standard diet.

Upon arrival, all animals were allowed to recover for one week before experiments. ApoE-/- and C57BL/6 strains were received at 7weeks of age and are sacrificed the following week. The ApoE-/- mice were sacrificed 13 weeks of age respectively. In-order to confirm the knockout of gene, 9 α7-/- mice were used for the study.

All mice were sacrificed by injecting intraperitoneally with pentobarbital (Apoteksbolaget, Sweden 0.9mg/g BW)

Harvesting of tissue

The connective tissue around the heart was removed and the blood is withdrawn using a syringe. A cannula was inserted into left ventricle as soon as the saline flow was turned on. The right ventricle is then cut free to rinse the blood out from the system. The perfusion was carried out until the liver turned pale and harvesting of Spleen, heart, aorta and carotid arteries were then carried out.

The heart was embedded for future lesion quantification. Aorta was considered as a primary tissue of interest in this study. All samples were stored at -80C for further use. The remaining C57BL/6 mice were sacrificed for in-vitro stimulation experiments

3.3 *RNA extraction and quality analysis*

Total RNA was extracted from aorta samples using Fibrous mini tissue kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. In brief, frozen aorta's were introduced into eppendorf tubes containing RLT lysis buffer and were completely disrupted and homogenized using tissue lyser® (Qiagen, Hilden, Germany) with a frequency of 20 Hz for 2 min's, for a period of 4 cycles of run. The arrangements of tubes were modified where the outermost tubes are placed innermost and vice versa to maintain complete homogenization. The RNA extraction was then carried according to standard protocol using fibrous mini tissue kit. A volume of 40 µl was extracted for RNA quality analysis and cDNA synthesis.

RNA concentration and quality was measured using Nano-drop® (Wilmington, USA) and Experion® (Bio-Rad, Hercules, USA). The measurement with Nano-drop was done by first tuning the machine to zero using MilliQwater, and then loading 1 µl of RNA sample for

analysis. Furthermore second detailed quality check was performed using Experion® (Bio-Rad, Hercules, USA). The Experion is an automated electrophoresis system which involves lab on chip micro fluidic technology, where the samples loaded onto a micro fluidic chip containing wells for liquid to be measured. The chip was then placed on electrophoresis station. The quality was then measured and a detailed report containing a virtual gel and an electropherogram were generated with a RNA quality indicator value (RQI) for each sample.

3.4 *Reverse Transcription*

Based upon quality check with Experion analyzer, samples having an RQI (RNA quality indicator) of 7.5 and above out of 10 were chosen for cDNA synthesis. About 90% of all samples crossed the barrier level with 2 C57BL/6, 2 ApoE samples failed to do so with an RQI value of 6.5. Single stranded cDNA were synthesized from 0.3 µg total RNA using Quanti-Tect Reverse transcription kit (Qiagen®) according to manufacturer's recommendations. The conditions for synthesis were 2mins at 42°C (for DNA elimination reaction), 15 mins at 42°C (c DNA synthesis), 3 mins at 95°C (to inactivate Quantiscript reverse transcriptase) and at least 5 mins at 4°C (Cooling)

3.5 *Real time quantitative PCR*

Reactions were setup using SYBR green PCR master mix (Qiagen, Inc, Hilden, Germany), a ready to use PCR mix containing all required compounds to initiate PCR. The gene expression was detected using CT value. A CT is the threshold cycle value which reflects specific cycle number at which the fluorescence produced in the reaction attains the threshold level [42].

CT values for control and experiemental groups were then utilized to calculate the mean difference between the groups using delta delta CT method [43]. The primer for chrna7 gene was purchased from Qiagen Inc, QuantiTect primer assay for SYBR green based real-time PCR. The cDNA was subjected to 55cycles of run using Light cycler 480 real time PCR system (Roche®, Basel, Switzerland) and was analyzed using Light cycler 480, software 1.5.0SP3. The number of cycles for each run was adjusted based upon the type of primer used. A typical set of cycling conditions were 95ºC at 10 s and 60ºC at 30 s.

The mouse strains used for validating receptor expression were C57BL/6, used as a positive control, ApoE-/- on high fat diet and standard diet to check during atherogenesis and α7R-/- for negative control of gene expression. A housekeeping gene was used to normalize the samples. Therefore in total three housekeeping gene run was performed in order to find a reference gene that is stable. The gene primers used were YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide), HPRT (Hypoxanthine phosphoribosyl transferase) and TBP (TATA box binding protein) but eventually YWHAZ was chosen, since the gene was less influenced by strain and diet compared to other two housekeeping gene's. All Samples were run in duplicates. Apart from detection of gene expression, the study was extended to inspect the expressional level variation between each experiemental groups.

Data analysis

All data were expressed as mean ± S.E.M. The difference was checked for statisitical significance ($p < 0.05$) using t-test and one-way analysis of variance (ANOVA) with Graph pad prism v5 statistical software (GraphPad Software, Inc, California, USA)

3.6 *In-vitro stimulation*

The prime objective of stimulation with LPS and nicotine was to examine the functionality of receptor mimicking an inflammation condition in-vitro. The released cytokine in the supernatant after subsequent stimulation was then evaluated for TNF-α cytokine release.

Total aorta was harvested and divided into 4 pieces A, B, C and D from C57BL/6 mice as illustrated in fig 6, i.e. the thoracic part is divided onto A, B and C and the whole abdominal part D is kept as such with bifurcations for some samples. Each tissue was then introduced into an eppendorf tube containing cell culture medium RPMI1640 (Sigma Aldrich, St. Louis, Missouri) supplemented with 10% FCS (Sigma Aldrich).

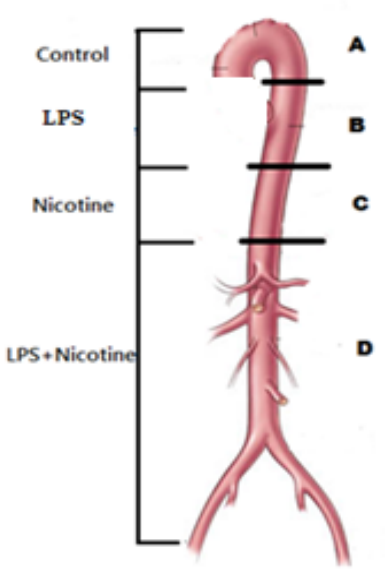


Fig 6: Illustrates the total divided aorta and type of stimulation performed on each. Thoracic part: A, B and C and abdominal part: D
Note: For some samples the bifurcations does not exist.
Picture adapted from http://my.clevelandclinic.org/heart/disorders/aorta_marfan/aortaillust.aspx

24 well tissue culture plates with flat bottom (SARSTEDT Inc, Nümbrecht, Germany) were used for in-vitro tissue stimulation. As illustrated in fig 6, four types of stimulation were carried out. A thorough cleaning of tissues was performed by washing 3-5 times with a new culture media. An LPS solution from E.coli-L4130 (Sigma Aldrich) with a final concentration of 100 ng/ml was added to cell culture medium RPMI1640. A final working concentration of

100 µg/ml of Nicotine, N3876 (Sigma Aldrich) was introduced into 500 µl of cell culture media with or without LPS. A well with only medium was used as a control. 100 ng/ml final concentrations of LPS were used for mimicking tissue inflammation. For inspecting receptor functionality two wells with a combination of LPS (100 ng/ml) and nicotine (100 µg/ml) were employed.

After appropriate stimulation, tissues were then washed with PBS and dried using paper towel. Each tissue was then weighed to normalize it with protein content. Finally all tissues were snap frozen in liquid nitrogen and stored at -70°C for later analysis.

The plate was incubated with standard condition of 37ºC and 5% CO2 in an enriched humidified atmosphere. In total four different stimulation runs were performed based upon varied incubation time such as 4, 15, 18 and 24 hours to verify optimal stimulating period needed for full receptor functionality.

3.7 *ELISA*

ELISA (Enzyme- Linked Immunosorbent Assay), a biochemical assay was performed to identify protein of interest using antibodies. In this project sandwich type ELISA was used to determine the amount of TNF-α protein released from stimulated tissues.

All experimental procedures were carried according to manufacturer's recommendations using ELISA MAX standard Biolegend kit, mouse TNF-α (Biolegend, London, UK)

Day 1: A 96well plate was coated with primary antibody (capture antibody) which then binds onto plate surface as shown in fig 7. The plate was later sealed and incubated overnight at 4°C

Day 2: Antigens (sample and standards) were incubated for 2 hours, which later binds to pre-coated capture antibodies.
A secondary detection antibody specific to antigen was then introduced, binding to the antigen, thereby forming a sandwich (primary antibody, antigen and secondary antibody)
An enzyme capable of binding to secondary antibody was added. Substrate specific to enzyme was then added resulting in formation of enzyme-substrate complex. This later emitted fluorescence upon enzyme-substrate reaction, fig 7.
The absorbance was read at 450 nm using Spectra Max M2 micro plate reader, Molecular device, (Sunnyvale, CA, USA)

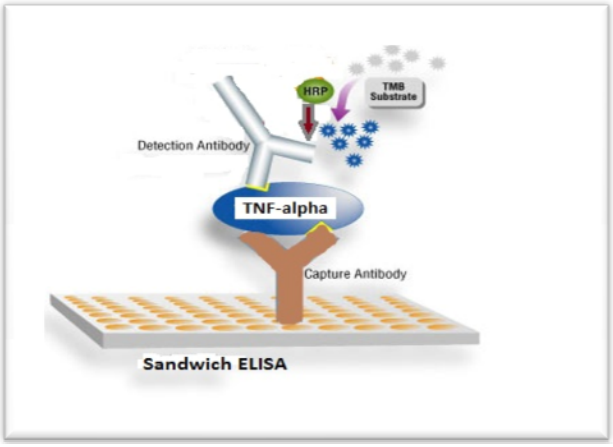


Fig 7: Illustrates the process behind sandwich ELISA. Picture modified from: http://www.neb-test.de/en/index.php?option=com_content&task=view&id=14&Itemid=20

3.8 *Pathway analysis*

Since the production of cytokines from macrophages and immune cells were regulated through different inflammatory pathways involving diverse key regulatory molecules, an attempt to learn and explore various pathways and molecules tuned during cytokine release was executed.

The bio-informatics online tools and resources such as NCBI GEO, DAVID pathway analysis, KEGG pathways were used respectively.

The NCBI GEO online database was used as a search tool, consisting of massive amount of publically available microarray datasets. Keywords related to α7nAChR were given as an input for data search to get a list of published article's supporting the work.

Paper of interest was selected from the hit list and 'Series matrix file' – an experimental data file which contains processed normalized data was then downloaded.

A minor adjustment in data such as removal of unwanted numbers and phrases on top and bottom part of datasets is done to avoid error. The data file is then directly loaded onto statistical software, MeV (Multi experimental viewer). Statistical and clustering analysis was done using the software to produce a list of significant genes.

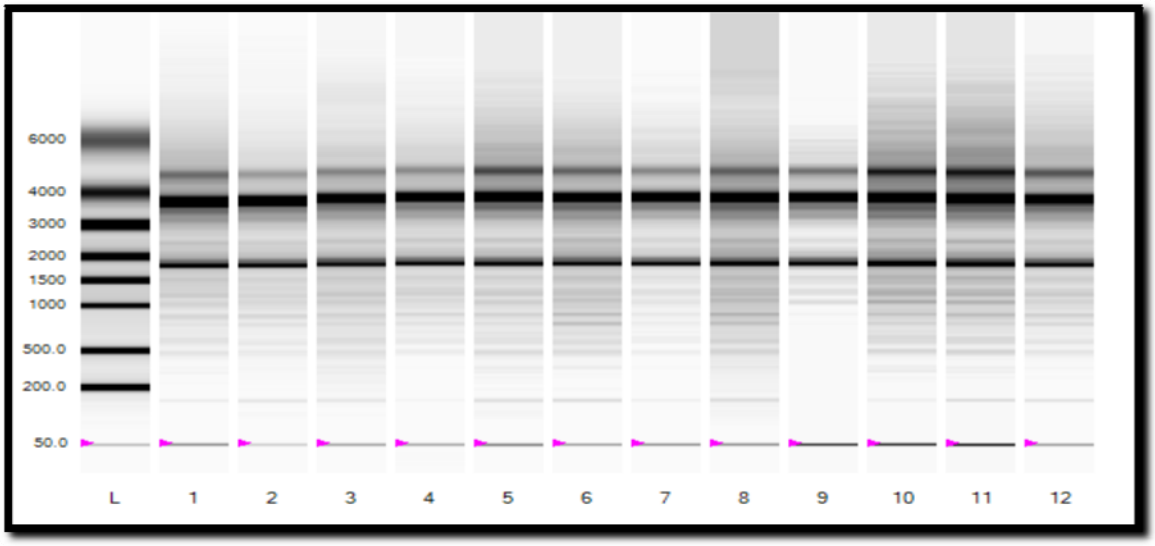
The significant gene list produced is then uploaded onto an online pathway analysis tool (David functional annotation tool) to obtain a number of pathways & molecules involved in regulation of cytokine release. A step wise explanation on use of DAVID tool is discussed in [44]

4. Results

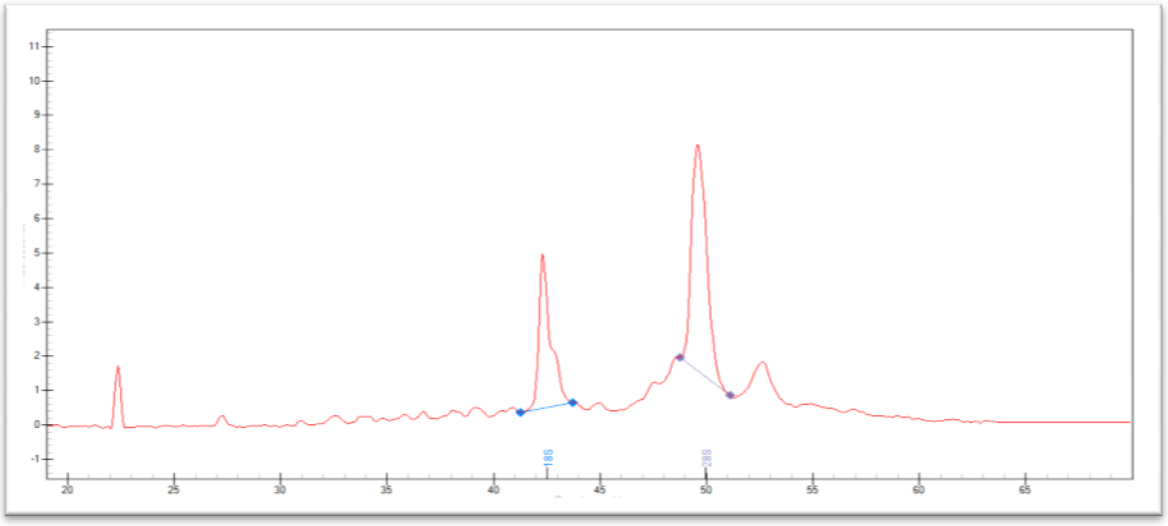
4.1 *RNA quality*

The RNA analysis results obtained from Experion (Bio-Rad) were in the form of both virtual gel image and electropherogram as shown in fig 8. As mentioned earlier, samples with RQI value of 7.5 and more were used for PCR run. The RNA quality was best with all samples free of protein contamination. The concentrations were in the range of 20 ng/µl to 60 ng/µl for wildtype and α7nAChR knockout mice. A range of 35 ng/µl to 90 ng/µl was obtained for ApoE-/- mice

On the negative side, results were quite enigmatic with an unexpected additional extra band found close to 28s band on all sample as shown in fig 8. We suspected that there might be a contamination during RNA extraction process or from the quality analyzer electrodes.



A



B

Fig 8: A) Virtual gel showing 18s and 28s ribosomal RNA band of an ApoE-/- mice aortic sample with an additional lighter band close to 28s band suspected as contamination

B) Electropherogram of an ApoE-/- mice aorta sample where the measured fluorescence directly reflects on amount of RNA. Image shows 18s and 28s ribosomal RNA slopes with a little slope close to 28s slope.

4.2 *Nicotine acetylcholine receptor α7nAchR is expressed on mouse aortic tissue*

α7nAchR mRNA expression were found in wild type, and ApoE-/- on Standard diet and High fat diet mouse(fig 9). The knockout samples failed to show-up for the receptor. However, the expression of the α7nAchR was unaltered across different strains, regardless of diet. Thus the outcome for receptor expression provided an insight in further investigating on expressional variation across groups.

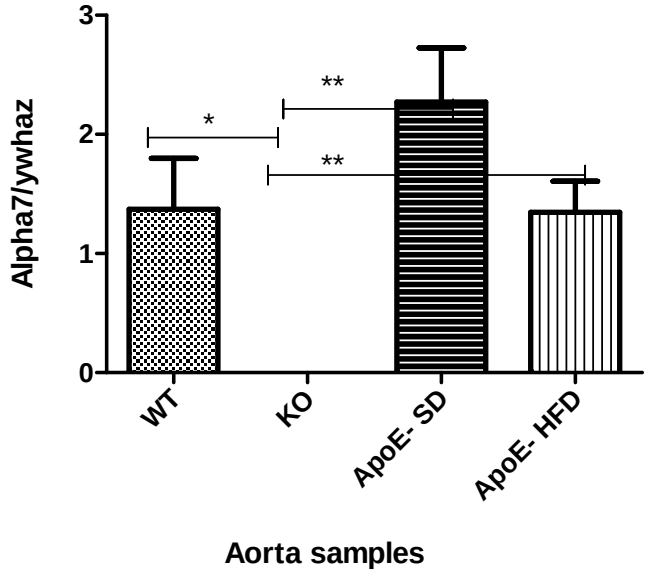


Fig. 9: α7nAChR mRNA expression for control and experimental groups in aortic samples. Data represents mean ±SEM values, (n=8) for ApoE standard diet and (n=6) for rest of the groups. WT- Wild type, KO- Knockout, SD- Standard diet, HFD- High fat diet. Alpha7/ywhaz: Target gene normalized with housekeeping gene ywhaz. * p-values ≤ 0.05, ** p-values ≤ 0.01.

4.3 *TNF-α cytokine analysis*

The ELISA results failed to provide evidence for anti-inflammatory function of α7nAchR in reducing pro-inflammatory cytokine release. The results were quite suspicious with no colour change on samples upon TMB substrate addition during ELISA experiment, with almost all sample concentration below detection level. Therefore no conclusion is drawn.

4.4 *Signaling Pathway Analysis*

The exploration for possible α7nAchR pathways was unsuccessful as there were constraints such as minimal datasets for the receptor and datasets with low significant gene hits. However some pathways for other nicotinic acetylcholine receptor were found such as leukocyte migration pathway for α4nAchR etc, although they are not an integral part of this project. The pathways found to have specific regulated molecules in a highlighted status but no further explanation is provided since it's considered away from current research.

5. Discussion

Our RT-PCR finding provided evidence on the expression of α7nAchR in mouse aorta; however the expression level was unaltered by strain or different diet. Moreover the knockout samples failed to show up confirming the lack of the receptor in knock-out animals. The assessment of RNA integrity and quality is an essential step for downstream assays, therefore we showed an overall good RNA quality with an excellent RNA integrity and protein free RNA, despite harvesting and handling mice aorta which is quite challenging.

In detail, the 28s and 18s rRNA bands were sharp and consistent for all samples. With 28s rRNA band approximately twice the size of 18s r RNA, which is a good indication of complete intact RNA.

To eliminate additional bump, which was considered as a possible contamination, DNAs treatment were performed on some samples to eliminate possible DNA contamination. Furthermore, the Experion electrodes, which come in contact with the sample, were cleaned thoroughly to ensure contamination free analyzer. The additional bump eventually still lasted after cleaning process; however it seems as it has not influenced during RT-PCR run.

On the other hand, we were unsuccessful in finding the postulated mechanism of α7nAchR in attenuating pro-inflammatory cytokine release through in-vitro stimulation and ELISA experiments.

As previously discussed, this was due to Samples under detection level despite multiple ELISA runs which is suspicious. Consequently, the substrate incubation time was increased to detect color change and the result remained the same. Therefore we performed multiple ELISA runs with a wide range of sample concentrations and further investigated with varying successive in-vitro stimulation time of 18 h and 22 h to ensure optimal TNF-alpha release. The results were again unaltered with all samples below detection level. Therefore we are unable to further conclude on receptor activity in attenuating TNF-α release. However samples incubated for 22 h had higher TNF-α level compared to sample with 4 and 18 hours stimulation. Therefore we are certain about good concentration of target protein TNF-α release in supernatant which were more than detectable mark, since various time stimulations are performed on tissues. We found 22 hrs stimulated time to be optimal compared to 4, 18 h and 24 h.

The pathway analysis for α7nAchR was also unsuccessful due to minimal data information. In some cases, microarray data sets were too few to obtain sufficient significant genes for further analysis. Therefore we hope more experimental microarray data for α7nAchR will be available in near future.

Advantage using pathway analysis:

•To compare the difference between healthy and diseased tissue samples.
•Check out whether interested pathway/Gene is regulated or not.
•Manually sorting out list.

6. Future direction

From previous studies [31, 36, 37] it is evident that α7nAchR plays a prominent role as an anti-inflammatory mediator. Therefore understanding latent immune mechanism paves the way for discovering novel therapeutic drugs. Intense research is under progress in discovering therapies implicating immune system as key player in both initiation and progression of diseases. My near future plan is to repeat experiments for validating the functional role of Receptor in reducing inflammation. The long term research perspective will be intended in expounding significance of the receptor in various time slots of atherosclerosis in reducing inflammation. It will also be necessary to check for more ligand which can be more specific than nicotine for α7nAchR target. As a starting point it is sensible to find the expressional variation of α7nAchR across different treated samples. Another goal will be to find additional layers of information that will be required to examine the pathways and molecules involved in cytokine regulation which might open a new insight to target specific molecules.

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