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The expression profile of WNT3A in venous insufficiency
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June, 2024

Approval

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

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ABSTRACT

The expression profile of WNT3A in venous insufficiency.

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Wnt3a, as a member of canonical Wnt ligands, regulates different cellular functions such as cell proliferation, differentiation, self-renewal and motility, It has been shown that Wnt3a activates both RhoA ATPase, as a component of non-canonical Wnt signaling, and Wnt/β-catenin signaling pathway in different cell types, A subsequent study reported that the Int gene and wingless gene in Drosophila were homologous genes Therefore, both genes were recognized as the Wnt gene These genes encode secreted glycoproteins that are rich in cysteine, Wnts can combine with cell membrane receptors that play a critical role in autocrine regulation and/or participate in paracrine modification by binding to adjacent cell membrane receptors. The signal transduction pathway mediated by Wnt genes is called the Wnt signaling pathway. The Wnt signaling pathway can be divided into canonical and noncanonical pathways. The canonical pathway is also called the Wnt/β-catenin pathway. A total of 68 WNT 3A samples, seven from the control and patients were collected. the real-time PCR settings used to measure the level of gene expression. Amplification conditions for the genes were optimized and primer dimers were obtained following real time PCR analysis. I have a total of sample 68 wnt3a: 1–29 are from our control (wnt3a venous insufficiency) and 30-68 patient wnt3a venous insufficiency the analysis yielded a two-tailed P value of less than 0.0007, which is considered extremely significant, and a t-value of < 0.05 with 27 degrees of freedom. The result gathered in this study according to the gene expression analysis of the WNT3a signal genes (WNT3A,) However, a decreased expression of WNT3A, and an increased expression in wnt3a when compared to the control (wnt3a venous insufficiency) and patient wnt3a venous insufficiency.

Keywords: Venous Insufficiency**,** Wnt Signaling Pathways**,** Wnt3a Protein**,** Protein**,** Endothelial Function**,** Gene Expression Analysis**.**

LIST OF ABBREVIATION

Wnt-Wingless+Int-1

Fz-Frizzled

Dsh-disheveled

DNA-Deoxyribonucleic acid

DKK1-dickkopf-related protein 1

ILC-Invasive Lobular Cancer

RNA-Ribonucleic acid

Cdk3-Cyclin-dependent kinase 3

β - catenin.

β- Beta

ODZ-outside development zone

ER-Endoplasmic reticulum

HLA-Human Leukocyte Antigen

APC-Adenomatous Polyposis Coli

GSK3-Glycogen synthase kinase 3

CK1α-Casein Kinase 1 Alpha

LRP5-Low Density Lipoprotein Receptor Related Protein 5

PCP- Polarity Cell Pathway

DAAM1- Dishevelled Associated Activator of Morphogenesis 1

CGM- Cyclic Guanosine Monophosphate

DVT-deep vein thrombosis

VI-venous insufficiency

Wnt 3-Wingless 3 family member

Wnt 4 - Wingless 4 family member

LEF-lymphoid enhancer-binding factor

TCF-T-cell-specific transcription factor

cDNA-complimentary Deoxyribonucleic acid

Bp-Base pair

EDTA-Ethylene Diamine Tetra-acetic Acid

TAE- Tris Acetic Acid EDTA ml-Milliliter mg-Milligram µl- Microliter PCR-Polymerase ChaiTAE- Tris Acetic Acid EDTA

Ml-Milliliter

Mg-Milligram

µl- Microliter

PCR-Polymerase Chain Reaction

QRT-Quantitative Real Time

Ct-Cycle threshold Reaction

DH2O- Deionized Water

Ct-Cycle threshold

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CHAPTER I

INTRODUCTION

Background of the study

The Int/Wingless family member 3A (WNT3A) is a canonical Wnt ligand (a secreted glycoprotein) that controls a variety of cellular processes, including motility, self-renewal, differentiation during embryonic development cell growth and proliferation. Wnt3a stimulates the canonical Wnt/β-catenin signaling pathway in several cell types as well as RhoA GTPase, a part of non-canonical Wnt/Calcium signaling (Pashirzad et al., 2019). Wnt3A signaling is integral to endothelial function and vascular remodeling (Reis & Liebner, 2013)

The Wnt gene was first found in mice as a new mouse proto-oncogene and has been termed the Integration 1 (Int 1) gene(Nusse & Varmus, 1982). Wnt was determined to be a homolog of the Drosophila wingless gene were found to be similar genes in a later investigation but in different manner as Int1 (Khramtsov et al., 2010) Consequently, it was determined that both genes were Wnt genes (Cadigan & Nusse, 1997)Later, The Wnt family, which is an acronym for "wingless-related integration site family," was formed by combining the letters int and wingless (Wg). Int1 became Wnt1, Wnt1, Wnt2, Wnt2b [Wnt13], Wnt3, Wnt3a, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a [Wnt14], Wnt9b, Wnt10a, Wnt10b, 11, 16, Wnt9b, Wnt10a, Wnt10b, 11, 16. Secreted glycoprotein growth factors, wnt proteins bind to the N-terminal extracellular cysteine-rich domain of the Frizzled (Fz) receptor family, of which there are ten Fz in humans. The extracellular Wnt signal initiates many intracellular signal transduction cascades(Khramtsov et al., 2010).

Members of the Wnt family of act in an autocrine and paracrine way or through activation of several signaling cascades in the target cells. The extra-cellular Wnt proteins bind to Wnt membrane receptors named the Frizzled (Fz) receptor family (approximately ten Fz in humans). The Wnt receptors are capable of joining with the receptors on the neighboring cell membrane that are both important for autocrine and paracrine action of signaling mechanisms. named "Wnt Signaling Pathway". Wnt family member 3 A (Wnt3A) Signaling Pathways, comprising both canonical (known as the Wnt/β-catenin pathway) (Nusse & Clevers, 2017)

Non-canonical pathways play a pivotal role in various cellular processes, including vascular development and disease. Wnt3A signaling is integral to endothelial function and vascular remodeling (Reis & Liebner, 2013)

Wnt family proteins are cysteine-rich, a modified-lipid (palmitoleic acid) attached proteins. This modified lipid by a palmitoyl transferase which acts as a binding motif for Wnt receptors in the canonical pathway.

In canonical pathway, Wnt proteins bind to a receptor complex of two molecules on the cell membranes: the Frizzled (Fz or FZD) receptor of the cell's surface, which is a 7-transmembrane protein with a cysteine rich domain and a single-pass transmembrane lipoprotein receptor-related protein co-receptor (LRP5/6). Wnt ligand interaction causes two receptors to dimerize and undergo conformational changes, which phosphorylates the LRP5/6 cytoplasmic domain. By several protein kinases including two Ser-Thr protein kinases (CK1α/δ and GSK3 α/β) on PPPSP motif on a serine. Several Wnt protein signaling pathway proteins such as beta-catenin, tumor suppressor proteins The Adenomatous polyposis coli (APC) and Axin, have the same motif. Upon the phosphorylation, the cytoplasmic tail of LRP recruits Axin. The cytoplasmic part of Fz receptor activates and binds the scaffold protein Disheveled (Dsh) that provides a stable platform for the binding of LRP to Axin (Nusse & Clevers, 2017).

The cytoplasmic protein β-catenin (Catenine family) have two functions: catenin binds to actin filaments intracellularly and anchors the cadherine glycoproteins, which mediates calcium-dependent cell-cell adhesion to the cytoskeleton; act as a transcription factor for the activation of the proteins promoting the cell growth. β-catenin is the key component in the canonical pathway, and (DC), complex. β-Catenin binds to the destruction complex that contains a large tumor suppressor protein Adenomatous polyposis coli (APC), which activates cell proliferations and are found in most sporadic human colon cancers (Kim et al., 2013)

Together, the scaffold proteins Axin1 and 2 with APC protein, β-catenin, CK1α/δ, and GSK3 α/β serine-threonine kinases form the destruction complex. The suppression of GSK3β phosphorylation causes β-catenin to separate from the destruction complex and phosphorylated β-catenin gradually stabilize in the cytoplasm. As a result, there is a total increase in β-catenin concentration within the cytoplasm. Afterward, it translocate into the nucleus; it forms a complex with T-cell-specific transcription factor and lymphoid enhancer-binding factor, then activates the target gene (Aberle et al., 1997).

The Wnt/β-catenin system is, on the other hand, one of the more conserved signal pathways within the evolutionary systems of organisms and represents an important pathway in embryonic development for the regulation of cell proliferation and differentiation. It also works with the formation of polarity in neural development and in cancer. Abnormal activation of the Wnt/β-catenin signaling cascade has been linked with the initiation and progression of an impressive spectrum of cancer forms. Also, β-catenin is known as one of the essential proteins for the regulation downstream target genes of Wnt/β-catenin. The recognition of the nuclear accumulation of β-catenin as a biomarker for cancer prognosis has been established (Franco et al., 2009).

Problem Statement:

The common vascular diseases include venous insufficiency (VI), which describes a condition of poor blood flow in the venous system. Venous insufficiency has some of its consequences usually as a result of edema and ulceration. The involvement of the majority of other signaling pathways in vascular health has been well-studied, and Wnt3As have been implicated in several vascular processes. Preliminary studies suggest a potential link between the aberrant expression of Wnt3a and the development of venous insufficiency, warranting further investigation (Rodriguez & Smith, 2019). However, especially Wnt3a role in venous insufficiency remain underexplored (Lee & Patel, 2020). Little has been done on the expression patterns of Wnt3a in venous insufficiency and the possible effects on key cellular and molecular functions (Kuchenbaecker 2014).

This ignorance presently hampers the elaboration of oriented therapeutic solutions by the ability to understand in full the molecular pathways which are at the basis of venous insufficiency. Accordingly, it is pertinent to identify the expression patterns of Wnt3a in venous insufficiency, assess their possible associations with the pathophysiology of the ailment, and unravel any downstream signaling pathways they might activate (Kim et al., 2021). We might learn more about the biology of venous insufficiency and perhaps even identify new diagnostic markers and novel therapeutic strategies by filling in these gaps (Thompson & Nguyen, 2022; Carthy et al., 2012; van Amerongen, 2012). This study aims to bridge this gap, offering new perspectives on the disease.

Objective of the study

In this study, the level of expression of Wnt3a between venous tissues in patients suffering from venous insufficiency will be determined. The comparison between the patient and control groups will be examined to explore the relationship between the severity of venous insufficiency and Wnt3a expression accordingly, whether Wnt3a can be used as biomarkers for

venous insufficiency prognosis and diagnosis will be assessed by analyzing the data.

Aim of the Study

This study intends to explore the Wnt3a roles in the VI pathophysiology and their potential as therapeutic targets.

The aim of the study was to establish the relationship in the venous insufficiency expression profile, wnt3a, this was done in order to determine the expression profile of the wnt3a genes related to venous insufficiency.

Research Questions Hypothesized

1. It is thought that the investigation of the expression levels of Wnt3a in the venous tissues of venous insufficiency patients could provide the possible roles of the Wnt3a in the pathogenesis of venous insufficiency?
2. The correlation between Wnt3a expression and venous insufficiency severity could be analysed and it could be contributed to understand the molecular pathways by which Wnt3a contribute to venous insufficiency. ? Finally, when it is applicable the data will be analysed to assess whether Wnt3a could be considered as potential biomarkers for the prognosis and diagnosis of venous insufficiency.

Limitation of the Study

The sample size, including the count of patients and control of WNT3A samples, could influence the study's statistical strength and its ability to be generalized.

CHAPTER II

LITERATURE REVIEW

Importance of Understanding *Wnt3a* in Venous Insufficiency

Key details that have been brought forth in relation to the Wnt family of proteins, especially Wnt3a, are vivid in vascular biology the definition remains precise, but evidently, based on initial research, the possible relationship of these proteins in the development of venous insufficiency has been noted. All these will be of great help in a detailed understanding of the molecular mechanisms that underline venous insufficiency through the assessment of activities of Wnt3a this information could lead to new understandings of the condition's diagnosis and course of therapy, potentially completely changing the way that care is provided. Wnt3a may also be indicators for early diagnosis and prognosis, which are essential for efficient management and treatment, (Geudens & Gerhardt, 2011)

Understanding Venous Insufficiency

The prevalence of venous insufficiency is higher in older adults, affecting around one in twenty people. If one has gone through any or all of the following, the risk of getting venous insufficiency increases: past deep vein thrombosis (DVT—blood clot) and varicose veins, limited mobility, either totally or partially, due to arthritis or paralysis, obesity, vein inflammation (phlebitis). This can happen for a variety of reasons, such as after certain illnesses, after surgery or after childbirth, leg fractures or certain orthopedic procedures, congestive heart failure prior experience with vascular procedures like vein ligations or varicose vein "stripping(Geudens & Gerhardt, 2011)

Venous Insufficiency

The primary characteristic of venous insufficiency, a vascular disorder, is the veins' incapacity to return blood from the lower limbs to the heart in an efficient manner. A range of symptoms are associated with this disorder, from pain and swelling in the legs to the formation of varicose veins and, in more severe cases, skin ulcers. Its incidence is notable, impacting a considerable

segment of the adult population; older people and specific occupational groups are more likely to experience it. Venous insufficiency has an effect that goes beyond just physical symptoms; it also causes psychological suffering and high medical expenses (Pourjafar & Tiwari, 2024).

Pathophysiology

Vascular insufficiency is a pathology that entails a complex interplay of genetic, cellular, and molecular components. These components interact to determine the disease's progress and symptoms. In particular, Wnt signaling pathways regulated by Wnt3 are crucial for vascular development and homeostasis; disruption of these pathways may be a factor in venous insufficiency. The canonical Wnt/β-catenin signaling pathway, which is crucial for cell differentiation, apoptosis, and proliferation, is mostly associated with Wnt3. In venous incompetence, these variations in the Wnt3 expression could transform the anatomical stability of walls and valves of the veins, impacting their operational efficiency. For example, the activation of pathological Wnt3 signaling may be related to pathological vascular remodeling, which is one of the primary causes of venous hypertension and venous valve dysfunction, here modifications might make the blood clot in the lower limbs worse, hastening the progression of the disease thus, from the gene expression profiles of Wnt3, it would seem that the role may lie in the processes underlying venous insufficiency. Reduced expression of these genes could express itself through lesser functioning of these mechanisms of healing, and a higher expression could associate with a compensatory response to repair the damaged venous tissue Although the exact contribution of Wnt3 to the process of venous insufficiency is not clear, they have been reported to be involved in vascular biology, therefore making them good candidates in playing a part in the disease Hence, the pathophysiological effect of the expression of Wnt3 is patent since it aids in identifying potential targets for treatment in venous insufficiency. Activation of these Wnt-signaling pathways could be manipulated as a therapeutic strategy, possibly for venous insufficiency, and alleviate symptoms in attempts for normal return to venous

function. Further research will, therefore, be needed so as to be able to fully elaborate on the exact mechanism that would lead to the venous insufficiency brought about by Wnt3 and even look at the therapeutic benefits that may be obtained on targeting these pathways (Tomanek, 2005)

Pathophysiology and Molecular Mechanisms

Several biological and mechanical components are engaged in an intricate pathophysiology of venous insufficiency. The major mechanical pathway includes malfunctioning venous valves, which result in venous hypertension. A number of biological processes include the alteration of expression of many proteins and signaling pathways, inclusive of endothelial dysfunction, inflammation, and the Wnt pathway. Growing evidence from molecular studies points to persistent inflammation and venous wall remodeling as causative role in the pathogenesis this disease (Arapatzi et al., 2022). Insufficient venous, especially in the lower limbs, is a condition that results because of the failure of the venous valves to function in the proper circulation of venous blood back to the heart, leading to venous stagnation.

General Mechanisms of Wnt Signaling Pathways

Wnt3a is a member of the Wnt family of proteins that are key components in the regulation of Wnt signaling pathways. The Wnt signaling pathways control a wide array of biological processes such as cell migration, angiogenesis, differentiation, and proliferation, and crucial in embryonal vascular development and vascular repair (Lagendijk & Hogan, 2015)

Wnt signaling network comprises two major pathways: the canonical (β-catenin dependent) and the non-canonical (β-catenin independent) pathways.

The canonical (β-catenin dependent) pathway:

The canonical pathway, primarily associated with cell proliferation and differentiation, has been studied in the context of vascular smooth muscle cell behavior and endothelial cell function. In the canonical pathway, Wnt proteins interact with the co-receptor LRP5/6, as well as the Frizzled receptors, and

activate the canonical pathway by stabilizing β-catenin and causing translocation into the nucleus. The translocation would affect gene transcription, hence affecting cell fate and its development. In such cases, however, β-catenin is dispensable for acting in mechanisms of activation participating in the non-canonical pathway, with mechanisms including planar cell polarity and calcium signaling, (Kotini et al., 2019).

The non-canonical pathways that are independent of β-catenin are: The non-canonical pathways, which include calcium (Wnt/Ca+2) signaling and planar cell polarity (PCP), are triggered without the need for β-catenin and are more associated with cell movement and organization, playing roles in endothelial cell alignment and vascular barrier integrity.

Rho is involved in the ATPase superfamily member RhoA, which mainly involves in the fibrile formation and cell focal adhesion and Jun Kinase (JNK) involves in cell growth control and apoptosis (Eelen et al., 2015)Profiling, small actin-binding proteins, are involved in cytokinesis, membrane trafficking and motility. Non canonical Wnt/Ca+2 pathway is less characterized comparing The canonical pathway the likely refers to the calcium signaling pathway, which plays a crucial role in various cellular processes, including muscle contraction, neurotransmitter release, gene expression, and cell growth and differentiation (Eelen et al., 2015).

Importance of Wnt Signaling in Vascular Development and Pathology

Wnt signaling is the critical element to the developmental, disease vascular biology of the embryo, including the regulation of different vascular systems' development and differentiation. In adult physiology, they take part in the maintaining of vascular homeostasis but are implicated in the pathophysiology of several vascular disorders, including atheroma, venous insufficiency, and arterial stiffness among the aberrations of normal Wnt signaling. Indeed, new research has supported this relationship, and modulation may be a promising avenue of treatment (Brown and Johnson, 2021).

Specific Functions of *Wnt3a* in Vascular Biology

The Wnt family of proteins, like Wnt3a, participate and plays a vital role in the vascular biology. In that sense, it has been well known that Wnt3a can have an action on the endothelium to get the angiogenic behavior of the endothelial cells, which ultimately leads to the phenomenon of angiogenesis, i.e., the splitting of generally, it interacts with the official Wnt signaling pathway. It contributes to the differentiation and proliferation of endothelial cells. The latter is important in the development of arteries in tissues, embryonic, and adult that are in need of repair. It was found to be more important in vascular remodeling and particularly during the endothelial-to-mesenchymal transition with respect to Wnt5a, although it is also engaged in angiogenesis (Olsen et al., 2017)

Evaluation of Current Therapies in Light of Wnt3a Research

The evaluation of current therapies within the context of Wnt3a research reveals critical insights and underscores the potential for innovative treatment strategies. Many physiological processes, including cell division and proliferation, depend on Wnt3a signaling, and its implications for diseases including cancer and degenerative disorders are better understood. It is feasible to reassess the efficiency of the medications now used to control this pathway and perhaps get better outcomes. Moreover, the discovery of Wnt3a's function opens up possibilities for further study that focuses on specific elements of this pathway. New agonists or inhibitors that precisely modify Wnt3a signaling may be developed as a result of these studies, offering targeted treatment options. Including these findings in clinical trials and adjusting treatment regimens appropriately could eventually improve patient outcomes and increase treatment efficacy (Zheng et al., 2019).

Canonical vs. Non-Canonical Wnt Signaling

It would, therefore, have to be an absolute must that these differences are fully understood, as the roots of giving the several roles it plays in both vascular health and illness. The function of endothelial cells and behavior of smooth muscle cells in the vascular system have been sought to relate to the canonical route that holds specific reference to the proliferation and differentiation of cells. The non-canonical pathways maintain the permeability of the vascular barrier and the aligning of the endothelial cells. These are most likely associated with the organization and motility of cells. Contrary to that, the vascular pathology is complicated in a way that both pathways are open to communication and not exclusive (Chae & Bothwell, 2018).

Gene Therapy and Molecular Interventions

Examine the viability, moral issues, and long-term effects of these strategies. RNA-based Therapies: Examine how RNA-based therapies such as RNA interference (RNAi) can be used to either increase or decrease the expression of Wnt3a. Think about how you could enhance results by combining these techniques with more conventional treatments (Liu et al., 2016)

CHAPTER III

MATERIALS AND METHOD

Permission to conduct the survey was given by the Scientific Research Ethics Committee at Near East University (YDU 2024/124-1840). Written informed consent was given by each respondent included in the study.

Collected tissue vein

Vascular material listing the patient and control group samples are obtained as a part of a procedure research study in an association from the Near East University Hospital, Department of Cardiovascular Surgery. For this, participants in this study the patient group were selected as a possible participant in this study because you require an operation or removal of a saphenous vein as varicose treatment operation. Control group were selected as a possible participant in this study because you require an operation or removal of a saphenous vein as coronary bypass operation. Aiming this research outcome would be useful in optimization of the treatment of vascular diseases. These samples were examined to determine the levels of expression Wnt3a in tissues and of venous insufficiency, and these levels were compared to the levels of expression of the housekeeping gene or the reference gene beta actin. Samples were taken in two separate batches and assigned to either the control group or the patient group.

This case-control study was conducted at Near East University Hospital (NEUH) involving 68 (39 patient and 29 control) great saphenous vein samples obtained from patients who underwent varicose vein and bypass surgeries

Equipment in the laboratory: Here in is the information on the materials and methods that were used in the experiment

- Laminar Air Flow Cabinet (Ankara, Turkey)
- PCR Cabinet (Ankara, Turkey).
- Nano-drop™ 2000/2000c Spectrophotometer (Thermo-scientific, Pittsburg, USA)
- Rotor Gene Real-Time PCR (Qiagen, Hilden, Germany)

Oligonucleotides

Utilized primers were obtained from hibrigen company (Turkey)

RNA Extraction

During this, the tissue samples were first mechanically ground, followed by homogenization in 1 ml of TRIZOL reagent for every 50-100 mg of tissue. The tubes in which the sample was carried were tightly capped, and 0.2 ml of chloroform was added to 1 ml of TRIZOL reagent. Subsequently, the tubes were again incubated after vortexing without an interval for 15 s for another 2 to 3 min at room temperature. Finally, incubation of the tubes was done for 15 min in the centrifuge under maximum force of 12,000 ×g. The temperatures during the process were in the range of 2 to 8°C. Centrifugation of the mixture yielded the biphasic solution that comprised the red-colored lower phase, the interphase, and the colourless upper phase, the aqueous solution. The top aqueous phase, which was where the RNA was located, was carefully transferred into fresh tubes without disturbing the interface. Isopropyl alcohol was added to allow the RNA to precipitate out from the aqueous phase. 0.5 millilitres of that solution were added to each 1-millimeter of the spent TRIZOL reagent. The resulting mix was then set aside, within an incubator, for between fifteen and thirty degrees Celsius and incubated for ten minutes. After centrifugation at a maximum force of 12,000 x g for 5 min at a temperature of 2 to 4°C, a pasty-gelatinous deposit composed of RNA was obtained. It was absent in the sample prior to centrifugation and had gathered aside and at the bottom of the centrifuge tube. Specifically, the obtained RNA

pellet was rehydrated in 75% ethanol. The tube was swirled vigorously at room temperature. At least 1 ml of 75% ethanol was added into the used TRIZOL reagent, vortexed, and centrifuged out. The mixture was centrifuged two times maximum speed of 7,500 x g for 5 minutes at the range of temperature between 2 to 8°C. This was done to make sure that all the ethanol that was remaining had evaporated. After that, the RNA pellet was dried by air, and in this case, it was avoided from staying for long to make sure that it did not overdose dry. The RNA was then eluted by applying 50 µl of DNase-RNase free water on to the pellet.

Complementary cDNA synthesis

cDNA synthesis was conducted at the Near East University Medical Genetic Diagnostic Laboratory in Nicosia, North Cyprus. The Hibrigen total nucleic acid isolation kit was used to treat wnt3a venous insufficiency in in line with the guidelines provided by the manufacturer (Hibrigen, Turkey). The cDNA was extracted from each sample. With the lysis of the cell buffer, the sample was further processed before the extraction of cDNA. The cDNA was added to chloroform after it was first incubated in ice to not be degraded. The solution was first vortexed and centrifuged, and then the DNA and chloroform were separated with ethanol. Finally, the solution was washed and centrifuged. The purity and concentration of the eluted RNA were measured through a Nano-drop spectrophotometer as stated by the manufacturer (USA, Pittsburgh, and Thermo-Scientific). With this method, C-DNA was synthesized for C-DNA synthesis kit (Hibrigen, Turkey). All these processes took place as standard cDNA synthesis. cDNA reverse transcription reactions were carried out with the use of reverse transcriptase, a cDNA template, a reaction mixture, and a manufacturer's recommendation to the latter without deviations.

Table 1. Describes the Protocol for cDNA Synthesis

Component	1x
Enzyme Mix	1 µL
Reaction Buffer	4 µL
Nuclease-Free dH20	10 µL
Total RNA	5 µL

Table 2. Showcases the Polymerase Chain Reaction Condition for cDNA Synthesis

Step	Temperature	Time
cDNA Synthesis Minutes	42°C	60
Inactivation of Kit Minutes	80°C	10

Analysis of gene expression: The Real-time PCR was performed on cDNA to measure the level of gene expression with **gene specific the primers. The real-time Polymerase chain reaction (RT-PCR) experiments:** The samples following nucleic acid extraction and cDNA (gel electrophoresis) synthesis were examined by real-time PCR. Was carried out by using Light Cycler 2X SYBR Green I Master KiT. The reverse and forward primers were used at a concentration of 0.5µL (as per the optimized protocol. In the PCR, the analysis of the melting curve will assist in giving a clear margin between the primer-dimer and the final product. The primers were designed in-house before the experiments were performed

PCR with gradients

The investigation of various annealing temperatures was carried out using gradient PCR in this work. Using a temperature gradient ranging from 56°C to 62°C, several annealing temperatures were evaluated in order to identify the most ideal temperature for the primers. This process makes it easier to figure out the ideal temperature for the target sequence's amplification to be most effective. This guarantees the choice of an annealing temperature that optimizes the PCR's effectiveness within the parameters of the experiment.

Table 3 Sequences of Gene Specific Primers

WNT3A	
Forward primer	GAGCAGGACTCCCACCTAAAC
Reverseprimer	AGACACTAGCTCCAGGGAGGA

Table 4 Specifies PCR Cycling Conditions Gradient PCR

	PCR procedures	Temperature ^{0}C/ Time	Cycles
	Initial Denaturation	96^{0}C/ 10 minutes	**1**
	Denaturation	96^{0}C/ 1:30 seconds	**35 cycles**
	Annealing	62 ^{0}C/ 30 seconds	
	Extension	**72 ^{0}C/ 45 seconds**	

TABLE 5.Gradients PCR CONDITION FOR PRIMER DIMER

PCR mix	Concentration
Forward primer	0.5 µMl
Reverse primer	0.5µMl
Master mix	12.5 µl
dH_20	6.5 µL
cDNA	10 ng/µl

The parts and corresponding volumes utilized in a polymerase chain reaction (PCR) experiment to maximize the conditions for WNT3 gene amplification are displayed above. Referred to as "Table 2: PCR optimization for WNT3," the following entries are included in this table: An example identifies the PCR's target, "Wnt3" in this instance, which most likely refers to a particular gene or transcript that is being amplified. Introduction: shows the reaction's forward primer volume, which is 0.5 µL in this case. DNA synthesis is started by short DNA sequences called primers.

Reverse Primer: Similarly, 0.5 µMl is the volume indicated for the reverse primer. A particular DNA segment between these primers can be amplified by using both forward and reverse primers. Deionized water, or dH2O, is utilized in this instance in a volume of 6.5 µL to get the reaction mixture up to the necessary final volume, guaranteeing appropriate enzyme activity and DNA handling. Complementary DNA, or cDNA, is employed in the procedure at a volume of 10ng. Made from mRNA, cDNA is a template for amplification that represents the expression of a gene.

Table 6 Optimized Conditions for gradient PCR

	PCR procedures	Temperature ^{0}C/ Time	Cycles
Steps	Initial Denaturation	96 ^{0}C/ 10 minutes	1
	Denaturation	96^{0}C/ 1:30 seconds	35
	Annealing	62 ^{0}C/ 30 seconds	
	Extension	72 ^{0}C/ 45 seconds	35

Real-Time PCR

The thermal cycling conditions for real-time PCR encompass the detailed temperatures and durations for all steps of the amplification process along with the number of cycles for each step.

Initial Denaturation: This step involves heating the reaction mixture to 96°C for 10 minutes. It is performed once (1 cycle) and is crucial for denaturing the double-stranded DNA template into single strands, allowing the primers to anneal in subsequent steps.

Denaturation: Following the initial denaturation, regular cycles of denaturation occur at 96°C, each lasting 1 minute and 30 seconds. This step is repeated for a specified number of cycles (not clearly indicated in the text but typically aligned with the number of annealing and extension cycles) to ensure that the DNA template strands are separated in each cycle to allow new strand synthesis.

Annealing: During this phase, the temperature is lowered to 62°C for 30 seconds, whereby primers bind or anneal onto the specific complementary sequences on the single-stranded DNA template. This step is one of the more critical phases that confer specificity to the amplification process, and as such, it is repeated 35 times.

Extension: Extension from the primer complementary to the DNA template strand takes place at 72°C for 45 seconds by synthesizing a new DNA strand by DNA polymerase enzyme. This step is also repeated for 35 cycles.

The table provides a comprehensive overview of the thermal cycling conditions necessary for the efficient and specific amplification of DNA in a gradient PCR setup, which is often used to identify the optimal annealing temperature for a given set of primers and template. The settings used were in measuring the quantity of gene expression. For the final primer concentration of 0.5 µl was used, the temperature of annealing reached, as shown in table 5. Primer dimers were observed. This led to a change in real-time PCR conditions.

PCR Optimization

Parameters PCR Setup Specific amounts and concentrations of the reagents involved in the reaction make part of the PCR setup. For The WNT3A gene, both the forward and the reverse primers are used at a final concentration of 0.2 µM. A volume and condition for the reaction was set with 6.5 µl of deionized water (dH2O). The amplification template is cDNA of 10 ng/µl. This optimization will also be crucial to ensure the effective and specific amplification of the WNT3A gene, which is very important for further analyses. It is needed that enough primer concentrations are used under the

appropriate reaction conditions so as to reduce the occurrence of non-specific amplification, and thus provide maximum yield of target gene production. All specified volumes and concentrations are optimized and confirmed to provide a real reliable and reproducible result in PCR experiments.

Table 7 CONDITIONS FOR qPCR

Reagents	***WNT3A***
SYBR- Green	12.5 µl
Forward primer	0.5 µM
Reverse primer	0.5 µM
dH_2O	6.5 µl
cDNA	10 ng/µl
Temperature	62℃ Annealing
Time	1:30 sec

CONDITIONS FOR qPCR

WNT3A PCR optimization" and enumerates the specific reagent volumes and conditions tailored the quantitative polymerase chain reaction (qPCR) amplification of the WNT3A gene. This table serves as a protocol guide for setting up a qPCR reaction, detailed as follows:

SYBR-Green: The table lists 12.5 µL of SYBR-Green, a fluorescent dye used to quantitatively measure the presence of double-stranded DNA, indicating the progression of DNA amplification during the PCR.

Forward Primer: A volume of 0.5 µMl of the forward primer is used to initiate DNA synthesis from the specific start point on the DNA strand that complements the target sequence.

Reverse Primer: Similarly, 0.5 µMl of the reverse primer is used to bind to the opposite strand of the DNA, allowing synthesis in the reverse direction, thus enabling the amplification of the target sequence.

dH2O: 6.5 µL of deionized water is added to the reaction mixture to achieve the necessary total volume and dilution for optimal enzyme activity.

cDNA: 10 ng of complementary DNA (cDNA) is used as the template for the reaction. cDNA is synthesized from mRNA and represents the gene expression profile that is being quantified.

Temperature: The optimal annealing temperature for the primers to bind to the cDNA is listed as 62°C.

Time: The duration for which the annealing temperature is maintained during each cycle is 1 minute and 30 seconds. This table is essential for researchers aiming to precisely replicate the qPCR conditions for studying the expression of the WNT3A gene, ensuring consistency and reliability in the results obtained across different experimental runs.

Analytical statistics

To do statistical analysis, Graph Pad Prism 10 version 10.1.2(324) was used. The mean plus standard error (SE) was used to express the data. The gene expression data were generated based on cycle threshold values, the number of cycles where a logarithmic graph of a PCR crosses a threshold line. In the present study, the relative expression of each gene was compared among different fat depots using the 2AACT method: AACT = CT of the target gene - CT of the housekeeping gene. Continuous variables will be the student's t-test for normally and abnormally distributed differences. In all statistics, a value of $p<0.05$ is considered significant.

CHAPTER IV

RESULT

In this chapter basically on the review of the results from the experiment. This provides the following numerical and graphical data on re-reviewing the results from real-time PCR analysis using the student's test statistical method: control.

Table shows the details of the *WNT3A* used in this project. DNA was successfully extracted from each sample and cDNA was synthesized. The concentration of cDNA samples is shown in tables.

Figure 1 qPCR

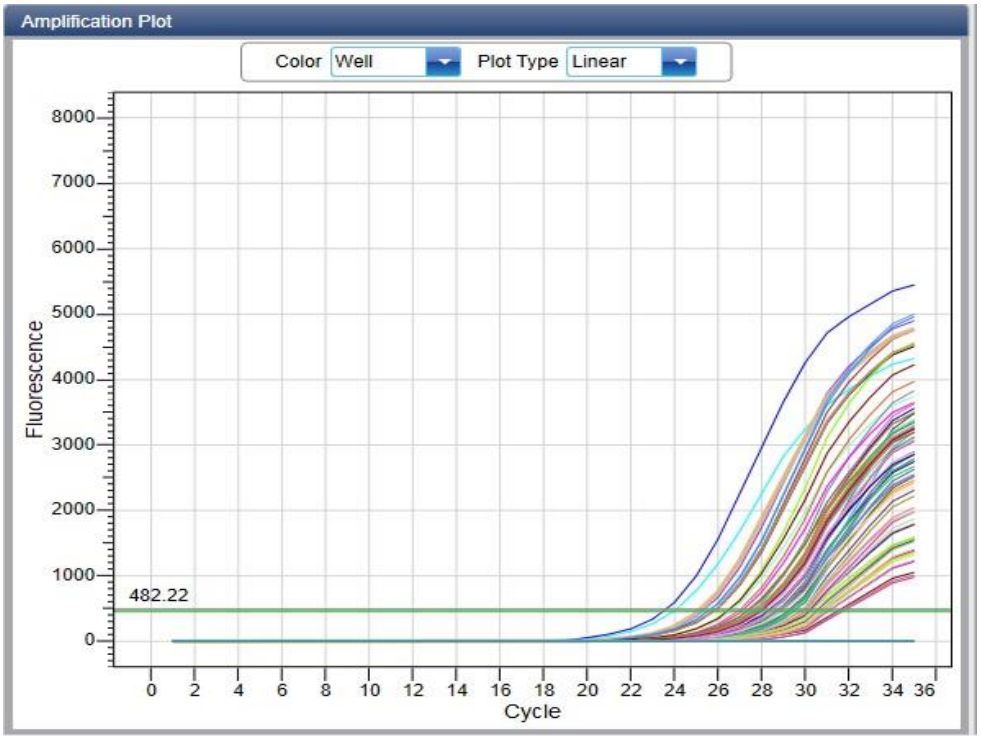


Table 8.

Correlation Analysis of Control Cases and Housekeeping Gene: Wnt3a

ACTB (Control Participants) vs. *Wnt3a* (Control Participants)	
Spearman r	
R	0.1609
95% confidence interval	-0.2293 to 0.5065
P value	
P (two-tailed)	0.4045
P value summary	Ns
Exact or approximate P value	Approximate
Significant (alpha = 0.05)	No
Number	29

The correlation analysis between ACTB expression levels in control participants and Wnt3a expression in the same cohort is quantitatively summarized using Spearman's rank correlation coefficient. The computed Spearman's r is 0.1609. Thus, there is a weak positive relationship between the two variables. However, this is not a statistically significant correlation because the p-value for a two-tail test of significance is 0.4045, which is far greater than the conventional alpha level of 0.05. The 95% confidence interval for r is (-0.2293, 0.5065). Encompassing zero, further suggesting that there is no statistically robust association between the expression of ACTB and Wnt3a in the control group. This lack of significance is supported by the p-value summary ("Ns" indicating 'not significant'), and the result is based on an approximate p-value calculation. The analysis includes data from 29 participants, providing a moderate sample size for this correlation study.

Figure 2

Correlation Analysis: Housekeeping Gene vs. Wnt3a Expression in Control *Participants*

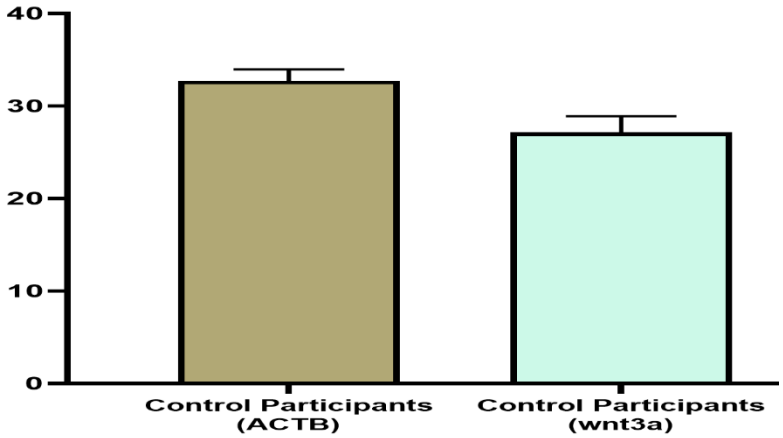


Table 9.

Correlation between Wnt3a and Housekeeping Gene among Patients

ACTB (Patients) vs. *Wnt3a* (Patients)	
Spearman r	
R	-0.3139
95% confidence interval	-0.5792 to 0.01142
P value	
P (two-tailed)	0.0516
P value summary	Ns
Exact or approximate P value	Approximate
Significant (alpha = 0.05)	No
Number	39

The correlation between ACTB and Wnt3a expression levels among patients was examined using Spearman's rank correlation coefficient. The analysis yielded a coefficient (r) of -0.3139, indicating a moderate negative correlation between the two gene expressions. However, the statistical significance of this correlation is marginal, as evidenced by the p-value of 0.0516. This p-value slightly exceeds the conventional significance threshold (=0.05α=0.05),

leading to a classification of the result as not significant (ns). The 95% confidence interval for *r* ranges from -0.5792 to 0.01142, narrowly crossing zero, which suggests the potential for a weak negative correlation, albeit not statistically confirmed.

The p-value was determined through an approximate method, and the analysis was conducted with a sample of 39 patients, providing a reasonable basis for the correlation estimation but failing to establish a statistically significant link between the expressions of ACTB and Wnt3a in the patient group.

Figure 3

Correlation Analysis: Housekeeping Gene vs. Wnt3a Expression in Patients

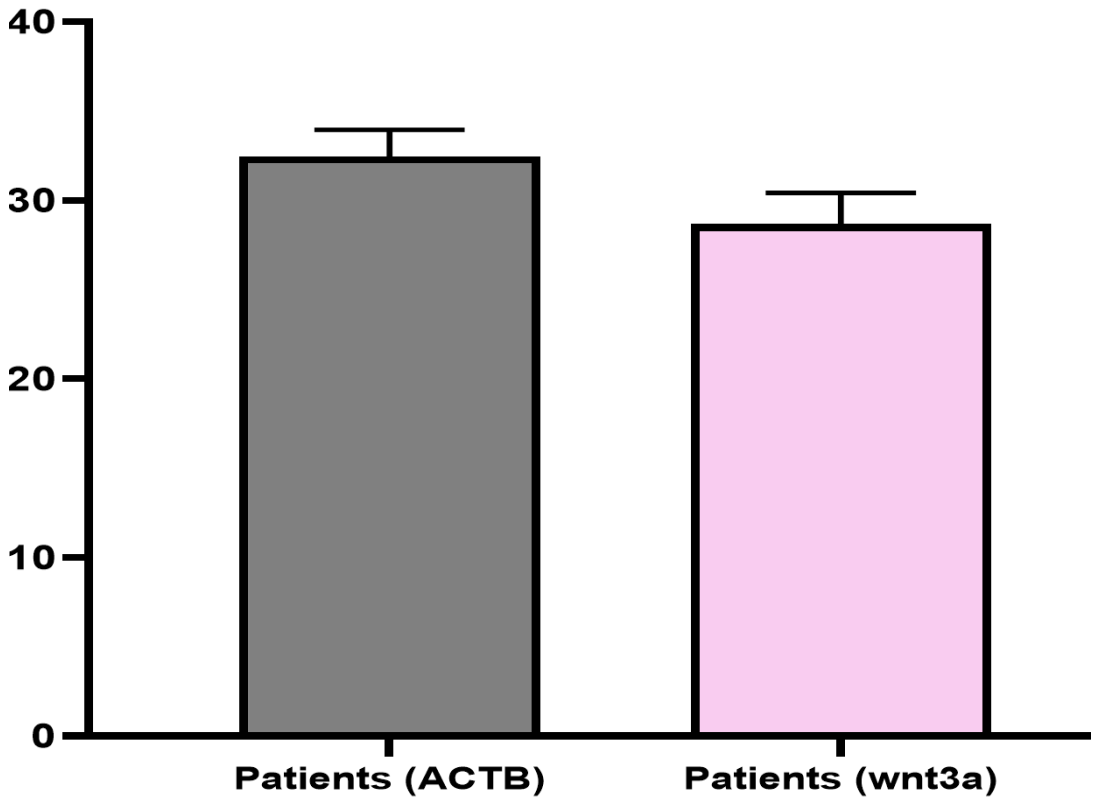


Table 10. T test

Wnt3a (Control Participants) vs. *Wnt3a* (Patients)	
Unpaired t test with Welch's correction	
P value	0.0007
P value summary	***
Significant (alpha < 0.05)	Yes
One or two tailed P value	Two tailed
Welch-corrected t, df	t=3.557, df=60.52
Difference between means	1.532 ± 0.4306
95% confidence interval	0.6706 to 2.393
R squared (eta squared)	0.1729

The comparison between Wnt3a expression levels in control participants and patients was statistically analyzed using an unpaired t-test with Welch's correction to account for potentially unequal variances between the two groups. The results indicate a significant difference in Wnt3a expression, as the p-value of 0.0007 is well below the alpha threshold of 0.05. This finding is emphasized by a p-value summary marked with three asterisks (***), denoting high statistical significance. The Welch-corrected t-statistic is 3.557 with degrees of freedom calculated as 60.52, supporting the robustness of the observed difference. The mean difference between the two groups is reported as 1.532, with a standard error of 0.4306, and the 95% confidence interval ranging from 0.6706 to 2.393, clearly not including zero, further verifying the significant expression disparity. Additionally, the calculated R-squared (or eta squared) value of 0.1729 suggests that approximately 17.29% of the variance in Wnt3a expression can be explained by the group difference (control vs. patients), highlighting a substantial effect size in the context of biological research.

Table 11.

Average Ct Values

Housekeeping (VVs				
	Housekeeping (Control)	Wnt3a Control	Housekeeping (VVs Patients)	Wnt3a Patients
N	29	29	39	39
Median	32.69	26.19	32.34	28.73
Mean	32.72	27.14	32.46	28.67
Std. Deviation	1.237	1.755	1.489	1.757

The table presents the average cycle threshold (Ct) values for housekeeping gene expression and Wnt3a gene expression across control participants and virus-vaccine (VV) patients. For both the housekeeping gene and Wnt3a, the number of samples (N) analyzed was consistent within groups: 29 for controls and 39 for VV patients. The median and mean Ct values for the housekeeping gene are relatively stable across control and patient groups, with only slight variations (Control: median = 32.69, mean = 32.72; Patients: median = 32.34, mean = 32.46). This stability suggests consistent amplification efficiency and gene expression level control across groups. In contrast, Wnt3a shows lower Ct values (indicating higher expression) in both control and patient groups compared to the housekeeping gene, with notably higher expression in patients (Control: median = 26.19, mean = 27.14; Patients: median = 28.73, mean = 28.67). The standard deviations for these measurements (ranging from 1.237 to 1.757) indicate moderate variability within each group. Overall, the table illustrates differential expression patterns of Wnt3a between controls and patients, with the patients exhibiting higher Ct values (lower expression) compared to controls, potentially highlighting differences in biological or disease states between these groups.

Figure 4

Compares the Expression Levels of ACTB and Wnt3a between Control Participants and Patients

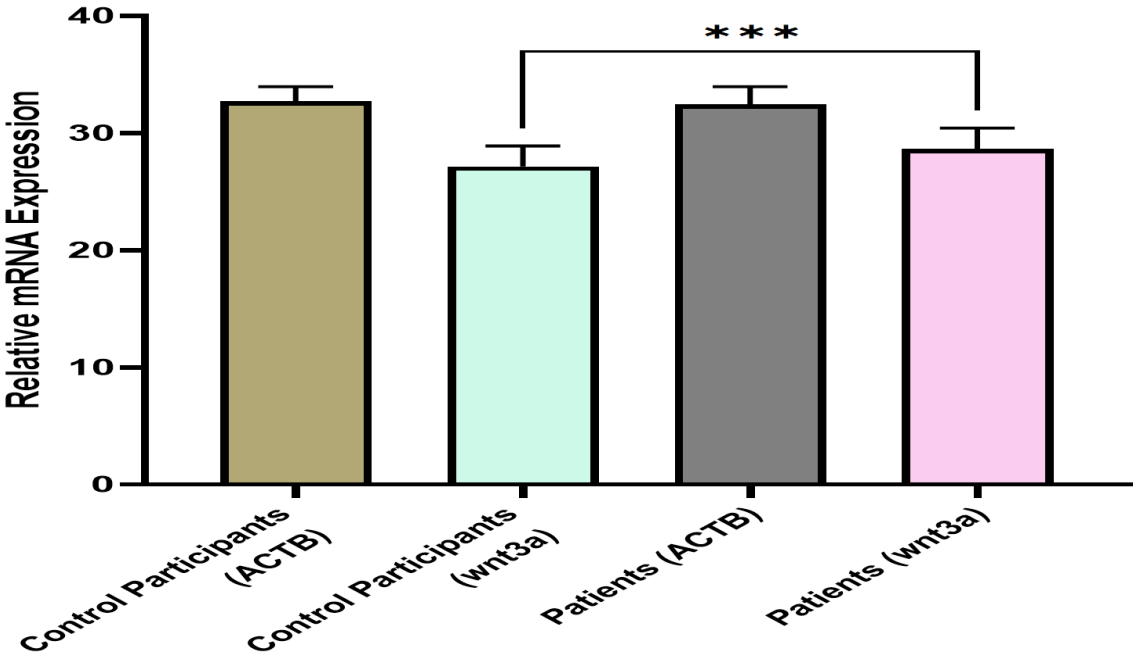


Figure 5

Difference between Means

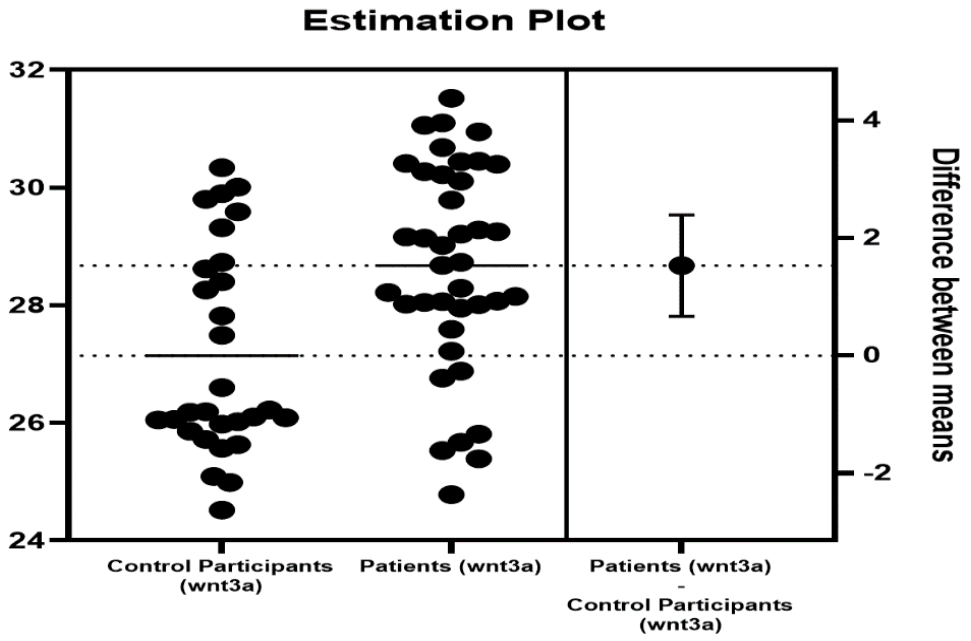


Table 12

Fold Change in Expression of Wnt3a

Fold Change Data	Control	Patient
Number of values	29	39
Median	1.457	0.2107
Mean	2.09	1.483
Std. Deviation	2.412	3.365
Coefficient of variation	115.40%	226.80%

The table summarizes the fold change in Wnt3a gene expression between control participants and patients, quantifying how the expression levels differ from a baseline or between groups. For controls, the median fold change is 1.457, suggesting a moderate increase above baseline, while the mean fold change is substantially higher at 2.090, influenced by larger values as indicated by the standard deviation of 2.412.

This variation is further highlighted by a high coefficient of variation (CV) of 115.4%, suggesting significant variability in expression changes within the control group. In contrast, the patient group exhibits a notably lower median fold change of 0.2107, implying reduced expression relative to baseline.

The mean fold change in patients is higher at 1.483, yet this average is skewed by extreme values, as reflected in a larger standard deviation of 3.365 and an even higher CV of 226.8%. This high variability in the patient group indicates a heterogeneous response to potentially varying clinical conditions or treatments. Overall, the data reveal distinct expression profiles between controls and patients, with more pronounced variability in expression changes among patients.

Figure 6

Fold Change in Expression of Wnt3a

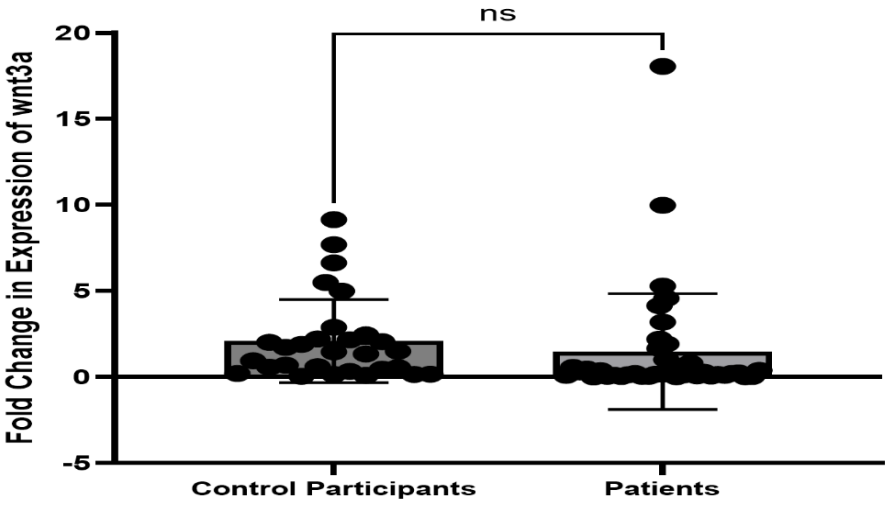
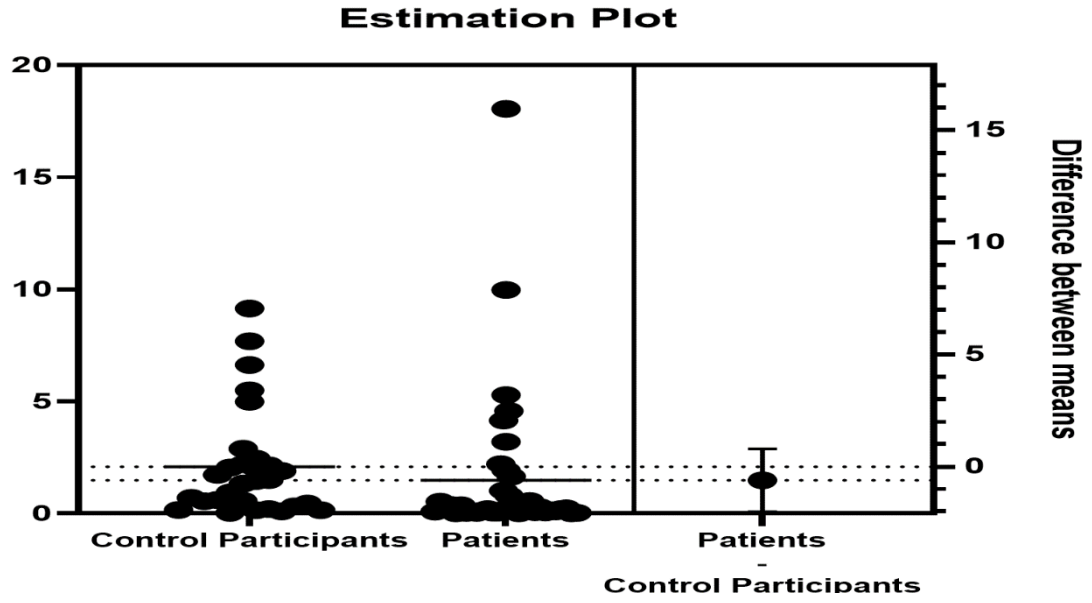


Figure 7

Difference between Means



Interpretation result

An amplification plot from a real-time polymerase chain reaction experiment is depicted in this picture. The main elements of this plot are explained as follows: Y-axis fluorescence: The fluorescent signal intensity found during the PCR process is represented by this axis. The signal is proportionate to the amplified amount of DNA (or RNA). (X-axis) cycle: The cycle number in the PCR process is represented by the x-axis. The amount of DNA should theoretically double with each cycle, resulting in exponential amplification.

Amplification Curves: Every line shows the amplification of a particular sample in a different PCR plate well. As the target DNA is amplified over multiple cycles, the curves display the change in fluorescence. Greater amplification efficiency is indicated by a steeper slope.

The horizontal line, or shold line: The cycle threshold (Ct) value is most likely found at the horizontal line set at a fluorescence value of 482.22. The cycle number at which the fluorescence exceeds this cutoff, signifying a substantial amplification of the target DNA, is known as the Ct value. It is an essential figure for calculating the quantity of raw materials.

The software appears to offer the option to filter the view based on either color (which might correlate to distinct fluorescent labels for different targets) or well (each well containing a separate sample or reaction mixture), based on the dropdown menus.

Plot Type: 'Linear' is the selected format from the dropdown menu that shows the various ways the plot can be shown. 'Logarithmic,' which is frequently used to better depict exponential data, could be another choice. Phase Linear of Amplification: The curves are primarily flat early in the plot because there is not enough fluorescence to distinguish it from the background. The curves show that the target DNA has been amplified successfully when they start to climb exponentially after a predetermined number of cycles.

Plateau Phase: As the reaction components are used up, the curves eventually level off, and the amplification achieves a plateau. Numerous applications, including genotyping, pathogen detection, and quantitative gene expression analysis, depend heavily on this kind of analysis. The precise number of curves and the points at which they begin can provide information about the initial amount of target DNA in each sample as well as the PCR reaction's effectiveness.

CHAPTER V

DISCUSSION

Some genes upregulate the Wnt pathway target genes, while others downregulate them and summarizes the essential characteristics of the Wnt pathway. For instance, when Wnt signaling is absent, most genes of the Wnt pathway that positively interrelated between their expression and PWV and that grouped as described above in factor 1, such as APC, AXIN1, and GSK3B, and, together with β-catenin-CTNNB1; also part of factor 1-form a protein complex inhibiting Wnt target gene transcription (Malsin et al., 2019).

As an example, the majority of Wnt pathway genes that have been negatively associated with PWV, including TCF4 While those grouped in factor 2, LEF1, TCF7, and AXIN2 encode proteins that, when Wnt signaling is on, form a cluster that allows translocation of transcription factors to the nucleus, where they upregulate Wnt pathway target genes(Guerriero, 2018).

The situation with Axin^2 is a little bit different since, although its protein form inhibits Wnt signaling (Badimon & Borrell-Pages, 2017), Wnt signaling also targets the gene AXIN2 (Malkov et al., 2009); It follows that there is a general correlation between TCF7 and LEF1 gene expression. Because our data is cross-sectional, it is difficult to determine whether the results are the result of a Wnt expression pattern, which indicates that Wnt signaling prevents the vessels from stiffening, or a negative feedback loop that downregulated Wnt signaling and caused the arteries to stiffen(Swafford & Manicassamy, 2015).

In summary, WNT signals via canonical and non-canonical pathways It follows that there is a general correlation between TCF7 and LEF1 gene expression. Because our data is cross-sectional, it is difficult to determine whether the results are the result of a Wnt expression pattern, which indicates that Wnt signaling prevents the vessels from stiffening, or a negative feedback loop that downregulated Wnt signaling and caused the arteries to stiffen. In summary, WNT signals via canonical and non-canonical pathways. In earlier

research, one or two circulating Wnt inhibitor proteins, such as Dkk1 or SOST (sclerosis), were examined in relation to the University of Pittsburgh Wnt pathway and arterial stiffness. SOST and Dkk1 inhibit the signaling of the Wnt pathway. But that is only one part of the intricate Wnt signaling pathway that they represent. It was demonstrated that there is a strong correlation between arterial stiffness and two Wnt genes, APC and TCF4. Little research has been done on the connection between APC and arterial stiffness, despite several studies linking APC to cardiac development and repair, hypertrophic cardiomyopathy, atherosclerotic fibrous cap stability, and cardio myocyte proliferation. Since the APC protein has never been assessed in a population-based study before, this is the first investigation of its in vivo expression and potential relationship to indicators of human vascular disease (Mikels & Nusse, 2006).

The majority of the APC protein is intracellular. Two Wnt genes, APC and TCF4, were shown to be substantially correlated with arterial stiffness. Though some studies have linked APC to cardiac development and repair, hypertrophic cardiomyopathy, atherosclerotic fibrous cap stability, and cardio myocyte proliferation, there has been little prior study on the relationship between APC and arterial stiffness. This is the first study of the APC protein's in vivo expression and possible correlation with markers of human vascular disease because it has not been evaluated in population-based studies before (Mikels & Nusse, 2006),(Yochum et al., 2007).

The APC protein is primarily intracellular. Conversely, rs7903146, a genetic variation found in TCF4, often referred to as TCF7L2, was discovered in 2007 to be a significant risk factor for diabetes mellitus and impaired fasting glucose. Numerous investigations have confirmed this genetic relationship and linked it to both ankle-brachial index and diabetic coronary artery atherosclerosis. As far as we are aware, no research has been done on the connection between arterial stiffness and genetic variations of TCF4. TCF4 appears to work in coronary artery disease By enhancing the proliferation and

flexibility of vascular smooth muscle cells, which, in turn, can affect the elasticity of the arterial wall. About sensitivity analyses, we observed that including diabetes mellitus in our global model somewhat mitigated [about 7%) the described correlations between the TCF4 expression and the arterial rigidity. Nevertheless, the strength of that association was still muscular (P=0.004; data not shown). Like APC, however, the TCF4 protein is predominantly intracellular and, hence has not been examined in population-based studies. The current paper, therefore, represents a new extension of the now-known associations between TCF4 and vascular disease to encompass arterial stiffness(Yochum et al., 2007).

This research is the first to thoroughly examine the relationship between human arterial stiffness and Wnt pathway gene expression. Fourteen of the forty-three Wnt mRNAs that were analyzed were discovered in the peripheral blood of the participants In our African ancestry study may implicate a role for these molecules in human circulation. After adjusting for several well-established CVD risk factors, two of the mRNAs we identified, APC and TCF4, were significantly associated with arterial stiffening, as measured by PWV. These same associations were also detected in exploratory factor analyses. These effects underscore the underlying biological complexity in the effects of the Wnt pathway on the circulatory system and, at the same time, the necessity for multiple elements of the path to be analyzed for the overall status of Wnt signaling to be determined. The ability to upregulate the Wnt pathway target genes with some genes but not others simply encapsulates the essence of this pathway. For instance, most of the Wnt pathway genes with positive correlations in their expression and PWV, such as APC, AXIN1, and GSK3B, which grouped in factor 1, form a protein complex that includes β-catenin that is also within factor 1. Wnt target gene transcription is inhibited in the absence of Wnt stimulation.34 The majority of Wnt pathway genes, however, have negative relationships between their expression and PWV, such as those grouped in factor 2 LEF1, TCF7, and AXIN2and TCF4, encode proteins which, in the absence of Wnt signaling, form a complex and prevent

translocation of transcription factors into the nucleus where they could upregulate Wnt pathway target genes (Liu et al., 1521). Axin-2 is a somewhat different situation because whereas the protein form serves to down-regulate Wnt signaling, this gene, AXIN2 itself, is also a target of Wnt signaling; hence, one might expect that gene expression would closely track LEF1 and TCF7. Due to the cross-sectional nature of our data, we can not conclude with certainty whether this Wnt expression pattern caused the arterial stiffness, thus implying Wnt signaling protects the vessels from stiffening, or these data may represent a negative feedback loop that down-regulated Wnt signaling to lead to stiffening of arteries. Last, Wnt signals via both canonical and no canonical pathways (Liu et al., 2016).

Most of the studies listed here focus on the canonical Wnt signaling cascade; however, no canonical signaling is raised in response to canonical signaling reduction, and can even be a key cause of CVD.38, 39 the results of this study, however, are the first to suggest a link between Wnt pathway activation and human arterial stiffness.

Previous studies investigating the relationship between arterial stiffness and the Wnt pathway frequently concentrated on one or two Wnt inhibitor proteins that are circulating in the bloodstream, such as SOST (sclerostin) or Dkk1. The Wnt pathway is inhibited by Dkk1 and SOST. 34 However, they only touch on a fraction of the complex cascade of Wnt signaling. Most of these investigations, but not all of them, discovered Consistent with the results of this investigation, circulating SOST was positively correlated with increased arterial stiffness. On the other hand, Dkk1's prior results were primarily null, albeit one indicated an unfavorable link. On the other hand, the current work (Schäfer et al., 2014) employed a pathway-wide strategy to acquire a more comprehensive understanding of Wnt pathway gene expression and its possible correlation with arterial stiffness Two Wnt genes, APC and TCF4, were shown to be substantially correlated with arterial stiffness. While there has been little prior study on APC and arterial stiffness, certain studies have

linked APC to hypertrophic cardiomyopathy, atherosclerotic fibrous cap stability, and cardiac development and repair. and. Circulating SOST showed a positive correlation with increasing arterial stiffness, which is consistent with the investigation's findings. Conversely, Dkk1's previous findings were mostly null, with one finding an adverse correlation. In contrast, the present study (Schäfer et al., 2014) used a pathway-wide approach to get a deeper comprehension of the expression of the Wnt pathway and its potential association with arterial stiffness. Research has demonstrated a strong correlation between arterial stiffness and two Wnt genes, APC and TCF4. There has been some research connecting APC to hypertrophic cardiomyopathy, cardiac development and repair, and the integrity of the atherosclerotic fibrous cap, despite the fact that APC and arterial stiffness have not been fully examined. According to research, TCF4 contributes to coronary artery disease by encouraging vascular smooth muscle cell proliferation and plasticity which may also have an impact On the stiffness of the arterial wall. In our entire model, the inclusion of sensitivity analysis by diabetes mellitus only slightly attenuated-prepared by molecular device≈7% though the magnitude of its relationship was similar and still significant-P=0.004; data not shown-the reported associations between TCF4 expression and arterial stiffness. Like APC, the TCF4 protein is primarily an intracellular protein and has not been assessed previously in population-based studies; this report will, for the first time, extend the known associations of TCF4 and vascular diseases to arterial stiffness. The paired t-test was employed to determine whether the differences in expression between CONTROL (ACTB) and *WNT3A* were statistically significant from zero. The analysis yielded a two-tailed P value of less than 0.0001, which is considered extremely significant, and a t-value of 8.662 with 27 degrees of freedom. The mean difference between the pairs was calculated to be 2.814, with a 95% confidence interval extending from 2.148 to 3.481. Despite these significant results, the correlation coefficient of 0.1108, accompanied by a one-tailed P value of 0.2872, indicated that the pairing was not effective, and suggesting that an

unpaired test may be more suitable for this analysis. Additionally, the assumption of normal distribution for the paired differences was affirmed, as evidenced by a Kolmogorov-Smirnov distance of 0.14 and a P value greater than 0.10, satisfying the criteria of the normality test with $P > 0.05$.

Studies have shown a significant relationship between arterial stiffness and the Wnt genes TCF4 and APC. Although the relationship between APC and arterial stiffness has not been thoroughly investigated, there has been some study linking APC to hypertrophic cardiomyopathy, cardiac development and repair, and the integrity of the atherosclerotic fibrous cap. TCF4 has been linked to coronary artery disease by promoting the proliferation and plasticity of vascular smooth muscle cells . This process may also affect the stiffness of the arterial wall. Sensitivity study revealed that incorporating diabetes mellitus into our complete Factors. Interestingly, the Wnt gene with the strongest individual association between its expression and arterial stiffness, TCF4, did not load onto any factor at ≥0.3, and it had pretty low levels of correlation to other Wnt genes (maximum, 0.26 with AXIN2; Table S2). This may suggest that a strong association of TCF4 with arterial stiffness could be operating through additional mechanisms other than canonical Wnt signaling (Pashirzad et al., 2019).

The two significant factors that came out of factor analysis appear to explain most of the variance in Wnt gene expression patterns. It would seem that while factor 1 is composed mainly of genes involved in Wnt signal transduction, factor 2 is composed primarily of genes involved in transcriptional regulation of Wnt signaling target genes. Furthermore, factor analysis also identified a third factor accounting for less than 10% of the variance in Wnt gene expression that appears to be primarily made up of genes encoding for cell surface Wnt signaling. Factors 1 and 2 were significantly related to arterial stiffness in minimally adjusted models but only factor 1 remained significant in models adjusted for CVD risk factors. Interestingly, the Wnt gene with the strongest individual association between its expression and arterial stiffness,

TCF4, did not load onto any factor at ≥0.3, and its levels of correlations with other Wnt genes were pretty low with a maximum of 0.26 with AXIN2; this may suggest that the strong association of TCF4 with AS may be operating through additional mechanisms other than the ones of canonical Wnt signaling.(Kaplan et al., 2003).

The data, which in this case are peripheral blood from men of African descent, are solely indicative of the tissue and population sample from which they were drawn, as is the case with every gene expression study. Since we were unable to sort or count the blood cells to ascertain the cellular makeup of the blood samples, the expression profiles of a variety of circulating cells are reflected in our data. Peripheral blood is a reasonable tissue source, nevertheless, because of its constant interaction with the vessel wall and noninvasive collection procedure, especially considering that arterial stiffness is our result of interest. We discovered that out of evaluated Wnt pathway genes did not exhibit any discernible expression, so we are unable to make any judgments regarding their possible correlation with vascular disease. We are likewise unable to remarkon Wnt pathway genes that were left off of this specially made array panel, which was made up of only the genes that had previously been shown to be expressed in the vascular wall or to be involved in processes related to CVD. Some of these genes might only be expressed and have an effect on the vessel wall, not the circulation. We could not have controlled for ethnicity, exposures, or physiological circumstances like inflammation, but it is also likely that different expression profiles would result from these factors. But since people of African descent have a heightened risk of vascular illness due to inadequate management of their hypertension, which includes these men, In terms of human molecular research, this constitutes a significant demographic segment. Future research is required to compare the current findings in women, as there may be a physiologic difference in Wnt expression profiles by sex, as previously described. Finally, the mechanism underlying the variations in Wnt gene expression is unknown (i.e., genetic variation, epigenetic variation, or a mix of both). According to Kaplan et al.

(2003), more research has to be done to determine how Wnt gene expression affects various subclinical and clinical illness types as well as their consequences.

However, even when controlled for the conventional CVD-related risk factors, two circulating genes of this pathway, APC and TCF4, were significantly related to arterial stiffness. Greater expression of APC, a gene that downregulates the expression of Wnt pathway target genes, was associated with greater arterial stiffness, while greater expression of TCF4, whose gene product upregulates the expression of Wnt pathway target genes, was associated with lower arterial stiffness. Based on these human-derived expression data, vascular-Wnt-pathway signaling perhaps contributes to vascular stiffness but is also a potential risk factor and pharmacological target for improving arterial health. Immunoassays analysis are some of the methods used to investigate its expression levels. The analysis of Wnt3a and expression levels in this instance makes use of patient tissue collection. By doing the following, the diagnostic group for this study is determined: Tissue Sample and Patient Selection: Examined will be a controlled group with normal venous function and a group with vent man insufficiency. Using patient consent and ethical considerations, venous tissues will be retrieved from each unique group. Put tissue samples together while preserving the integrity of the proteins for cDNA, immunoassays, and RNA extraction for investigations of gene expressionCombination: For high-quality RNA, extract total RNA from the tissue samples using a dependable extraction technique. For qPCR, create complementary DNA (cDNA) from the recovered RNA by synthesizing it (Kaplan et al., 2003).

It is recommended to utilize quantitative real-time PCR for the design and validation of Wnt3a specific primers. By using housekeeping genes as internal standards for normalization, the expression levels of Wnt3a mRNA can be quantified by qPCR.

Gene expression levels, which are frequently determined by measuring mRNA, do not necessarily precisely correspond to protein activity for a

variety of reasons. For instance, even though a gene's mRNA is produced at high quantities, the protein product may have low real activity in the cell if it is broken down quickly. The absence or inhibition of the modifying enzymes may also indicate that, despite high levels of mRNA and even protein expression, the active form of the protein is not present at high levels if a protein needs particular post-translational modifications (such as phosphorylation) to be active. It is frequently required to evaluate not just the levels of mRNA expression but also the levels and activity of proteins accurate picture of a gene's functional activity. Numerous techniques, including enzyme tests for protein activity and Western blotting for protein amount, can be used to accomplish this.

Examining your graph, which displays the expression levels of WNT3A and ACTB in a patient relative to a control, reveals the findings. It could be wise to test the protein levels of ACTB and WNT3A as well as their activities in the patient and control samples if we are talking about a condition where mRNA levels do not correlate to protein activity.

The two sets of mRNA expression data will be combined with a third, independent data set for protein level and activity to create a cohesive picture of the overall functional implications of the observed expression levels.

The majority of developmental processes depend on appropriate and functioning Wnt signaling, and the Wnt3a gene is one of the Wnt genes whose malfunction is linked to the pathophysiology of numerous disorders. Venous stasis may be caused by a progressive decline in venous wall and/or valvar function as a result of a lack of fully functional Wnt/β-catenin pathway. This condition makes it difficult to return blood from the lower extremities against gravity and can cause discomfort, edema, and skin changes. This could be because endothelial function regulation and venous wall remodeling may be inhibited in cases of venous insufficiency. Dysregulation of Wnt3a expression in conjunction with endothelial cells is one of the main modulators of venous disorders. The pathogenic process of venous hypertension and valve failure may be exacerbated by overexpression of Wnt3a alone, which may also

exacerbate proinflammatory responses, endothelial cell proliferation, and regulation of vascular smooth muscle cells (Kaplan et al., 2003).

Conversely, a decrease in Wnt3a signaling may prevent angiogenesis and vascular repair processes that are necessary for maintaining the integrity of veins. Thus, to maintain vascular homeostasis, Wnt3a expression needs to be maintained at the proper ratios. According to this study, patients with venous insufficiency may have a distinct Wnt3a expression profile than healthy controls. For instance, this increased expression level might be correlated with the severity of venous changes, and as a result, it would function as a biomarker for the disease's advancement.. Therefore, Wnt3a therapeutic modulation may offer a novel approach to addressing the consequences of venous insufficiency. This can be accomplished by adjusting the upward or downward modulation of Wnt3a activity to either enhance or repair endothelial function, or to suppress it in order to lessen inflammation and vascular proliferation.

Therefore, future studies should determine Wnt3a's involvement in venous illness and evaluate it further as a potential therapeutic target. Clinical research aimed at modifying Wnt3a signaling may offer new venous insufficiency treatment options that improve patient outcomes and quality of life. Moreover, longitudinal studies assessing Wnt3a expression over the course of the disease could provide insight into the role played by the protein in the onset and persistence of chronic venous insufficiency (Gloviczki et al., 2011).

Findings significant contribute clinical parameters to assess the relationship between gene expression levels and the severity of venous insufficiency and also discuss the potential role of Wnt3a and in the pathophysiology of venous insufficiency, considering both the literature and the findings from the study, Evaluate whether Wnt3a could serve as biomarkers for venous insufficiency or as targets for therapeutic intervention.

Statistically Analyse qPCR data statistically to compare the expression levels of Wnt3a a between patients with venous insufficiency and the control group and used real time PCR analysis. I have a total of 68 wnt3a: 1–29 are from our

control (wnt3a venous insufficiency) and 30-68 patient wnt3a venous insufficiency.

Conclusion: However, the finding gathered in this study, based on gene expression of WNT3a signal genes, showed a lower expression of WNT3A and higher expression in Wnt3a than wnt3a in the patient of venous insufficiency and control of venous insufficiency, respectively.

The current study aims to delineate the expression profiles of Wnt3a within venous tissues of individuals suffering from venous insufficiency. Employing a cohort of patients alongside a control group with normative venous function, venous tissue samples will be harvested and subjected to rigorous RNA extraction. Subsequent cDNA synthesis will facilitate quantitative real-time PCR (qPCR), assessing mRNA levels of the target genes. A dual-layered understanding of gene expression will be made possible by the addition of protein expression analysis by Western blotting and immunohistochemistry methods to this molecular data, a statistical comparison of the expression levels in the patient and control groups will be conducted, and connections with clinical indicators of the severity of the illness will be investigated.

CHAPTER VI

CONCLUSION

Some intriguing findings have been obtained by examining the expression profile of Wnt3a in connection to venous insufficiency, Patients with venous insufficiency had tissues expressing levels of Wnt3a significantly altered from healthy controls. Wnt3a may, therefore, be implicated in the pathophysiological mechanisms of venous insufficiency disease due to an unequal amount of its expression. Besides, changes in Wnt3a signaling are noted to be the other cause of the aberrant remodeling of vasculature and dysfunction of endothelium, which are both common findings of the said condition, Wnt signaling pathways, and Wnt3a's specific function is yet unclear Nevertheless, these preliminary findings suggest that Wnt3a might function as a biomarker for the development of the illness and might potentially be a target for treatment It is necessary to do additional study to investigate the molecular pathways associated with Wnt3a and to evaluate the potential therapeutic benefits of modifying its activity in the management of venousinsufficiency.

Accumulating evidence indicates that the WNT3A product is a key regulator not only for the cell differentiation process but also in relation to human growth and development. To date, it has become quite reasonable to put forward that WNT3A and its signaling pathway are on the verge of becoming molecular targets for disease diagnosis and therapy. Both canonical and non-canonical WNT3A signaling pathways are involved in the progression of cell differentiation, hence unfolding all these WNT3A signaling pathways in future, as they are well established through myriad studies. On the contrary, higher expression of WNT3A protein may lead to renal, hepatic, and lung fibrosis. Another question is whether higher expression of WNT3A by venous insufficiency could result in part of the known functional differences. Yet, another puzzle is how wnt3a can have different functions in the same cell at different stages.

We would therefore hypothesize that is supposed to activate either the canonical or non-canonical pathway, basing on the different conditions, but still, we have more experiments that should be done before assuming its role. Wtn3 protein has been tried successfully in wound healing, nerve repair, improvement of bone mineral density, and assisted reproduction as a treatment application.

However, further studies may be needed for overall body safety evaluation. In conclusion, the result has been collected in this study according to the analysis of gene expression in the WNT signaling gene (WNT3A) shows a decrease in the expression of WNT3A and an increase in WNT5A expression in an abnormal karyotype when compared to a normal karyotype in spontaneously aborted materials, thus, further research should be conducted with the protein expression levels of the genes studied to actually confirm the changes detected in gene expression really correlate at the protein level, ideally with a larger cohort.

However, in this study, RNA concentration measurements were not freshly taken in the samples. To express these factors more clearly, further studies are needed in relation to the Wnt signal genes and implantation in spontaneous abortion. Moreover, a better understanding of the role of Wnt genes during implantation can be implored and also with the help of IVF techniques, errors can be rectified.

To evaluate the effectiveness of existing therapies for venous insufficiency in the context of WNT signaling, a comprehensive approach should be employed, Clinically measure the symptoms and progression of venous insufficiency in patients before and after treatment, utilizing standardized tools like the Venous Clinical Severity Score (VCSS) and the CEAP (Clinical, Etiological, Anatomical, and Pathophysiological) classification, Molecular Analysis: Examine the expression levels of key WNT pathway components (such as WNT5A, WNT7A, and WNT7B) in venous tissue samples from patients undergoing therapy.

Techniques such as qPCR, Western blotting, and immunohistochemistry can be used, Correlation with Treatment Outcomes: Correlate the changes in WNT signaling molecules with clinical outcomes. Determine if improvements in venous insufficiency symptoms align with the modulation of WNT pathway activity, Conduct in vitro and in vivo studies to understand how treatments may modulate WNT signaling. Investigate the downstream effects of altered WNT signaling on venous wall remodeling, valve function, and inflammation. Meta-analysis: Perform a meta-analysis of existing research literature to evaluate the consensus on the impact of venous insufficiency therapies on WNT signaling.

By integrating clinical outcomes with molecular changes within the WNT pathways, the effectiveness of current therapies can be assessed not only in terms of symptomatic relief but also in their capacity to correct the underlying molecular aberrations associated with venous insufficiency. This will aid in refining existing treatments and potentially developing targeted therapies that specifically address the WNT-mediated aspects of the disease.

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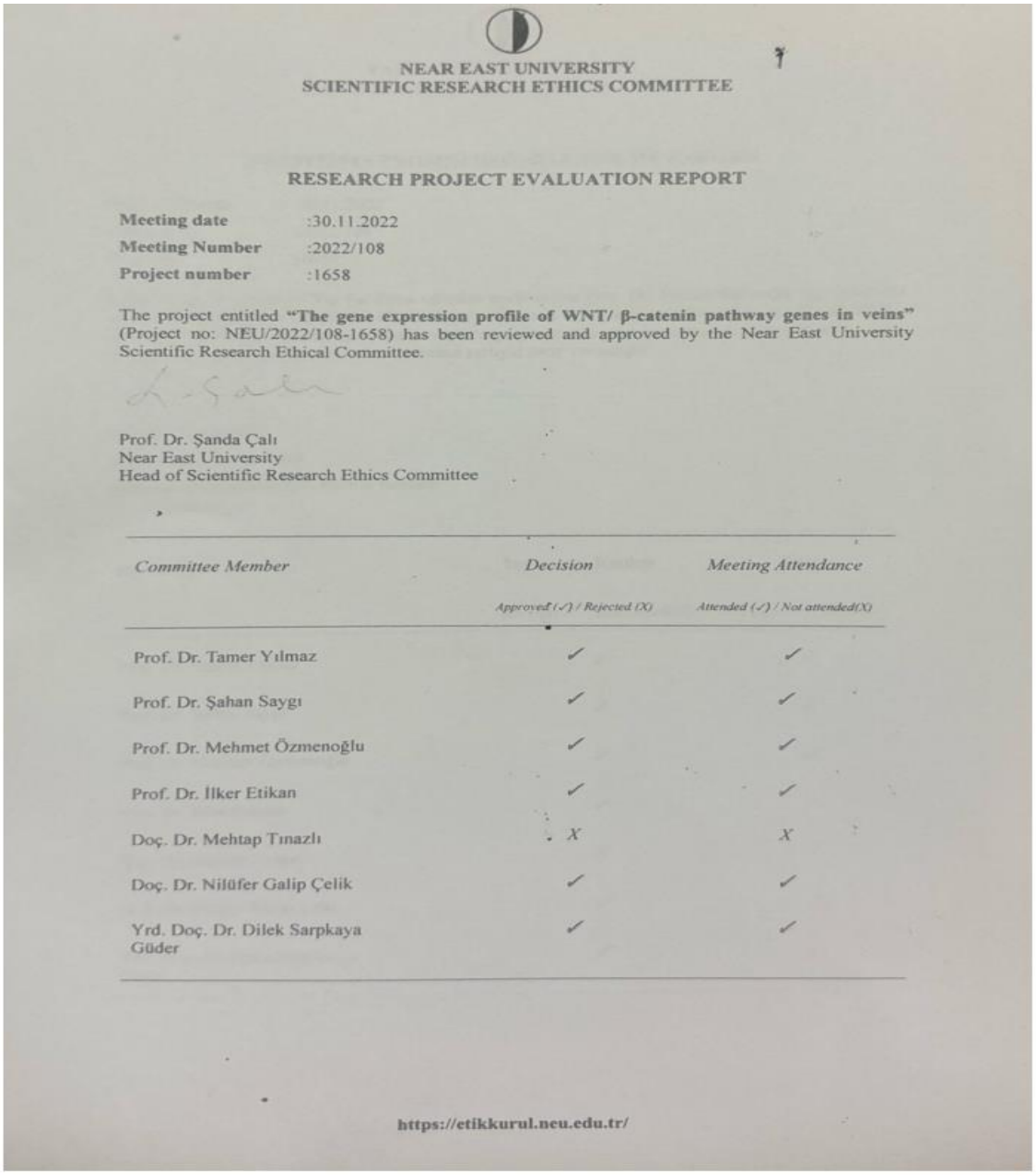
APPENDICES

Appendix A



NEAR EAST UNIVERSITY
SCIENTIFIC RESEARCH ETHICS COMMITTEE

RESEARCH PROJECT EVALUATION REPORT

Meeting date :30.11.2022
Meeting Number :2022/108
Project number :1658

The project entitled **"The gene expression profile of WNT/ β-catenin pathway genes in veins"** (Project no: NEU/2022/108-1658) has been reviewed and approved by the Near East University Scientific Research Ethical Committee.

Prof. Dr. Şanda Çalı
Near East University
Head of Scientific Research Ethics Committee

Committee Member	*Decision* Approved (✓) / Rejected (X)	*Meeting Attendance* Attended (✓) / Not attended(X)
Prof. Dr. Tamer Yılmaz	✓	✓
Prof. Dr. Şahan Saygı	✓	✓
Prof. Dr. Mehmet Özmenoğlu	✓	✓
Prof. Dr. İlker Etikan	✓	✓
Doç. Dr. Mehtap Tınazlı	X	X
Doç. Dr. Nilüfer Galip Çelik	✓	✓
Yrd. Doç. Dr. Dilek Sarpkaya Güder	✓	✓

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