Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Androgen regulated protein and pyruvate dehydrogenase kinase 4 in severe erectile dysfunction: A gene expression analysis, and computational study of protein structure

Elham Kazemi[1], Javaad Zargooshi[2*], Mozhgan Fatahi Dehpahni[2], Hamid-Reza Mohammadi Motlagh[3*], Marzieh Kaboudi[4], Danial Kahrizi[5], Habibolah Khazaie[6], Behzad Mahaki[7], Youkhabeh Mohammadian[8] , Leila Yazdani[2]

[1] Fertility and Infertility Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
[2] Department of Sexual Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
[3] Medical Biology Research Center, Health Technology Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran
[4] Department of Marital Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
[5] Department of Plant Production and Genetics, Razi University, Kermanshah, Iran
[6] Sleep Disorders Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[7] Department of Bio-Statistics and Epidemiology, School of Health, Kermanshah University of Medical Sciences, Kermanshah, Iran
[8] Department of Clinical Psychology, Kermanshah University of Medical Sciences, Kermanshah, Iran

[*]**Correspondence to:** zargooshi@gmail.com, mohammadimotlagh@gmail.com

Received February 25, 2021; **Accepted** August 14, 2021; **Published** August 31, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.2.13

Abstract: Erectile dysfunction (ED) is one of the most common sexual disorders in men. During the past 30 years, there has been no new drug development for ED. Thus, exploring the genetic basis of ED deserves further study, in hope of developing new pharmacological treatments for ED. In this study, Real-Time PCR analysis was used to assess the expression of androgen regulatory protein (Andpro) and pyruvate dehydrogenase kinase 4 (Pdk4) genes in ED. For this purpose, the experiment was performed on 20 men with severe ED and 20 potent men. IIEF-15 was used to determine the ED severity. The study was conducted in the Department of Sexual Medicine of the Kermanshah University of Medical Sciences, Kermanshah, Iran. The EDTA-Na vacuum blood tube was taken from ED patients and controls. Informed consent was obtained from all participants. After blood sampling, RNA was extracted from whole blood. Then cDNA was synthesized. The gene expression was analyzed through the qPCR method. The ß-actin was used as a reference gene. To further study these two proteins, their three-dimensional structures were predicted through I-TASSER. Compared with controls, in ED patients, the expression of the Andpro gene decreased, while the expression of the Pdk4 gene increased (p<0.01). Predicting the structure of the protein showed that Pyruvate Dehydrogenase Kinase 4 had a double subunit and androgen-regulated protein had a single subunit.

Key words: Andpro; Pdk4; Severe erectile dysfunction; Real-Time PCR; Protein structure.

Introduction

Erectile Dysfunction (ED) in Men is the permanent or transient inability to achieve or maintain an adequate erection for sexual activity (1). In addition to affecting a person's self-esteem, ED can also lead to disharmony or conflict with sexual partners (2-4). Some diseases such as diabetes, hypertension, atherosclerosis, thyroid dysfunction, trauma and depression can cause ED (5).

The prevalence of ED in men with diabetes is 2 to 3 times higher than in non-diabetics (6) and 10 to 15 years earlier in diabetic men than in non-diabetics (7). The mechanism of erection is the intense pumping of blood into the cavernous bodies of the penis and preventing it from coming out by closing the arteries. Therefore, in vascular ED, there may be two scenarios. First, reduction or interruption of arterial flow in which blood does not enter the penis well. Second, non-closure of venous pathways and leakage of blood entering the penis from the outside (8). There have been many studies on the genetic cause and many candidate genes have been introduced (9).

There are several techniques to estimate gene expressions, such as Northern Blot analysis (10), ribonuclease (RNase) protection assay (10), quantitative real-time PCR (qPCR) (11), serial analysis of gene expression (12), and microarray analysis (13, 14). The qPCR is one of the most convenient methods for studying RNA transcripts, and can generally be used for total RNA extracted from any biological source (15).

In many studies that have examined the relationship between the expression of certain genes and ED, the animal model of diabetic mice has been used. In 2013, Lacchini et al. (16) investigated VEGF polymorphism and its association with ED. In a study of 126 patients, they reported three polymorphisms [2578C> A (rs699947), -1154G> A (rs1570360) and -634G> C (rs2010963)] for the VEGF gene promoter. Finally, they stated that genotype-1154AA has a significant relationship with ED. Liu et al. (17) linked the decrease in VEGF gene expression to ED (17). Then, ED correlated with TNF-α (18), GNB3 (19), IGFBP-3, (20), FGF2 (21), VEGF (22), DDAH2 (23), hNGFβ (24), NOS3 (25), Ad-COMP-Ang1 (26) and HMGCS2 (27) genes have been investigated. In 2013, Zhang et al. (28) in a meta-analysis study showed that there was no significant relationship between angiotensin-converting enzyme (ACE) and ED gene expression. In 2014,

Chen et al reported significant ED association with the expression of PTAFR, IL27, CD37, CD40, IL7R, PSMB9, and CXCR3 genes (29).

In 2014, Kovanecz et al. (30), Kam et al. (31), Pan et al. (32), and Vishnubalaji et al. (33) used microarray technology to study the expression of ED-related genes. In 2015, Dai et al. examined the eNOS G894T gene polymorphism and reported that there was generally no significant relationship between this polymorphism and ED, but this association could be significant depending on the geographical area (34). In 2015, Pan et al. examined the effect of long non-coding RNAs or lncRNAs on ED and stated that the expression of some of these genes affects ED (35).

Also in 2018, Ben Khedher et al. (36) investigated the association of ED with polymorphism of GNB3 C825T, eNOS T-786C and eNOS G894T genes. In 2019, Segura et al. (37) studied the relationship between ED and eNOS gene polymorphism at positions T786C, 4VNTR, and G894T. In 2018 the association of ED with PCSK9 gene polymorphism was investigated by Mostaza et al. (38). Experiments have shown that decreased expression of the MEG3 lncRNA gene reduces ED (39).

Structural bioinformatics is a branch of bioinformatics that deals with the analysis and prediction of the three-dimensional structure of biomolecules such as proteins, RNA, and DNA. It deals with structural/functional relationships, which are performed both through empirically derived structures and computational models. The term "structural" has the same meaning as in structural biology, and structural bioinformatics can be considered as part of computational structural biology. The main purpose of structural bioinformatics is to develop new methods for processing large biomolecule data in order to solve problems in biology and produce new knowledge (40-45).

The structure of a protein is directly related to its function. The presence of certain chemical groups in specific locations allows proteins to act as enzymes and suppress several chemical reactions (41, 44, 45).

The aim of this study was to assess the expression of the androgen-regulated protein (Andpro) and the pyruvate dehydrogenase kinase 4 (Pdk4) genes in ED and controls. The three-dimensional structure of the proteins encoded by these genes was also predicted and designed.

Materials and Methods

This study was conducted in the Department of Sexual Medicine of the Kermanshah University of Medical Sciences, Kermanshah, Iran. In this experiment, the expression of two candidate genes in ED including androgen-regulated protein (Andpro) and pyruvate dehydrogenase kinase 4 (Pdk4) was examined. For this purpose, we studied 20 men with severe ED and 20 potent men as controls. The mean age of ED cases was 56 years and the mean age of controls was 38 years.

IIEF-15 was used to determine the ED severity. No patient had an IIEF-15 score of more than seven, and none of the controls had an IIEF-15 score of less than 27.

RNA extraction

Three ml of blood was taken from patients and controls, using the 3 ml- EDTA-Na vacuum blood tubes. After blood sampling, RNA was extracted from blood. To extract RNA from whole blood, 500 µl of blood sample was mixed with 1 ml of RBC lysis buffer and kept at room temperature for 5 min. It was then centrifuged at 500 xg for 3 min. The cell plate was washed with 300 µl of RBC buffer and then centrifuged at 500 xg for 3 min. One ml of RNX plus (Cat. No. : EX6101, Sinaclon Company, Iran) was added to the cell plate and kept at room temperature for 5 min. Then 200 µl of cold chloroform was added and kept on ice for 5 min. It was then centrifuged for 15 min at 12000 rpm at 4 °C. The supernatant was separated and the same volume of isopropanol was added, then kept at -20 °C for 20 min. It was then centrifuged again for 15 min at 12,000 rpm at 4 °C. The supernatant was discarded and add 1 ml of 75% cold ethanol to the precipitate and then centrifuge for 8 min at 7500 rpm. The supernatant was discarded and wait for the pellet to partially dry. The pellet was then dissolved in 30 to 40 µl of DEPC-treated water, 10 min was kept in a water bath at 60 °C, and the extracted RNA was stored in the freezer for later use. Quantification of the extracted RNA was performed by Nanodrop spectrophotometer and 1% agarose gel electrophoresis was used to determine the quality of the samples.

cDNA synthesis

The first-strand cDNA was synthesized with Easy™ cDNA Synthesis Kit)Parstous Company, Iran) using the following steps. The 2X RT-premix solutions contains MMLV RTase 200 units/10 µL, 100 mM Tris-HCl (pH 8.3), 20 mM DTT, 150 mM KCl, 2 mM dNTP mixture, 6 mM MgCl2, RNase Inhibitor 20 units, RTase Stabilizer and cDNA. First, for the synthesis of the first strand of cDNA, a mixture of 1 µl Oligo dT (primer), 1 µl dNTPs, 3 µl RNA, and 5 µl nuclease-free water was added (final volume was 20 µl). The mixture was incubated at 47 °C for 60 minutes in a water bath (Model S 100, Hanyang, South Korea). Then, to stop the reaction, it was placed at 85 °C for 5 min and then placed on ice for 2 min.

Gene expression analysis

The real-time PCR technique was used to analyze the expression pattern of two candidate ED-related genes. Quantitative evaluation was performed with qPCR GreenMaster with a low ROX kit (BioFACT™ Company, South Korea). To perform the Real-time PCR reaction, 100 ng of synthesized cDNA, specific primers for the studied genes and ß-actin (as reference) gene (Table 1 were used.

For analysis of gene expression by the real-time-PCR method, the SYBR green fluorescence dye method replications were used. In this method, for performing this reaction, a 48-well plate for Real-Time PCR (Rotor-Gene 6000, Corbet Research Australia) with a final reaction volume of 20 µl, including the materials mentioned (Table 2), was used. The temperature program used included enzyme activation at 95 °C for 15 min and a 40-cycle reaction including denaturation at 95 ° C for 15 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 sec. Finally, the

Table 1. Primers designed for Real-time PCR reaction

Primer name	Sequence (5′→3′)	Nucleotide No.	Annealing Tem (°C)	Amplicon length (nt)
Andpro	F: GACTTGGATCTTGGGCCTTTG	21	60	124
	R: AAATCCTGGGCCAAAAGGTTG	21		
Pdk4	F: AGAGGTGGAGCATTTCTCGC	20	60	138
	R: ATGTTGGCGAGTCTCACAGG	20		
ß-actin	F: CTGGAACGGTGAAGGTGACA	20	60	140
	R: AAGGGACTTCCTGTAACAATGCA	23		

Table 2. Chemicals and values used in Real-Time PCR reaction.

Chemical	Amount	Final concentration
cDNA	100 ng	
qPCR GreenMaster with lowRox	100 µl	1X
Primer F	0.8 µl	0.5 µM
Primer R	0.8 µl	0.5 µM
RNase free H2O	20.0 µl	

melting point test was performed in a temperature cycle including 95 °C for 15 seconds, 60 °C for 1 min and an increase of 0.3 °C to 95 °C to plot the melting diagram of the PCR product.

Analysis of real time-PCR data and study of relative expression of each gene was performed based on the relative standard curve method according to Equation 1 (46) and with Bio-Rad computing software and Excel software.

Equation 1:

$$\text{Gene expression} = 2^{-(C_T\ \text{Gene-C}_T\ \text{Ref) Case}} / 2^{-(C_T\ \text{Gene-C}_T\ \text{Ref) Control}} = 2^{-\Delta C_T\ \text{Case}} / 2^{-\Delta C_T\ \text{Control}}$$

Predicting the three-dimensional structure of PDK4 and Andpro proteins

To predict and design the three-dimensional protein structure, first, the protein sequences were received in an NCBI database (https://www.ncbi.nlm.nih.gov/). The three-dimensional structure of PDK4 Andpro predicted by Iterative Threading ASSEmbly Refinement (I-TASSER) (https://zhanggroup.org/I-TASSER/).

Statistical analysis

Data analysis and comparison of means were performed by LSD method and SAS software version 9.1 at a 5% probability level.

Results and Discussion

The main purpose of this investigation was to determine the quantitative expression levels of Andpro and Pdk4 genes in severe ED and potent men.

Performance analysis of qPCR primers

In this study, 20 ED cases and 20 potent men were examined to evaluate Andpro and Pdk4 genes expression. The results of gel electrophoresis showed that the quality of the extracted RNA was high, and the three rRNA-related bands were observed well (Fig 1). The two sharp bands are related to 28S and 18S and the weak band is related to 5S ribosomal RNA.

Results of melting showed a single peak graph by the designed primers for qPCR and there were no primer-dimer with the extra band (Fig. 2). The results of agarose gel electrophoresis confirmed the absence of primer-dimer and additional band. The PCR efficiency ranges from 96% (Andpro) to 110% (Pdk4). Calculation of the efficiency and taking it into account to determine the initial template concentration is essential to get an accurate estimation.

Andpro and Pdk4 gene expression

The qPCR was performed for evaluation of expression of Andpro and Pdk4 genes in the ED and controls. The results showed that there were significant differences ($p<0.01$) between cases and controls for Andpro and Pdk4 genes. Compared to controls, expression of the Andpro gene decreased and the Pdk4 gene increased in ED patients ($p<0.01$). The expression level of Andpro was between 0.05-0.15 (±0.033) fold changes and in

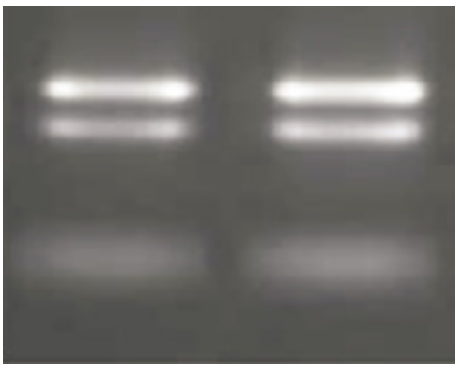

Figure 1. Two samples of gel electrophoresis of extracted RNAs. The two sharp bands are related to 28S (the heaviest) and 18S (middle) and the weak (the lightest) band is related to 5S ribosomal RNA.

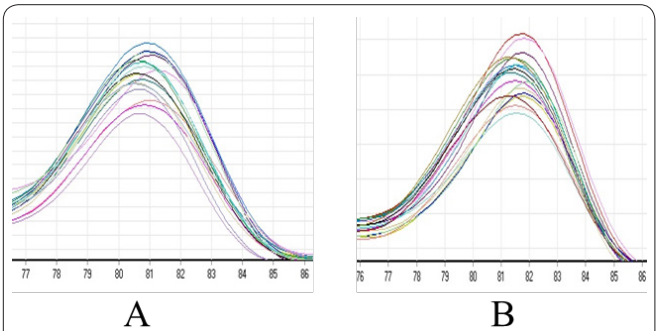

Figure 2. Dissociation curves for study genes in qPCR. A: Andpro gene and B: Pdk4 gene.

Pdk4 ranged 4.80-8.96 (±1.12) fold changes (Fig. 3).

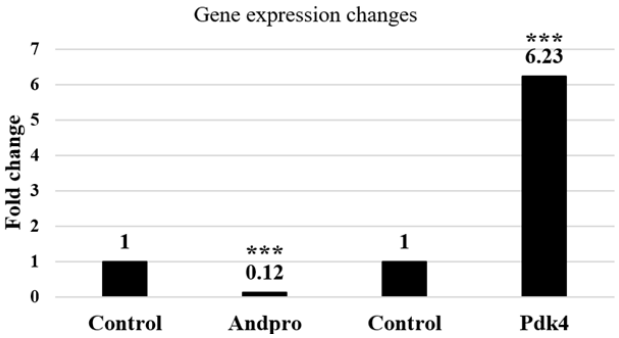

Figure 3. Changes in Andpro and Pdk4 genes expression in ED samples compared to controls. Where *** indicates $p<0.001$.

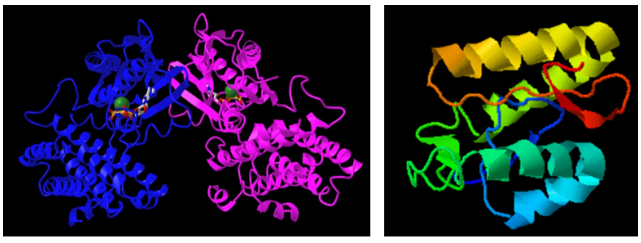

Figure 4. Predicting the three-dimensional structure of studied proteins. A: Pyruvate Dehydrogenase Kinase 4 (PDK4). B: androgen-regulated protein (Andpro).

Predicting the three-dimensional protein structure

The predicted three-dimensional protein structure of Pyruvate Dehydrogenase Kinase 4 (PDK4) and androgen-regulated protein (Andpro) is shown in Fig.4.

Prediction results showed that PDK4 protein has two subunits and Andpro protein has one subunit.

The mitochondrial pyruvate dehydrogenase complex (PDC) biocatalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA (PDK1-4). PDC activity is strictly regulated by four members of a family of pyruvate dehydrogenase kinase isoforms (PDK1-4) that phosphorylates and inactivates PDC (47).

The androgen-regulated protein (Andpro) gene (Accession NM_006685.4) also known as SMR3B or submaxillary gland androgen-regulated protein 3B, is located on the long arm of human chromosome 4. It has three exons, is 7167 bp in length, and its coding sequence is 231 bp (Table 3). It is also known as SMR1B, P-B; PBII; PRL3; PROL3 (9).

Androgen receptor (AR), is a nuclear receptor that is activated in the cytoplasm by binding to the androgenic hormones testosterone and dihydrotestosterone. In humans, the receptor is encoded by the "AR" gene, which is located on the long arm of the X chromosome (48). The androgen receptor is closely related to the progesterone receptor, and high doses of progestin can block it (49). The main role of the androgen receptor is to serve as a DNA-binding transcription factor that regulates and modulates gene expression (50). Genes regulated by androgens (male sex hormones) play an important role in the formation and perpetuation of the male sexual phenotype (51).

Maintaining a balance between energy requirements and availability is critical to maintaining good health. In this process, pyruvate dehydrogenase kinase (PDK4) plays an important role in maintaining energy homeostasis (52).

Competition between fatty acids and glucose for entry into metabolic oxidation pathways in tissues occurs mainly at the pyruvate level (pyruvate dehydrogenase compound function) resulting in derivatives of carbohydrate and lipid (53).

The pyruvate dehydrogenase kinase 4 (Pdk4) gene (Accession NM_002612.4) is located on the long arm of human chromosome 7, has 11 exons, is 13117 bp in length, and its coding sequence is 1236 bp (Table 3) (9).

A research team in San Francisco conducted a study of the genomes of 1 million men and found that different variations at the SIM1 locus on chromosome 6 were significantly associated with an increased risk of ED. Although previous studies of twins have shown that at least one-third of the risk of ED is hereditary, they have not yet determined the location of the risk in the human genome. In a community of nearly 37,000 people, studies of SIM1 locus have shown that this locus is linked to ED through a new mechanism (54). However, variations of SIM1 locus are associated with many other abnormalities, diseases dysfunctions and failures, especially cancers, and should not be seen as a simple failure (55-56).

The performed qPCR for assessment of Andpro and Pdk4 genes expression in the ED and controls indicated that there were significant differences between cases and controls for these genes. We observed down-regulation in the Andpro gene and up-regulation in the Pdk4 gene in the ED cases compared to controls. It was predicted that Pdk4 protein and Andpro proteins have two and one subunits, respectively.

A limitation of our study was the age discrepancy of ED cases and controls, with the controls being remarkably younger than the ED cases. However, it can be argued that age was not significantly involved in the expression of genes. Another limitation of our

Table 3. The sequence of studied proteins including Andpro (Accession: AAP97234.1 and GI: 33150712) and Pdk4 (Accession: NP_002603.1 and GI: 4505693).

Protein name	Sequence
Andpr	MMLFKVLVITVFCGLTVAFPLSELVSINKELQNSIIDLLNSVFDQLGSYRGTKAPLEDYTDDDLSTDS EQIMDFTPAANKQNSEFSTDVETVSSGFLEEFTENTDITVKIPLAGNPVSPTS
Pdk4	MKAARFVLRSAGSLNGAGLVPREVEHFSRYSPSPLSMKQLLDFGSENACERTSFAFLRQELPVRL ANILKEIDILPTQLVNTSSVQLVKSWYIQSLMDLVEFHEKSPDDQKALSDFVDTLIKVRNRHHNV VPTMAQGIIEYKDACTVDPVTNQNLQYFLDRFYMNRISTRMLMNQHILIFSDSQTGNPSHIGSIDPN CDVVAVVQDAFECSRMLCDQYYLSSPELKLTQVNGKFPDQPIHIVYVPSHLHHMLFELFKNAM RATVEHQENQPSLTPIEVIVVLGKEDLTIKISDRGGGVPLRIIDRLFSYTYSTAPTPVMDNSRNAPLA GFGYGLPISRLYAKYFQGDLNLYSLSGYGTDAIIYLKALSSESIEKLPVFNKSAFKHYQMSSEADDW CIPSREPKNLAKEVAM

study is the small number of participants. Despite these limitations, we think that our preliminary study is useful in helping other researchers to focus on these two genes in their larger scale, more rigorous studies.

Author Contributions

Software, Elham Kazemi Mozhgan Fatahi Dehpahni; supervision, Javaad Zargooshi and Marzieh Kaboodi; writing, review and editing, Javaad Zargooshi, Elham Kazemi, Leila Yazdani and Danial Kahrizi; investigation, Elham Kazemi and Hamid Reza Mohammadi Motlagh; validation, Habibolah Khazaei; Advisor, Javaad Zargooshi and Habibolah Khazaei. All authors have read and agreed to the published version of the manuscript.

Funding

This research as a part of the Ph.D. thesis of the first author was funded by Kermanshah University of Medical Sciences, Kermanshah, Iran.

Acknowledgments

This research is a part of the first author's Ph.D. thesis that has been supported by Kermanshah University of Medical Sciences, Kermanshah, Iran.

Ethical consideration

This study was reviewed by the ethics committee of Kermanshah University of Medical Sciences and approved with the ethics committee number IR.KUMS.REC.1397.925. The informed consent form was completed and signed by all clients before attending the study.

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

ACE (Angiotensin-converting enzyme), Andpro (Androgen regulatory protein), Ang1 (Angiopoietin-1), AR (Androgen receptor), CXCR3 (C-X-C Motif Chemokine Receptor 3), DDAH2 (Dimethylarginine Dimethylaminohydrolase 2), DEPC (Diethyl Pyrocarbonate), dNTP (Deoxynucleoside triphosphate), ED (Erectile dysfunction), EDTA (Ethylenediaminetetraacetic acid), eNOS (Endothelial nitric oxide synthase), FGF2 (Fibroblast Growth Factor 2), GNB3 (Guanine nucleotide-binding protein), HMGCS2 (3-Hydroxy-3-Methylglutaryl-CoA Synthase 2), hNGFβ (human nerve growth factor beta), IGFBP-3 (Insulin-Like Growth Factor Binding Protein-3), IIEF (The international index of erectile function), IL27 (Interleukin 27), lncRNA (Long non-coding RNAs), MEG3 (Maternally Expressed 3), MS (Multiple sclerosis), NOS3 (Nitric Oxide Synthase 3), NPT (Nocturnal penile tumescence), PCSK9 (Proprotein convertase subtilisin/kexin type 9), PDC (pyruvate dehydrogenase complex), Pdk4 (pyruvate dehydrogenase kinase 4), PSMB9 (Proteasome 20S Subunit Beta 9), PTAFR (Platelet Activating Factor Receptor), qPCR (Quantitative Polymerase Chain Reaction), SMR3B (submaxillary gland androgen-regulated protein 3B), TNF-α (Tumor Necrosis Factor Alpha) and VEGF (Vascular endothelial growth factor).

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