Identification of Serum Biomarkers in End Stage Liver Disease

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Abstract: *Background*: Progressive fibrosis and cirrhosis, clinically presenting as end-stage liver disease are common outcomes in alcoholic hepatitis as well as non-alcoholic fatty liver disease(NAFLD). In these processes, a series of changes occurs in liver tissues leading to cell death, remodeling, fibrosis and regeneration. The aim of this study is to identify potential novel biomarkers for non-invasive diagnosis of cirrhosis due to alcoholic etiology or NAFLD.

Methods: Serum from patients with biopsy proven end-stage liver disease of various etiologies, namely NAFLD(n=9), alcohol(n=5), and other end-stage liver diseases(n=6), who underwent liver transplant during the first six months of 2007 were utilized for retrospective analysis. Serum samples were also collected from a group of healthy volunteers (n=7). The samples were analysed using Luminex technology or ELISA for 27 biomarkers that are known to be involved in pathologic processes such as cell death, regeneration and fibrosis.

Results: Of the 27 serum markers examined, 16 were elevated in the serum in all groups with end-stage liver diseases compared with the control group. They include adipokines, apoptosis and inflammatory mediators and growth factors. Interestingly, the serum of NAFLD patients showed significantly elevated HGF levels and trend towards increase in sFAS, TGFβ1, TNFR-1, TNFR-2 and leptin. The level of serum markers showed excellent correlation with each other indicating a complex interdependent pathogenetic mechanism.

Conclusions: The data from this study indicate that a large number of serum markers are altered in end-stage liver diseases. A panel of such markers may potentially be useful in assessing advanced fibrosis and cirrhosis in patients with chronic end stage liver diseases.

INTRODUCTION

Progressive fibrosis and ultimately cirrhosis are common features in a number of chronic end-stage liver diseases. In these processes, a series of changes occur in liver tissues leading to cell death, remodeling, regeneration and fibrosis. During this process of cellular death and regeneration, a series of alterations take place at the molecular and cellular level, leading to the alteration of a vast number of molecules. Hepatic stellate cells (HSCs) have been revealed as key components in hepatic healing and fibrosis [1]. There is emerging evidence that the fibrogenic function of HSCs may be influenced by cytokines, growth factors, death receptor ligands, and even adipokines [2-4].

From the diagnostic and prognostic aspect, percutaneous liver biopsy is considered the gold standard for the assessment of fibrosis. However, this procedure is expensive, invasive, amenable to sampling and interpretive error, and poses a considerable risk of complications including pneumothorax, hemorrhage, and puncture of other viscera. As a consequence to these limitations, a great deal of attention has been given to institute safe and reliable surrogate markers of liver fibrosis. In some of the earlier studies, alterations of serum levels of the molecules such as pro-collagen peptides, FAS, TRAIL, and metalloproteinases have been demonstrated to correlate with degree of liver fibrosis [5-8].

Furthermore, in an end stage cirrhotic liver, histologic findings in conditions such as alcoholic liver diseases and non-alcoholic fatty liver disease (NAFLD) are similar. Hence, etiologic differentiation based on histologic findings is extremely difficult. Along with alcoholic liver diseases, NAFLD is becoming more common worldwide, possibly due to lifestyle alteration, increased incidence of obesity and metabolic syndrome. In the past 10 years, the rate of obesity has doubled in adults and tripled in children [9]. Both alcohol and NAFLD may ultimately lead to progressive cirrhosis and fibrosis. It is important to distinguish NAFLD from alcoholic liver disease as management between these two etiologies vastly differs.

Within the last decade, studies have attempted to evaluate non-invasive diagnostic tests for advanced liver disease such as fibrosis and cirrhosis using echography, transient elastography, magnetic resonance imaging, and biochemical tests [7-10]. Serum markers of fibrosis have the potential of either replacing the need for liver biopsy or identifying those patients with more advanced liver disease who may benefit from a liver biopsy and other subsequent intervention. Ideally, such markers should possess the ability to discriminate between degrees and etiologies of fibrosis and may even have the ability to determine response to various therapies and evaluate not only disease progression but also possible regression.

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The aim of this study was to identify potential novel serum biomarkers which may be useful in the diagnosis of advanced fibrosis and cirrhosis and specifically, to evaluate whether they are helpful in differentiating the etiologic factors such as alcohol or NAFLD.

METHODS

Patient Population

To determine the levels of the potential markers of liver fibrosis in patients with various liver diseases, we carried out a retrospective analysis. Pre transplantation serum from patients with biopsy proven end-stage liver diseases (n=20) of various etiologies, who underwent consecutive liver transplants, during the first six months of 2007 were collected and stored at -80^{o}C. These samples were utilized for analysis. They were grouped as alcoholic cirrhosis (n=5), cirrhosis associated with NAFLD (n=9). The other group consists of end stage liver disease of various etiologies not related to alcohol or NAFLD(n=6). Histopathological findings of the explanted liver were reviewed in a masked fashion by two pathologists, to confirm the diagnosis. All patients' charts were reviewed for possible clinical correlations with other relevant data such obesity, diabetes, and liver enzyme levels. No additional inclusion/exclusion criterias were used as this study was retrospective and exploratory in nature. Serum from healthy volunteers (n=7), similarly collected and stored, were also analyzed. The volunteer (control) group consisted of 4 male and 3 females with age range of 32-55 years. They didn't have any known liver diseases and no history of alcoholism. They didn't show any sign of acute illness during the time of blood collection. Liver enzyme assessment or biopsies were not performed for this group.

Quantification of Biomarkers

We used Luminex^{TM} (Luminex Corp., TX), fluorescence bead-based system to carry out simultaneous analysis of multiple biomarkers. Luminex uses a bead-based technology. Each bead set is coated with a antibody specific to a particular molecule. Within the analyzer, laser excites the internal dyes that identify each microsphere particle, and also captures any reporter dye during the assay. In this way, xMAP^{TM} technology allows multiplexing of up to 100 unique quantitative assays within a single sample.

Serum was analyzed for levels of (1) inflammatory cytokines: interleukin (IL)-1ra, IL-4, IL-6, IL-8, IL-10, TNFR-1, TNFR-2, TNF-α, IFN-α and C-reactive protein (CRP); (2) proteinases: MMP-1, MMP-2, MMP-9; (3) adipokines: adiponectin, leptin, resistin; (4) angiogenic and growth factors: HGF, bFGF, PDGF-bb, TGF-b, VEGF; (5) apoptosis markers: soluble Fas, Fas ligand (sFasL) using multiplexed biomarker immunoassay kits according to manufacturers' instruction (Biosource, Medicorp, Que; Linco*plex*, Millipore Corp, MA; R&D$_{TM}$ Systems Inc., MN; Bio-Rad Laboratories, CA). A Bio-Plex^{TM} 200 readout System was used (Bio-Rad). Levels were automatically calculated from standard curves using Bio-Plex Manager software (v.4.1.1, Bio-Rad). Levels of TGF-β were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, ON).

Statistical Analysis

Statistical analyses were performed using Wilcoxon rank-sum test with post-hoc (bon-ferroni correction) analysis and Spearman correlation analysis.

RESULTS

The aim of this study was to identify potential serum markers that are altered in end-stage liver diseases. In addition we tried to identify whether the levels of any such markers are different in cirrhosis of various etiologies namely alcohol and NAFLD.

The demographic and the clinical data of the patient population are outlined in Table **1**. The age of the healthy

Table 1. Clinical Characteristics of the Patients in this Study

Etiology	Age	Sex	Risk Factors	ALT	AST	Alk.phos	Bilirubin
Alcohol	66	Male	None	17	43	52	60
Alcohol	50	Male	Obesity (BMI:34.8)	717	2114	90	71
Alcohol	58	Male	None	36	62	143	117
Alcohol	65	Male	None	30	70	127	272
Alcohol	41	Female	DM, obesity (BMI: 31)	13	24	86	57
NAFLD	59	Male	DM, obesity (BMI: 31)	36	78	119	154
NAFLD	58	Male	DM	404	652	202	165
NAFLD	40	Male	DM, obesity	348	487	104	166
NAFLD	65	Male	Obesity (BMI: 38.4)	21	56	74	643
NAFLD	62	Female	DM, obesity	33	48	96	27
NAFLD	67	Male	DM, obesity (BMI: 28.8)	33	64	93	171
NAFLD	46	Male	Obesity (BMI: 44.8)	44	80	151	71
NAFLD	61	Female	Hypothyroidism, obesity	33	53	119	33
NAFLD	47	Female	obesity (BMI: 36.6)	24	37	122	37
Budd-Chiari	35	Male	Methadone abuse	26	56	163	34
Acetaminophen	17	Female	None	4006	2032	211	86
Polycystic liver	53	Female	None	20	34	138	12
Sarcoidosis	56	Female	Obesity (BMI:31.7)	15	24	138	52
A-1 antitrypsin	65	Male	DM	31	64	174	71
Cryptogenic	50	Male	None	44	80	151	72

Table 2.

GROUPS													
BIO-MARKERS	**Units**	**NAFLD**				**ALCOHOL**			**OTHERS**			**CONTROL**	
		MEAN	***MEDIAN***	***SD***	***MEAN***	***MEDIAN***	***SD***	***MEAN***	***MEDIAN***	***SD***	***MEAN***	***MEDIAN***	***SD***
Apoptosis related													
sFas	pg/ml	15072.7	14756.3	4194.4$	10320.3	7786.6	3085.4	11213.1	10796.1	4034.1	4559.7	4676.2	473.8*
sFas ligand	pg/ml	347.8	196.8	307.9	240.5	199.2	199.2	96.9	62.70	115.7	0	0	0*
Growth Factors													
TGF-β 1	ng/ml	3.9	2.6	3.4$	1.5	1.5	1.9	2.6	0.9	3.4	0	0	0*
TGF-α	pg/ml	149.3	42.6	296.8	1187.4	30.3	2804.9	156.7	45.64	280.5	68.7	57.8	56.6
HGF	pg/ml	753.6	767.9	165.4**	523.7	478	127.8	680.3	503.4	453.7	131.8	124.5	28.5*
Basic FGF	pg/ml	12.1	0	15.7	8.5	0	20.9	14.7	0	23	17.6	8.8	22.5
PDGF-bb	pg/ml	2664.5	2933.1	1585.8	2357.7	1466.8	1466.8	4041.6	4673.5	1840.7	1597.2	1414.4	880.3
VEGF	pg/ml	247.4	147.7	134.8	99.7	53.5	99.6	136.9	94	127	39.5	38.1	22.8
Inflammatory mediators													
IFN-α	pg/ml	27.7	7.7	19.3	32.6	0.5	55.2	25.6	0	52.5	15.2	0	26
TNFR-1	pg/ml	7175.3	4209.9	6586.2$	2632.5	2552.2	1164.9	2612.9	2388.3	1124	621.4	614.5	161.5*
TNFR-2	pg/ml	6784.7	6824.8	4025.3$	3472.8	2816.5	1756.6	4011.4	3833.1	1200.2	1265.1	1248.1	339.1*
IL-1 β	pg/ml	16	0	25.3	6	0	14.7	0	0	0	0	0	0
IL-1ra	pg/ml	360.4	359.1	171.5	235.2	186.7	208	135.1	74.4	168.3	0	0	
IL-1 α	pg/ml	1041.6	31.6	2322.9	1054.1	424.7	1857.9	987.5	811.9	342.6	2569.6	2405.4	2703.8
IL-4	pg/ml	2	2	1.1	2	1.3	0.9	2.4	2.2	1.6	0.2	0	0.5*
IL-6	pg/ml	360.5	109.3	498.2	233.2	136.4	304.8	49.4	38.4	44.4	0	0	0*
IL-8	pg/ml	1518.9	858.9	2070.8	2027.6	496.9	4144.4	241.4	126.5	253.6	2	0	5.4*
IL-10	pg/ml	0	0	0	0	0	0	0	0	0	0	0	0
IL-13	pg/ml	2.9	2.8	1.9	2.2	2.2	2.7	5.9	1.9	7.9	1.6	0	3
TNF-α	pg/ml	11.4	0	25.9	18.8	0.3	32.1	20.5	0	50.3	4	0	10.6
CRP	ng/ml	20221.2	23000	9075.7	15712.8	15712.8	11289.3	23000	23000	0	7090.5	1300.1	10877.5*
MMP-1	pg/ml	3959.8	3335.9	2555.9	4617	2955.8	3618.8	4628.9	4187.8	2903.8	1484.6	1402.2	679.4*
MMP-2	ng/ml	336.1	297	85.4	329.9	329.9	71.7	264.3	250	73.8	177.2	173.7	20.6*
MMP-9	ng/ml	321.1	285.3	86.8	357.1	326.4	147.3	355.3	273.9	248.8	44	41.7	17*
Adipocytokines													
Leptin	pg/ml	18986.3	7976.1	6318.9	25055.7	24300.2	16426.8	23994	20002.2	12792.7	15508	7406.2	17651.5
Resistin	pg/ml	17802.8	17468.5	4908.3	25640.4	17599.5	17599.5	12025.2	10376.5	4259.3	6548.1	6731.5	1801.8*
Adiponectin	ng/ml	15856.3	17245.3	7337.8	19153.8	18706	6478	19567.9	21092.33	5109.7	8484	8562.7	1837.2*

* = significantly different compared to all other groups or Alcohol and NASH group combined, ** = significantly different compared to alcohol group, $ = p<0.2 compared to alcohol group.

volunteers ranged from 29-51 (M=4, F=3). None of the volunteers had any history of diabetes, liver disease or alcoholism.

Of the 27 biomarkers examined, 16 were significantly elevated (2.7-to >2000 fold in mean value) in the serum of the patients with all end-stage liver diseases (irrespective of etiology) were compared with the control group (Table **2**). Similar statistical significance was also observed when control group were compared with cirrhotic patients of combined alcohol and NAFLD group. These biomarkers include adipocytokines, markers of apoptosis, inflammation and growth factors. Both sFas and sFas ligands were significantly elevated. Among the growth factors, TGFβ1 and HGF were significantly elevated. No alteration in TGFα and bFGF were seen. Although both VEGF (4.3 fold) and PDGFbb (1.9 fold) were elevated, the p values fell short of significance ($p<0.06$ and <0.07 respectively). Several of the inflammatory cytokines were significantly elevated in the cirrhotic livers. These include TNFR1, TNFR2, IL1ra, IL-4, -6, -8, MMP-1,

-2, -9 an CRP. No significant alterations were seen in IL-1α, IL-10, -13. In the adipocytokine group both adiponectin and resistin were significantly elevated in all groups with end-stage liver diseases, whereas no significant alterations in serum leptin levels were observed. Very high levels of upregulation were seen in sFas ligand, IL-1ra and IL-6 in these groups compared to controls. It is of further interest to note that some of the investigated molecules such as TGFβ1, IL-6, IL-1B and IL-1ra were undetectable in the control population.

A further intention of the study was to observe if any patterns of markers were able to differentiate two specific common etiologies of cirrhosis. In this respect, the serum of NAFLD patients showed significantly ($p<0.01$) elevated HGF levels. It is however to be noted that several biomarkers, such as sfas, TGFβ1, TNFR-1, -2 and leptin, were different in these two groups. However these failed to reach a level of statistical significance and they demonstrated a p value ranging from <0.1 to <0.2.

The correlation analysis showed excellent correlation of various biomarkers with each other. Except for leptin and IL-10 (undetectable in any group), all other biomarkers showed significant correlation with one or more markers from same and other groups (Table **3**).

DISCUSSION

Liver biopsy utilized to stage liver diseases has recently become increasingly being criticized because of its invasiveness and expense, despite the associated low incidence of morbidity and mortality [10]. The major drawback of liver biopsy is, however, the potential for sampling error. A liver biopsy samples a miniscule portion of liver; and even with a 2.5 cm biopsy, inaccurate staging can occur in up to 20% of biopsy samples [11].

Currently, there are multiple non-invasive methodologies proposed to evaluate liver fibrosis and all appear to perform reasonably well and with a similar diagnostic accuracy. The FibroTest (FibroSure, LabCorp.USA) and FIBROSpect II

Table 3. Spearman Rank Correlation

Biomarkers	Correlates significantly ($p<0.05$) with
Apoptosis related	
sFas	sFas ligand, TGF-β1, HGF, VEGF, TNFR-1, TNFR-2, IL-1B, IL1ra, IL-4, IL-6, IL-8, MMP-2, resistin, adiponectin
sFas ligand	S Fas, TGF-β 1, HGF, TNFR-1, TNFR-2, IL-8, MMP-2,
Growth Factors	
TGF-β 1	S Fas, sFas ligand, HGF, IFNα, TNFR-1, TNFR-2, IL1ra, IL-4, IL-6,MMP-2
TGF-α	basic FGF, IL-1α, adiponectin
HGF	S fas, sFas ligand, TGF-β1, VEGF, TNFR-1, TNFR-2, IL-6, IL-8, MMP-2, MMP-9, resistin, CRP, adiponectin
Basic FGF	Il-10, IL-13, TNFα
PDGF-bb	VEGF, IL1ra, MMP-1, MMP-9
VEGF	sFas, HGF, PDGF-bb, IFNα, TNFR-1, TNFR-2, IL-6, IL-8, MMP-9, resistin, CRP
Inflammatory mediators	
IFN-α	TGF-β1, PDGF-bb, VEGF,
TNFR-1	sFas, sFas ligand, TGF-β1, HGF, VEGF, TNFR-2, IL1ra, IL-4, IL-6,MMP-2, MMP-9, leptin, resistin
TNFR-2	sFAS, sFas ligand, TGF-β1, HGF, VEGF, TNFR-1, IL1ra, IL-6, MMP-9, leptin, resistin
IL-1 β	IL-6, IL-8
IL-1ra	sFas, TGF-β1, HGF, TNFR-1, TNFR-2, IL-4, IL-6
IL-1 α	TGF-α
IL-4	sFas, TGF-β1, PDGF-bb, TNFR-1, IL1ra, IL-6,IL-13, MMP-1, MMP-2, MMP-9, resistin, adiponectin
IL-6	sFas, TGF-β1, HGF, VEGF, TNFR-1, TNFR-2, IL-1 β,IL1ra, IL-4, IL-8, MMP-2, MMP-9, resistin, CRP, adiponectin
IL-8	S Fas, sFas ligand, HGF,VEGF, TNFR-1, TNFR-2, IL-1 β,IL1ra, IL-4, MMP-1, MMP-2, MMP-9, resistin, CRP, adiponectin
IL-10	-
IL-13	basic FGF, IL-4, TNF-α
TNF-α	basic FGF, IL-1ra MMP-1
MMP-1	PDGF-bb,IL-4,IL-8, TNF-α
MMP-2	S Fas, sFas ligand, TGF-β 1,HGF, TNFR-1,IL-1ra,IL-4,IL-6,IL-8, IL-9, resistin, CRP, adiponectin
MMP-9	HGF, PDGF-bb, VEGF, TNFR-1,TNFR-2,IL-1ra,IL-4, IL-6,IL-8, MMP-2, MMP-9, resistin, CRP, adiponectin
CRP	HGF, VEGF, TNFR-1,TNFR-2, IL-6,IL-8, MMP-2, MMP-9, resistin, adiponectin
Adipocytokines	
Leptin	-
Resistin	sFas, HGF, VEGF, TNFR-1,TNFR-2, IL-1ra,IL-4, IL-6,IL-8, MMP-2, MMP-9,CRP
Adiponectin	sFas, HGF, TG F-α, IL-4, IL-6,IL-8, MMP-1,MMP-2, MMP-9,CRP

(Prometheus Laboratories Inc., San Diego, CA) are commonly utilized tests that combine multiple markers for predictive value, including hyaluronic acid, tissue inhibitor of metalloproteinase-1 (TIMP-1), haptoglobin, α_2-macroglobulin, apolipoprotein A1, and total bilirubin [12]. A recent study from Rosenberg and the European Liver Fibrosis Group also used HA, procollagen peptide III and TIMP-1 to stage fibrosis in a variety of liver diseases with promising results [13]. Moreover, the SHASTA index (using Serum HA, AST, Albumin) was also shown to predict fibrosis [14].

The data from this study indicate that the large number of serum markers, involved in various processes such as apoptosis, fibrosis and angiogenesis, are altered in cirrhosis; and a panel of such markers may potentially be useful in assessing end-stage liver damage and cirrhosis in patients with chronic liver diseases. Use of luminex technology provides an unique opportunity to quantify multiple molecules from a small sample and provides a novel avenue to explore a large number of molecules. This technology also allowed us to measure previously undetectable levels of proteins. Such technology has previously been used in other disease process [15]. Although several of the molecules we studied may mediate several cellular processes, based on some of their major functions we selected to study specific markers as they relate to specific pathogenetic processes of interest in cirrhosis. We found alterations of several biomarkers. The results of this study are in keeping with the complexity of the pathogenetic mechanisms, such as cell death, inflammation, angiogenesis and fibrosis leading to cirrhosis. The correlation analysis further suggests interactions of these factors and complexity of the process regardless of its etiology. Some of our data are in keeping with the previous findings whereas others are not. Previous studies have shown increased TGFβ1, IL-10, TNFR-2, IL-8, MMP-1, MMP-2, MMP-9 in cirrhosis [7, 16-19]. However, majority of such studies were carried out in hepatitis C patients. In our study we found increased TGFβ1, TNFR-2, IL8, MMP-1,-2,-9 in all cirrhotic patients regardless of etiology. We didn't detect increased IL-10 in any groups in this patient population of non-viral end-stage liver disease. Some investigators have also demonstrated increased IL-8 and IL-6 in alcoholic liver disease and suggested that this can be used as a marker for such etiology [20, 21]. None of these studies however, compared the levels of these markers with that in the cirrhosis of non-alcoholic etiology. In our study, these biomarkers were elevated in end stage liver disease irrespective of the etiology. Hence although, they may be useful to assess extent of the liver damage (as in normal volunteer, they were very low or undetectable), they may not be useful as etiology-specific marker. Similarly increased serum adiponectin and resistin levels have previously demonstrated in NAFLD and have been proposed to be a potential markers of these diseases [22, 23]. However, these studies also didn't compare these data with liver damage induced by other etiologic factors. In our study such increase were seen in all end stage liver diseases. Hence these markers may not so be etiology specific.

Based on this study measurement of a panel of markers including sFas and sFas ligands, TGFβ1, HGF, TNFR-1, TNFR-2, IL-1ra, IL4, IL-6, IL-8, MMP-1, MMP-2, MMP-9, CRP, adiponectin and resistin may be useful in the diagnosis of end stage liver disease such as cirrhosis. However, a smaller panel including the molecules such as sFAS ligand, TGFβ1, IL-1ra and IL-6 which are either undetectable in normal population or highly elevated in cirrhosis may be a practical alternative. However, larger studies are necessary to confirm such notion. It is also interesting to note that levels of HGF were significantly different in Alcohol and NAAFLD group. Increased HGF has previously been demonstrated in alcoholic hepatitis [24]. Similar to our study, in chronic liver disease, it has been shown to correlate with CRP [25]. In addition, we have also observed that several other biomarkers such as sFas, TGFβ1, TNFR-1, -2 and leptin showed a trend to increase in alcoholic cirrhosis compared to NAFLD group. . Although, these didn't reach the level of statistical significance ($p<0.1$-$p<0.2$), possibly due to a small sample size, further studies are warranted. Hence in is tempting to speculate that a panel including such marker may be useful for future studies to consider, which may potentially be helpful in differentiation between these two etiologic factors. We refrained from carrying out additional analysis to identify predictive values of the markers identified in this study as we recognized the limitation, i.e, small sample size. However, larger prospective studies in patients with chronic liver disease may be conducted to confirm the accuracy of these markers. Such studies are already underway in our laboratories. Nevertheless, the present study may represent a first step towards the identification of a panel of such non-invasive serum markers. As the knowledge of biomarkers and noninvasive tests for fibrosis increases and long-term studies become available, it will be interesting to study if these tests have the same prognostic value as liver biopsy in relation to the risk of disease progression and clinical outcomes.

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ABBREVIATIONS

AST	=	Aspartate transaminase
ALT	=	Alanine transaminase
bFGF	=	Basic fibroblast growth factor
CRP	=	c - reactive protein
DM	=	Diabetes mellitus
ELISA	=	Enzyme-linked immunoabsorbant assay
IFN	=	Interferon
IL	=	Interleukin
MMP	=	Matrix metalloproteinase
NAFLD	=	Non-alcoholic fatty liver disease
PDGF-bb	=	Platelet derived growth factor –bb
sFas	=	Soluble Fas
TGF-β	=	Transforming growth factor β
TNF	=	Tumour necrosis factor
TNFR	=	Tumour necrosis factor receptor
VEGF	=	Vascular endothelial growth factor

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