Gene polymorphism of Il-18 as a pro-inflammatory mediator and susceptibility of diabetes type 1in Egyptian children.

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ABSTRACT

Type 1 diabetes (T1D) is a chronic immune-mediated disease and is the most common autoimmune disorder in childhoodbut the disease may become manifest at any age. It is characterized by selective loss of insulin-producing β-cells in the pancreatic islets in genetically susceptible subjects.Interleukin-18 (IL-18) is a proinflammatory cytokinethat was originally identified as an interferon-γ inducing factor.It plays a critical role in chronic inflammation, in autoimmune diseases, and in a number of infectious diseases. Functional variants of IL18 gene has been reported as associated with T1D.the purpose of this was to investigate the role of IL-18 gene polymorphism (-607 A/C)in the prediction ofT1D in the Egyptian children. One hundred and four patients with T1D and 116 healthy controls, IL-18 promoter genotyping at -607 (rs1946518) position was analyzed with sequence-specific PCR(PCR/SSP).Therewas no significant association between polymorphisms in SNPs of IL-18 (rs1946518A/C) and T1D risk in Egyptian population,However, the results suggest an association of genotypes with age at onset of T1D.Our preliminary data suggested that polymorphisms in the IL-18 gene may not contribute to T1D susceptibility. To the best of our knowledge, this study is the first one that deals with IL-18 gene polymorphism in T1D patients in Egypt. Although, further studies with large sample size should be conducted to validate these results in Egyptian population

Key words: T1D; IL-18; polymorphism; PCR/SSP

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-secreting pancreatic b-cells are destroyed by autoimmune attack. Although the etiology of T1D is not fully understood, it has been suggested to involve interactions between a susceptible genetic background and environmental factors. ***(Noble and Erlich2012; Lee et al., 2009).*** **Age** in type 1 diabetes at symptomatic onset is no longer a restricting factor. ***(Leslie, 2010).*** Polydipsia, polyphagia, and polyuria (the classic trio of symptoms associated with disease onset).

T1DM is associated with the appearance of autoantibodies many months or years before symptom onset. These autoantibodies are not thought to be pathogenetic but serve as biomarkers of the development of autoimmunity. Characteristic autoantibodies associated withT1DM are those

that target insulin (***Ziegler et al., 1999; Ilonen et al.,2013; Krischer et al.,2015).*** Individuals with specific HLA genotypes (which encode MHC proteins) — that is, HLADR and HLADQ genotypes (HLADRDQ) — have an increased risk of developing two or more autoantibodies and T1DM***.(Krischeret al., 2015; Ziegler et al., 2013)***

The inflammatory pathway has an important role in the development and complications of T1D (***B. Baumann and Salem2012).*** In inflammations events, several interleukins (ILs) could be produced in order to activate specifics immune components and present itself as a potential factor in association to T1D pathogenesis.***(Hulme et al., 2012; Kunz and Ibrahim 2009; Reddy 2004).***

Interleukin-18 (IL-18), a member of the IL-1 family, is produced by monocytes, macrophages, and dendritic cells. ***(Srivastava et al., 2010).*** IL-18 promotes Th1 responses through stimulating interferon (IFN)- g production by T lymphocytes and natural killer cells in synergy with IL-12 ***(Nakanishi et al., 2010).*** Because of the important role of the Th1/Th2 balance in immunological homeostasis, the expression of IL-18 has been correlated with several autoimmune and inflammatory disorders ***(Shan et al., 2009a)***

IL-18 is now recognized as an important regulator of innate and acquired immune responses, including potent stimulation of interferon (IFN)-γ production, enhancement of cell cytotoxicity of natural killer cells, and stimulation of Th cell differentiation (***Garlanda et al., 2013; Dinarello et al., 2013; Sedimbiet al.,2013)***. It plays a critical role in chronic inflammation, in autoimmune diseases, and in a number of infectious diseases ***(Tanaka et al.,2001; Tucciet al.,2006; Aachouiet al.,2013; Motavafet al.,2014)***.

The human *IL-18* gene is located on chromosome 11q22.2–q22.3, and the production and activity of IL-18 is regulated by the *IL-18* promoter gene *(*Thompson and Humphries 2007***; Smith and Humphries 2009;** *Barker***et al., 2011)***.* Accordingly, polymorphisms in the *IL-18* promoter gene are attractive candidates due to their impact on pro-inflammatory cytokine production and subsequently on inter-individual disease susceptibility.

The existence of SNPs was investigated at the *IL-18* promoter gene *(*Thompson and Humphries 2007***; Smith and Humphries 2009;** *Barker***et al., 2011)**Recently, these promoter SNPs have been found associated with genetic susceptibility to many autoimmune diseases, such as systemic lupus erythematosus, type 1 diabetes, and rheumatoid arthritis ***(Dong et al., 2007; Sa´nchez et al., 2009; Wen et al., 2014).***However, divergent results of association have been found in different populations, being necessary replications studies in population with different genetic background.

MATERIALS AND METHODS

Subjects

In the present study, 104 diabetes type 1 patients and 116 healthy individuals as control group were enrolled. (56 males; 48 females). The mean age was 11.08±4.24 with the range of 4-18 years. All patients were examined for the biochemical and diabetic parameters. Demographic and biochemical data of both patients and healthy controls were summarized in Table (1). The diabetes type 1 patients were positive for diabetes. control cases with no history of diabetes in them family. This study was approved by Health and Human Ethical Clearance Committee guidelines for Clinical Researches and following recruitment, the subjects gave informed consent for genetic analysis.

DNA extraction

2 ml of peripheral venous blood was collected from all the patients after an overnight fasting in EDTA tube. Genomic DNA was extracted from whole blood by using the Gentra Puregene Blood Kit (Qiagen Company, Hilden, Germany) according to manufacturer 's instructions. The tubes were centrifuged at 1500 rpm for 10 min. Plasma was separated, aliquoted, and stored at and frozen at- 20^{o}C. Extracted DNA was applied to 1% agarose gel to confirm its integrity. The concentration of DNA in all samples was measured by using a Nano drop 2000c (Thermo scientific).

Genotyping of IL-18

Polymorphism at position -607**(**rs1946518**)** in the promoter region of IL-18 gene was analyzed by polymerase chain reaction withsequence specific primers (PCR-SSP) method ***(Zhang P-Aet al 2005)***. For the position -607 A/C specific PCR, a common reverse primer and 2 sequence specific primers were used. An amplified product of 196 bp was detected. A control forward primer was used to detect an amplified product of 301 bp fragment and acts as an internal positive amplification control (Table 1).

PCR reactionwas done in two tubes, one for each allele. Each reactionmixture was consisted of DreamTaq Green Master Mix (2×) (Fermentas, Thermo Fisher Scientific Inc.), by addition of 10 pmol of each allele-specific primer, 10 pmol of reverse primer, and 150 ng of template DNA reaction mixture. In addition, internal positive control primer was added to the reaction mixture. Therefore, for every individual two PCR reactions were performed. All polymerase chain reaction (PCR) were performed in the 2720 thermal cycler (Applied Bio systems). **PCR conditions** were as follows; denaturation at 94 C for 2 min, followed by seven cycles for 20 s at 94 C, 40 s at 64 C and 40 s at 72 C and 25 cycles for 20 s at 94 C, 40 s at 57 C, 40 s at 72 C and 72 C for 5 min. PCR products were examined by 2% agarose gel electrophoresis stained with ethidium bromide and documented.

Table 1. Primers for IL-18 gene promoter polymorphism.

SNP	primer	Sequence	Product size
-607 A/C-specific PCR	Forward primer	5-GTTGCAGAAAGTGTAAAAATTATTAC-3	196-bp
	Reverse primer	5-GTTGCAGAAAGTGTAAAAATTATTAA-3	
	Control forward primer	5-CTTTGCTATCATTCCAGGAA-3	301-bp
	Common reverse primer	5-TAACCTCATTCAGGACTTCC-3	

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 19 (IBM Corporation, USA). Comparisons were made using independent test and results were presented as mean ± SD. Chi-square tests were used to test for differences in allele and genotype distribution between different groups. Odds ratios (OR with 95% CI) were calculated to measure the relative risks in both control and CHB patients. The correlation between variables was determined using Spearman's correlation test. The online tool SNPstats (http://bioinfo.iconcologia.net/SNPstats) was used to perform Haplotype reconstruction from population genotype data, LinkageDisequilibrium parameters (D ′ and r2) and to identify departure from the Hardy-Weinberg equilibrium. All values were two-tailed, and $p < .05$ were considered to be statistically significant. P values that remained below .05 after correction for the number of variables (Pc) were considered significant (Bonferroni correction).

RESULTS

Patient's characteristics

In the present study, 104 diabetes type 1 patients and 116 healthy individuals as control group were enrolled. (56 males; 48 females). The mean age was 11.08±4.24 with the range of 4-18 years. All patients were examined for the biochemical and diabetic parameters. Demographic and biochemical data of both patients and healthy controls were summarized in Table (1). The diabetes type 1 patients were positive for diabetes. Levels of HBA1Cand BMI showed significant increase in the patients group (P<0.001). Remarkable elevation in HBA1C level was observed in patients compared with controls (r=0.293 and P<0.01). Remarkable elevation in BMI level was observed in patients compared with controls (r= 0.679 and P<0.01). Remarkable elevation in age at diagnosis level was observed in patients compared with controls (r=0.293 and P<0.01).

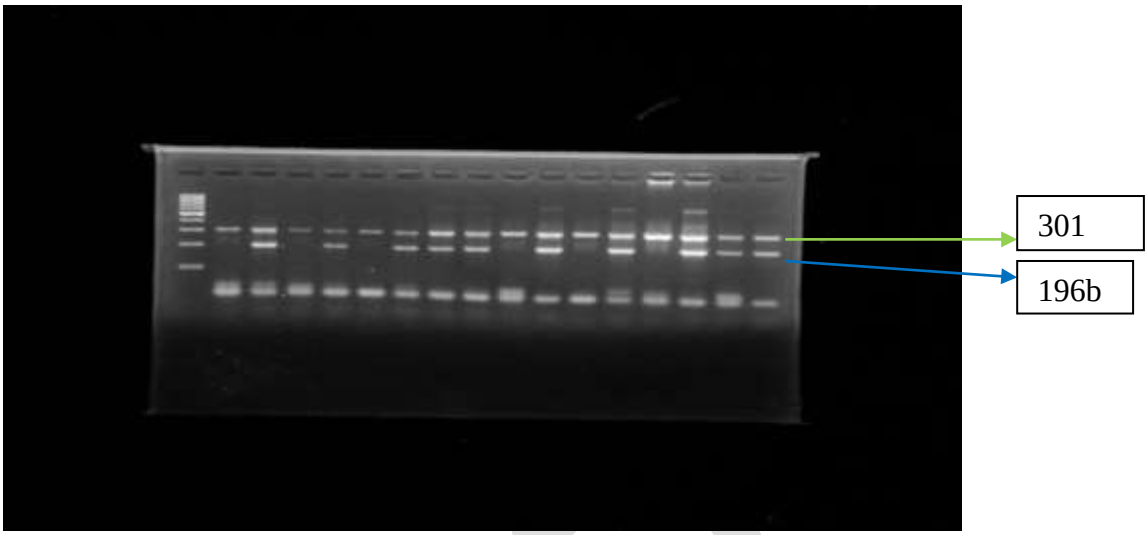

Fig.a. PCR product of IL-18 (-607A/C)for eightcontrolsamples**.**Lane (1): 100bp DNA ladder. Lane (2,4.6.8,10.12.14and 16): give A of PCRcontrolcases. Lanes (3 ,5,7,9,11,13and 15): give C ofPCR controlcases**.**

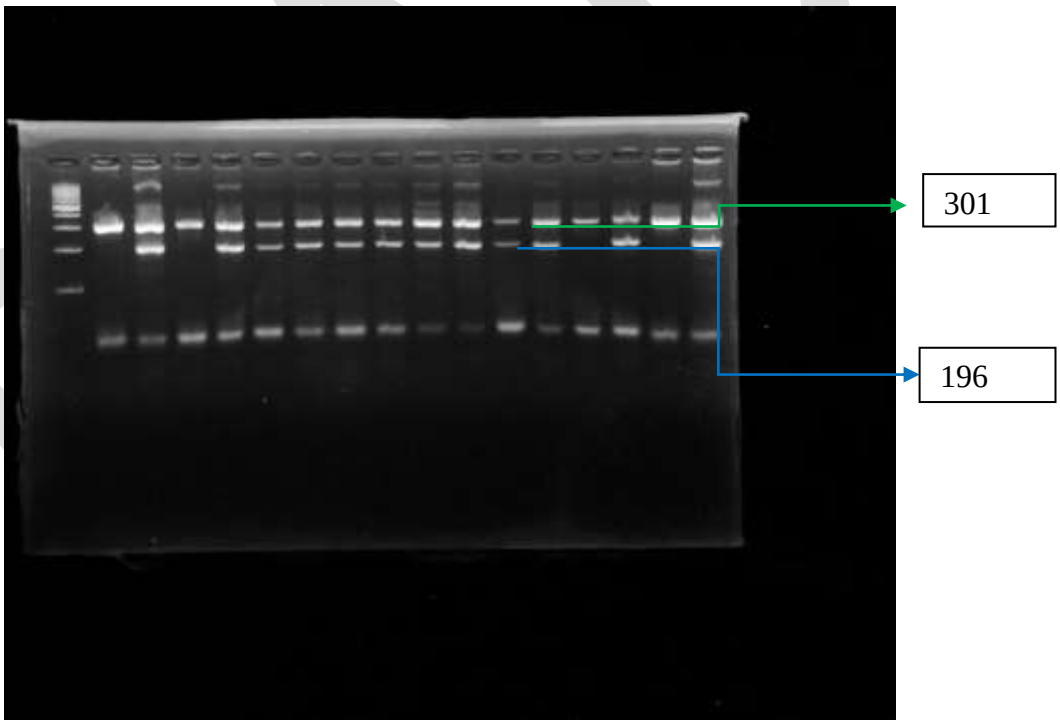

Fig. b.PCR product of IL-18 (-607A/C) foreightpatient samples**.**Lane (1): 100bp DNA ladder. Lane (2,4.6.8,10.12.14and 16): give A of PCRpatient cases. Lanes (3 ,5,7,9,11,13and 15): give C of PCR patient cases**.**

Table 2. Demographic and biochemical characteristics of diabetic patients and healthy controls.

Characteristics	Control group N= 116	Patient group N= 104	P	Correlation with disease
Age	14.96±5.19	11.08±4.24	P<0.01	r= -0.378 P<0.01
Gender (Male ♂/ Female ♀)	54/62	56/48	NS	
Age at diagnosis	--	8.16±3.74	P<0.01	r=0.293 P<0.01
BMI (unit)	10.43±4.99	19.71±1.08	P<0.01	r= 0.679 P<0.01
HBA1C (unit)	--	9.52±2.42	P<0.01	r=0.293 P<0.01

All data are presented as mean±SD, body mass index (BMI), not significant (NS),glycosylatedhemoglobin (HBA1C)

Genotyping of IL-18 607A/C SNP (rs1946518)

IL-18P-607 (A/C) was genotyped by SSP -PCR. The PCR products were measured in comparison to 100bp DNA ladder. The size of PCR product for -607A/C of control cases (**Figure a**)andThe size of PCR product for -607A/C of patient cases(**Figure b**) were 196 bp and control band at 301bp IL-18 p - 607 SNPs allele and genotype frequencies of both controls and patients are shown in Table (2).The genotype frequencies of IL-18 **(**-607A/C) SNPin patients were in HWE (observed data were similar to the predicted data) while controls deviated significantly from the HWE [AA, AC, CC were 1.7%, 57%, 41.3%, respectively (observed)] versus 1%, 60.5%, 38.5 %, respectively non-significant. Table (2) showed that there was significant association between the demographic, biochemical characteristics of diabetes type 1 patients and the difference in their (-607A/C) genotypes. Table (2) showed that there was significant association between the demographic, biochemical characteristics of diabetes type 1 patients and the difference in their (-607A/C) genotypes The analysis of haplotype distribution of SNPs in the IL-18 gene promoter region showed no significant association of any haplotype with susceptibility to diabetes (Table 3). To analyze the association of SNPs in IL-18 gene with the patient's clinical presentation (age at onset, HbA1c) the whole patients group was divided in genotype-based subgroups. Patients with the minor allele -607A of IL-18 gene developed diabetes at earlier age at onset than patients with major allele (C) at the same positions, the difference reach significant level (Table 4). However, the minor alleles at each of the two IL-18 loci (-607A) were associated with the disease occurrence at significantly younger age compared to carriers of major alleles (-607C) (Table 4).

Table 3. The genotype and allele frequencies of IL-18 -607 A/C gene polymorphisms in diabetic patients and controls

Polymorphisms	Control (N= 116) N (%)	Patients (N= 104) N (%)	95% Confidence Interval OR (lower-upper)	p-value
IL-18 (-607 A/C) genotype (N, %)				
AA	2 (1.7)	1 (1)	0.553 (0.049-6.194)	NS
AC	66 (57)	63 (60.5)	1.206 (0.704-2.064)	NS
CC	48 (41.3)	40 (38.5)	0.885 (0.516-1.521)	NS
AC/CC vs AA	114 (98.3)	103 (99)	1.807 (0.161-20.224)	NS
A	60 (30.2)	65 (31.3)	1.170 (0.682-2.008)	NS
C	162 (69.8)	143 (68.7)	1.807 (0.161-20.224)	NS

Table 4. Demographic Data of diabetic Patients of IL-18 -607 A/C Genotypes.

Demographic data	IL-18 -607 A/C			
	AA	**AC**	**CC**	**p-value**
Sex **Male** **Female**	0 1	33 30	23 17	**NS**
Age	5.50±0	11.52±4.29	10.52±4.11	**NS**
Age at onset	1.50±0	8.78±3.91	7.34±3.18	**P<0.05**
BMI	14.0±0	19.72±5.08	19.85±5.13	**NS**

DISCUSSION

In the present study we demonstrated that IL-18-607 C/A (rs1946518) polymorphisms in Egyptian patients, are not associated with the susceptibility for the development of type 1 diabetes mellitus. in order to, the contradictory results have been obtained by studying the SNPs of IL-18 gene promoter-607C/A in different populations with autoimmune diabetes. To our knowledge, only a few case–control studies reported the significant association of these SNPs with T1DM. In Polish population, the significant association of genotypes with T1DM was found at this loci (***Kretowski et al., 2002***). In the Chinese (***Dong et al., 2007***) or Japanese (***Ide et al., 2004***) populations, significantly different distribution of the -607 genotypes in T1DM patients compared to controls appears due to decreased frequency of patients carrying the AA genotype. In contrast, many studies, including this one, have demonstrated no association of IL-18-137 and -607 polymorphisms with autoimmune diabetes (***Martin et al., 2005; Mojtahedi et al., 2006;***

Novota et al., 2005; Szeszko et al., 2006). A meta-analysis (6075 cases and 5744 controls) showed that the IL-18 -607 C/A polymorphism may be associated with susceptibility to T1D in Asians, but not in Europeans. 2015 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc.

In our study, we found no association of IL-18-607 genotype, allele or haplotype with T1DM development in Egyptians. However, the results suggest an association of genotypes with age at onset of T1DM.

CONCLUSION

This study observed that there was no significant association between polymorphisms in SNPs of IL-18 genes and diabetes type 1 risk in Egyptian population. Therefore, larger prospective studies are needed to confirm our findings especially in Egyptian populations. In the light of these data, further studies concerning other IL-18 gene polymorphisms will contribute to a better understanding of the pathogenesis of the disease.

REFERENCES

Aachoui, Y., Sagulenko, V., Miao, E. A. & Stacey, K. J (2013). Inflmmasome-mediated pyroptotic and apoptotic cell death, and defense against infection. *CurrOpin Microbiol* 16, 319–326

Kretowski A, Mironczuk K, Karpinska A, Bojaryn U, Kinalski M, Puchalski Z, Kinalska I (2002). Interleukin-18 promoter polymorphisms in type 1 diabetes. Diabetes. 51:3347–3349.

Altinova AE, Engin D, Akbay E, et al (2010). Association of polymorphisms in the IL-18 and IL-12 genes with susceptibility to type 1 diabetes in Turkish patients. J. Endocrinological. Invest.451–4.

B**. Baumann, H.H. Salem, B.O(2012).**Anti-inflammatory therapy in type 1 diabetes CurrDiab Rep,12,pp.499-509

Barker, B. R., Taxman, D. J. & Ting, J. P (2011). Cross-regulation between the IL-1beta/IL-18 processing inflmmasome and other inflammatory cytokines. CurrOpin Immunol 23, 591–597

Boraska V, Terzic J, Skrabic V, et al (2006). NeuroD1 gene and interleukin-18 gene polymorphisms in type 1 diabetes in Dalmatian population of Southern Croatia. Croatian Med. 571–8.Diabetes, 3347-3349

Dong GP, Yu ZS, Liang L, Zou CC, Fu JF, Wang CL (2007). IL-18 gene promoter -137C/G and -607C/A polymorphisms in Chinese Han children with type 1 diabetes mellitus. Int. J. Immunogenet.75–9.

Giedraitis V, He B, Huang WX, Hillert J (2001). Cloning and mutation analysis of the human IL-18 promoter: a possible role of polymorphisms in expression regulation. J. Neuroimmunol.146 -52.

Hadzija MP, Korolija M, Jemin N, et al (2013). Polymorphisms in the IL-18 and IL-12B genes and their association with the clinical outcome in Croatian patients with Type 1 diabetes. Gene 477–81.

Ilonen, J. et al (2013). Patterns of β-cell autoantibody appearance and genetic associations during the first years of life. Diabetes 62, 3636–3640 .This is the first investigation to dissect the temporal pattern of the first-appearing β-cell-targeting autoantibody as a biomarker of T1DM.

Ide A, Kawasaki E, Abiru N, et al (2004). Association between IL-18 gene promoter polymorphisms and CTLA-4 gene 49A/G polymorphism in Japanese patients with type 1 diabetes. J. Autoimmun.73–8.

Kawakami, K (2002). Interleukin-18 and host defense against infectious pathogens. JImmunother 25Suppl 1,S12–19

Krischer, J. P. et al(2015). The 6-yearincidence of diabetes associated autoantibodies in genetically at-risk children: the TEDDY study. Diabetologia58,980–987

Lee HJ, Her SH, Im YS, et al (2009). Significance of inflammatory markers in diabetic patients with stable coronary artery disease. Korean J. Intern. Med.;24(3):212–9.

Leslie RD (20 10). Predicting adult-onset autoimmune diabetes: clarity from complexity. Diabetes; 59: 330–31.

Kunz M, S.M(2009). IbrahimCytokines and cytokine profiles in human autoimmune diseases and animal models of autoimmunity Mediators Inflamm pp. 979258-979278

M.A. Hulme, C.H. Wasserfall, M.A. Atkinson, T.M(2012). BruskoCentral role for interleukin-2 in type 1 diabetes Diabetes, 61, pp. 14-22

Martin RJ, Savage DA, Carson DJ, Maxwell AP, Patterson CC (2005). Interleukin 18 promoter polymorphisms are not strongly associated with type I diabetes in a UK population. Genes Immun.171–4.

Mojtahedi Z, Naeimi S, Farjadian S, Omrani GR, Ghaderi A (2006). Association of IL-18 promoter polymorphisms with predisposition to type 1 diabetes. Diabetic Med.: J. British Diabetic Assoc.235–9.

Motavaf, M., Safari, S. &Alavian, S. M (2014). Interleukin 18 gene promoter polymorphisms and susceptibility to chronic hepatitis B infection: a review study. Hepat Mon 14, e19879

Nakanishi, K., Tsutsui, H., and Yoshimoto, T. (2010). Importance of IL-induced super Th1 cells for the development of allergic inflammation. AllergolInt 59, 137–141.

Noble JA, Erlich HA (2012). Genetics of type 1 diabetes. Cold Spring Harb Perspect Med.;2(1):a007732.

Novota P, Cejkova P, Cerna M, Andel M (2004). Genetic risk factors in autoimmune diabetes mellitus,

their significance and function. Casopislekaru ceskych159–63.

P. Reddy (2004), Interleukin-18: recent advances CurrOpinHematol, 11 pp. 405-410

Pizarro, T. T. et al (1999).IL-18, a novel immunoregulatory cytokine, is upregulated in Crohn's disease: expression and localization in intestinal mucosal cells. J Immunol162,6829–6835

Sahoo, M., Ceballos-Olvera, I., del Barrio, L. & Re, F (2011). Role of the inflmmasome, IL-1beta, and IL-18 in bacterial infections.Scientific WorldJournal11,2037–2050

Shan, N.N., Zhu, X.J., Peng, J., Qin, P., Zhuang, X.W., Wang, H.C., and Hou, M. (2009a). Interleukin 18 and interleukin 18 binding protein in patients with idiopathic thrombocytopenic purpura. Br J Haematol 144, 755–761.

Smith, A. J. & Humphries, S. E (2009). Cytokine and cytokine receptor gene polymorphisms and their functionality. Cytokine GrowthFactor Rev 20, 43–59

Srivastava, S., Salim, N., and Robertson, M.J. (2010). Interleukin-18: biology and role in the immunotherapy of cancer. Curr Med Chem 17, 3353–3357.

Szeszko JS, Howson JM, Cooper JD, et al (2006). Analysis of polymorphisms of the interleukin-18 gene in type 1 diabetes and Hardy–Weinberg equilibrium
testing. Diabetes 559–62.

Tanaka, M. et al (2001). Mature form of interleukin 18 is expressed in rheumatoid arthritis synovial tissue and contributes to interferongamma production by synovial T cells.JRheumatol28,1779–1787

Tavares NAC, Santos MMS, et al (2013) . Interleukin 18 (IL18) gene promoter polymorphisms are associated with type 1 diabetes mellitus in Brazilian patients. Cytokine 286–9

Thmpson, S. R. & Humphries, S. E (2007). Interleukin-18 genetics and inflmmatory disease susceptibility. Genes Immun8,91–99.

Tucci, M., Quatraro, C., Dammacco, F. &Silvestris, F (2006). Interleukin-18 overexpression as a hallmark of the activity of autoimmune inflammatory myopathies. Clin. Exp. Immunol.146, 21–31

Zhang P-A, Wu J-M, Li Y, Yang X-S (2005). Association of polymorphisms of interleukin-18 gene promoter region with chronic hepatitis B in Chinese Han population. World J Gastroenterol WJG 1594–8.

Ziegler, A. G.et al(2013). Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 309, 2473–2479.

Ziegler, A. G.,Hummel, M.,Schenker, M& Bo nifacio, E(1999). Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. Diabetes 48,460–468