İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EPIGENETICS ANALYSIS OF BEHÇET SYNDROME TWINS

M.Sc. Thesis by
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BEHÇET SENDROMLU İKİZLERİNDE EPİGENETİK ANALİZLER

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TABLE OF CONTENTS

Page

ABBREVIATIONS

BS : Behçet Syndrome
RA : Rheumatoid Arthritis
MS : Multiple Sclerosis
IDDM : Insulin-Dependent Diabetes Mellitus
RNA : RibonucleicAcid
HPA : Hypothalamic-pituitary-adrenal axis
DNMT : DNA- methyl transferase
TIDM : Type 1 Diabetes
TNF : Tumor Necrosis Factor
LD : Linkage Disequilibria
MIC : Major Histocompatibility Complex
mRNA : Messenger Ribonucleic Acid
HLA : Human Leukocyte Antigen
DNA : Deoxyribonucleic Acid
PCR : Polymerase Chain Reaction
EDTA : Ethyl-enediaminetetra-acetic Acid

LIST OF TABLES

Page

LIST OF FIGURES

Page

EPIGENETIC ANALYSES OF BEHÇET SYNDROME TWINS

SUMMARY

Behçet syndrome (BS) is a multisystem vasculitis characterized by skin and mucosa lesions and musculoskeletal, ocular, gastrointestinal, neurological and major vessel involvement. It is seen mainly in the Mediterranean basin, Middle East and the Far East. Turkey has the highest prevalence which is 8 to 42 per10000 populations, while the frequency is lower in western countries. The disease is more severe among young males and the severity diminishes with age. The ultimate etiology of Behçet's syndrome remains unknown. Current evidence suggests that interplay of genetic and environmental factors may be responsible. Abnormal neutrophil function, alterations in lymphocytes, and some infectious agents are also proposed to play a role in Behçet syndrome. Strong association between BS and HLA-B51 indicates the importance of genetics in the etiology of the disease. On the other hand, deficient concordance in monozygotic twins and sporadic BS in families with no disease background points out also the importance of environmental elements.

Along with stochastic epigenetic changes related to age, epigenetic analyses in discordant monozygotic twins are considered to be able to explain the changes that are due to environmental factors.

This study aimed to analyze global methylation, an important epigenetics mechanism of BS twins. To this end, methylation profile of 4 BS monozygotic (MZ) (n= 8) and 4 dizygotic (DZ) (n= 8) twins along with healthy controls (8 twins, n=16) were investigated; MZ twins whom one of them is BS and the other one is healthy (BS-healthy twins), MZ twins who both are BS, and DZ twins whom one of them is BS were compared with the MZ and DZ healthy controls. Global methylation value was obtained by injecting digested DNA samples to HPLC (HPLC- High Performance Liquid Chromatography) machine and separating nucleosides including dC and 5-mdC. The area below the peaks was recorded. Tests were triplicated, 5-mdC: dC ratio for each of the samples was calculated (%RSD= 7%). The average values were compared between groups using student T test analyses.

It was concluded that there is a smaller difference between methylation pattern of the twins in healthy control group compared to BS twins (P= 0.02876). MZ BS twins versus MZ healthy controls (P= 0.04133) and DZ BS twins versus DZ healthy controls (P= 0.09551) were analyzed separately. Results showed significant relationship between MZ twins, but not for DZ twins based on Student T test analyses as expected. Correlation results showed a stronger relationship among MZ twins ($\rho \sim 1$) compared with DZ twins in discordant BS twins ($\rho = 0.73$).

Main limitation of this study is the small sample size. More data is needed to confirm our preliminary findings. Future studies would be to examine the impression of methylation profile differences on gene expression. As a conclusion, we would be able to define the effect of environmental epigenetic differences on gene regions.

BEHÇET SENDROMLU İKİZLERİNDE EPİGENETİK ANALİZLER

ÖZET

Behçet sendromu deri ve mukoza kezyonları ile ayırt edilen muskuloskeletal, oküler, gastrointestinal, nörolojik ve bir çok ana damarları kapsayan vücuttaki birçok doku ve organ sistemini etkileyebilen multisistem vaskülit bir hastalıktır. Hastalık özellikle Akdeniz, Orta Doğu ve Uzak Doğu bölgelerinde yaygın olarak görülmektedir. Türkiye'de 10bin'de 8-42 görülme sıklığı ile bu oran en yüksek iken, batı ülkelerinde oran düşüktür. Hastalık özellikle genç erkeklerde daha şiddetli olarak görülürken yaş ilerledikçe şiddet azalmaktadır. Behçet sendromunun etiyolojisi kesin olarak bilinmemekle birlikte, günümüzde genetik ve çevresel fakörlerin hastalığın en önemli sebepleri olduğu tahmin edilmektedir. Nötrofil fonksiyonlarındaki bozukluklar, lemfositlerdeki değişiklikler ve bazı enfeksiyon ajanlarının da Behçet sendromunda rol oynadığı öne sürülmüştür. BS'nin birden fazla dokuyu ve organı etkileyen patolojisi ise tam olarak anlaşılamamıştır. BS ile HLA-B51 geni arasındaki güçlü ilişki hastalığın etiyolojisinde genetik faktörün önemini ortaya koymaktadır. Öte yandan özellikle monozigot ikizlerde hastalığın oluşumunda bir ilişki bulunmaması ve hastalık geçmişi olmayan ailelerde hastalığın az sıklıkla da olsa görülmesi çevresel faktörlerin önemine de dikkat çekmektedir.

Yaşa bağlı stokastik epigenetik değişiklerin yanı sıra, özellikle hastalıklar açısından uyumlu olmayan tek yumurta ikizlerindeki epigenetik analizlerin hastalıklardaki çevreye bağlı değişikleri açıklayabileceği düşünülmektedir.

Bu projede BS ikizlerde önemli bir metilasyon mekanizması olan globalmetilasyon düzenlerinin analiz edilmesi amaçlanmıştır. Bu amaçla, 4 monozigot (MZ) BS (n=8) ve 4 dizigot (DZ) BS ikizler (n=8), sağlıklı kontrol grubu ikizleriyle (8 ikiz, n= 16) karşılaştırılmıştır. Buna ek olarak biri BS olan ikizler ile, ikisi de BS olan ikizler; ve bir tanesi hasta olan dizigot ikizler ile sağlıklı kontrol grubu ikizleri (monozigot ve dizigot ikizlerden oluşan) karşılaştırılmıştır. Global metilasyon değerleri enzimlerle kesilmiş DNA numunelerinin HPLC (Yüksek Performanslı Likit Kromatografisi) kullanılarak dC ve 5-mdC dahil olmak üzere nükleozitlerin ayrılmasına dayanmaktadır. Elde edilen pikler altındaki alan kaydedilmiştir. Her bir numune için üçlü tekrar yapılmıştır (%RSD= 7%) ve bu tekrarlardan aritmetik ortalama bir değer elde edilmiş ve bu değerler T test ile karşılaştırılıp, analiz edilmiştir.

Yapılan student T test istatistiksel analizlere göre, sağlıklı kontrol grubu ikizlerinin bireyleri arasındaki metilasyon düzenleri arasındaki farkın biri BS biri sağlıklı olan ikizlere göre daha düşük olduğu tespit edilmiştir (P= 0.02847). MZ BS ikizleri ile MZ sağlıklı ikizler arasında (P=0.04133) ve DZ BS ve DZ sağlıklı ikizler arasında (P=0.09551) da ayrı analizler yapılmıştır. Sonuçlar MZ ikizlerde oran ile önemli bir ilişkiyi işaret ederken, DZ ikizlerde bu tür bir ilişki tespit edilememiştir. Biri hasta olup biri sağlıklı olan MZ ve DZ ikizleri bireyleri arasnıdaki korelasyon analizi sonuçları DZ ikizlerde (ρ ~1) MZ ikizlere gore (ρ =0.73) daha yuksek bir korelasyonu işaret etmektedir.

Bu çalışmadaki ana kısıtlayıcı etken numune sayısının az olmasıdır. Çalışmada elde edilen ön sonuçların doğrulanması için daha fazla veriye ihtiyaç duyulmaktadır.Bir

sonraki aşama bu farklılıkların gen ekpresyonu açısından etkilerinin incelenmesi olacaktır. Sonuç olarak çevresel koşullar sonucu ortaya çıkan epigenetik farklılıklarının belirli gen bölgelerindeki etkileri de görülebilecektir.

1. INTRODUCTION

1.1 Behçet Syndrome

Behçet's Syndrome (BS) is a multi-systemic inflammatory autoimmune disorder. It was first reported in 1937 as a syndrome with oral and genital ulceration and ocular inflammation (1, 2). Four major symptoms which characterize Behçet Syndrome are consisted of oral aphthous ulcers, ocular symptoms, skin lesions and genital ulcerations. Inflammation in tissues and organs throughout the body including the vascular system, lung, kidney and joints are probable among other BS symptoms (2).The diagnostic criteria have developed significantly over the decades reflecting the complexity and diverse demonstrations of this multi-organ systemic disease (3).

Although BS is rare in many parts of the world, the prevalence of it is increasing in eastern Mediterranean countries, the Middle East, and East Asia (4). Aphthous oral ulcers are usually the first and most continual clinical feature of Behçet's syndrome. On the genitals as well, apthous ulcers appear oftentimes (e.g., the scrotum or vulva) (5).

Eye involvement is the most serious manifestation of BS and affects about 50 % of the patients (6, 7).Inflammation of the arteries (arteritis) in patients with Behçet's syndrome can lead to death of the tissues whose oxygen supply depends on these vessels (7). Swelling, stiffness, warmth, pain, and tenderness of joints can be seen in patients with Behçet's syndrome who endure join inflammation (arthritis) (7).

1.2 Genetic Epidemiology

Genetic risk factors of BS have been investigated in several family and twins studies which are explained further.

1.2.1 Incidence of the Disease

BS is prevalent in Middle Eastern countries with the highest rate (4 in 1000 individuals) found in Turkey. A genetic contribution to BS is supported by the high sibling recurrence risk ratio, estimated from 11.4 to 52.5 in the Turkish population (8).

1.2.2 Classic Genetic Studies

Classic genetic studies of BS have been performed in family and twins studies:

1.2.2.1 Family Studies

In order to look for a possible familial aggregation of Behçet's syndrome or of its component features, the first-degree family members of patients with Behçet's syndrome were investigated (9). In a family study among Armenians, Familial Mediterranean Fever (FMF) has been used as a comparator disease for BS. The genetic load for FMF is significantly higher among the Armenians when compared to the load for BS among the same ethnic group. On the contrary, the rather low frequency of BS among the Armenians when compared to the frequency among the general population living in the same environment is further evidence for a genetic predisposition to BS. HLA- B51 does not seem to play a dominant role in the said predisposition (10).

1.2.2.2 Twins Studies

Human monozygotic (MZ) twins show different degrees of concordance for complex diseases, such as cancer, cardiovascular diseases, or autoimmune disorders (11). On the other hand concordance rates close to 100% in identical twins are used to prove coinheritance of mutant genes that are dominant. Most diseases or traits manifest a concordance in identical twins in the broad range of 5%–75% (12). Most of the twin-based studies have focused on the concordance between siblings that has led to the revealing of aspect specific genes (13), although not much awareness has been paid to the degree of discordance, which suggests the participation of factors other than pure genetic changes (14).

Genetic factors are not mentioned as absolute on their own, since only one pair of monozygotic twins concordant for BS has been reported (15). Genetic contribution

for some autoimmune diseases can be concluded due to generally higher disease concordance rate in monozygotic relative to dizygotic twins or other family members indicates (16). Nonetheless, autoimmune disease concordance in identical twins is incomplete most of the times, showing a requirement for additional factors, presumably from the environment (17).

Case reports on MZ twins with Behçet's syndrome have been few. Frequency of MZ and dizygotic (DZ) twin births in BS has been sought and compared to healthy controls. Concordance rate among the MZ and DZ twins has also been looked in previous studies; the pair wise concordance rate for BS was 2/6 for MZ and 1/8 for DZ twins ($p=0.538$) (18). The frequency of MZ twin births in BS is not different than that in the general population while the DZ twins were seen less frequently among the BS patients. Genetic predisposition has been concluded according to the higher concordances for BS in MZ compared with DZ twins. Nevertheless, persistence of discordance among the MZ twins demands further research to understand non-genetic factors in causation of BS (18). The degree of similarity between identical twins brought up in the same home would indicate to the fact that all their genes are identical and that they share a common family environment. If identical twins can be located in different environment (adopted twins), the effect of environment on the variation among individuals can be investigated. This kind of experiments had been carried out for autoimmune diseases (19, 20) such as Multiple Sclerosis, but not for BS yet.

1.2.3 Molecular Genetics Analyses

The strongest marker found to be associated with BS is HLA-B51 ($P= 0.00063$) (1); in several studies, association between BS and HLA-B51 has been corroborated in patients of many ethnic groups; HLA-B51 and B52 were present in 63% ($p<00001$) and 21% ($p<0.05$), respectively, of the patients compared with 9% of the control group for both cases (21). The fact that HLA-B51 may play a role in the pathogenesis of BS, cannot confirm its utilization as a predictive value for the occurrence of organ involvement. Eye inflammation is exceptional in this case since in the presence of B51, the risk of eye involvement is superior (22). The fact that the disease is in association with HLA allele itself and/or some HLA-linked genes play in role in controlling the susceptibility to the disease according to the strong linkage

disequilibria (LD) observed among alleles of genes in the HLA region is yet to be discussed (23).

MIC-A is also thought to be a possible additional marker of BS. The phenotype frequency of the MICA-TM A6 allele was significantly increased in the BS patients (50.0% in control subjects versus 86.8% in BS cases, P = 0.0012) (15, 24). Novel single nucleotide polymorphisms (SNPs) in five genes (KIAA1529, CPVL, LOC100129342, UBASH3B, and UBAC2) that encode proteins with both known and unknown functions are identified by Fei and coworkers (Odd Rations are: 2.04, 2.26, 1.84, 1.71, and 1.61, respectively). (25). According to recent studies done by Gül A. et al., independent association within the MHC Class I region in BS patients were found. IL10 ($P = 3.54 \times 10^{-18}$) and the IL23R-IL12RB2 locus ($P = 6.69 \times 10^{-9}$) associations were also identified in the same study (26). In Touma Z et al. study, polymorphisms in the tumor necrosis factor (TNF) gene at specific locations have been investigated in various ethnic groups and showed probable association with BS (27). A familial incidence also approves genetic factors contributing to the development of this syndrome (28, 29). Carrying IL-1beta-511T allele ($p = 0.01$) were found to be a significant risk factors for higher periodontal scores in Turkish population (30).

Transforming growth factor-beta (TGF-beta) has been shown to play a role in cartilage regeneration and is increased in patients with BS. Two functional polymorphisms of the TGF-beta pathway, TGFBR1*6A and TGFB1*CC, have also been found that are associated with risk of malignancy. The probable difference in the incidence of these polymorphisms in BS patients compared with healthy controls were tested and it has been concluded that lower incidence of TGFBR1*6A in BS patients may play a protective role against development of malignancy since BS cases had lower incidence of TGFBR1*6A compared with controls (11.3% versus 13.3%, respectively) (31).

The stimulation of Interleukin (IL) 12, IL2, Tumor Necrosis Factor α (TNF- α) exert strong control on the Th1/Th2 balance, which is important in the regulation of the inflammatory disease such as BS. TNF- α and IL10 may be regulated at the transcriptional level and several SNPs in the promoter regions of both genes have been associated with changes in expression level (32).

1.2.4 Environmental Effect

Exposure to environmental factors (such as sunlight, diet, allergens, infectious agents or environmental toxins) in an individual with a susceptible genotype can lead to initiation of an autoimmune process. Critical modulating factors that can make the discrepancy between disease expressions include sex hormones that can act bilaterally with hormones of the hypothalamic-pituitary-adrenal axis (HPA) or sympathetic nervous system. Immune responses are affected by all these factors and it responses to self and foreign antigens through modulation of cytokine creation and effectors cell function. Outcome of the immune response is dictated by the nature of the antigen and the character of the immune response—that is, TH1 (T-Helper 1) or TH2 (T-helpe 2) (33). Human epidemiologic researches as well as increasing animal models show that maternal nutrition can "program" gene expression patterns in the embryo that remain into adulthood and impart metabolic disease (34).

In addition to nutrition, the effect of sex hormones on appearance of complex diseases has been studied. Investigating DNA and histones' epigenetic modification at specific gene regulatory regions may indicate the underlying mechanisms of sex hormone action leading to gender differences in susceptibility to complex diseases such as asthma, diabetes, lupus, autism and major depression (35).

1.3 Epigenetics

Epigenetics refers to a heritable change in the pattern of gene expression without changing the primary nucleotide sequence. Functionally, an additional layer of transcriptional control is provided by epigenetics that plays a crucial role in normal physiological development, as well as in pathological conditions (27). DNA methylation by DNA methyltransferases (Dnmts), histone modifications such as methylation, acetylation, and phosphorylation, structural modifications of chromatin, and microRNAs as well as other noncoding regulatory RNA were epigenetic machanisms which happen during the full course of human life span (36). CpG islands -DNA hypomethylation in CpG- rich, promoter associated regions, and acetylated histones allow active transcription, while DNA hypermethylation and histone hypoacetylation elevate gene silencing (37).

1.3.1 Methylation

Covalent modification of cytosines by addition of a methyl group to a 5' carbon of the cytosine ring located within CpG dinucleotides which leads to the occurrence of DNA methylation, is up to now the most studied epigenetic mechanism. More than 85 % of CpG dinucleotides expanded in the genome and located in repetitive sequences are heavily hypermethylated/ transcription-silenced in the normal cells, a state critic to the integrity of the chromatin structure of the genome. The remaining approximately 15 % of CpG dinucleotides are clustered within the short DNA regions called "CpG islands", which account for 1 % of the genome. Nearly 40 to 50 % of the genes have CpG within or around the promoters and are unmethylated in the normal somatic cells to a great amount (38). The methylated CpG dinucleotides within promoter regions can intervene with transcription factor binding; recruitment of methyl- CpG binding domain (MBD) proteins leads to gene expression to get repressed. The methylation condition of the genome is remained by three methyltransferases (DNMTl, DNMT3a and DNMT3b) and S-adenosyl-methionine as methyl donor.

1.3.1.1 Methylation Changes

Genetic Methylation changes are divided into two separate groups which are “hypomethylation” and “hypermethylation” that refer to decrease and increase in Cytosine and Adenosine methyl groups respectively.

DNA is often hypomethylated in cancer at the global level. The studies of Seifert et al. (39) strongly support the hypothesis of early global hypomethylation in bladder carcinomas. Hypomethylation can result in activation of the normally silent regions of the genome and as a consequence, expression of repeat elements or genes that would normally be silent during development.

Another class of methylation alteration is distinguished by local hypermethylation of individual genes, associated with aberrant gene silencing. The tissue-to-tissue differences exists in CpG islands methylation except with regard to the type of the gene methylated, also with regard to the methylation frequency of the specific gene and the overall methylation extent (40).

The researches related to global DNA methylation patterns in the neurodegenerative disorders have elucidated according to the following findings related to schizophrenia example: no difference in global genome DNA methylation of peripheral blood leukocytes between schizophrenia (SZ) patients and control subjects in addition to no association between global leukocyte DNA methylation and homocysteine levels was discovered, nevertheless the homocysteine levels were higher in SZ patients than in controls (41); on the other hand, the tendency to lower content of methylated deoxycytidine (mdC) of leukocyte DNA was emerged in male patients with SZ in comparison with controls, this shows a significant effect of age because this difference was more prominent in younger individuals. In females, thus far, no effect of age or disease status on mdC content was observed. The findings shows that there is a consequential sex-dependent difference in the mdC content of human peripheral leukocyte DNA in SZ patients (42); the decrease of methylation in entorhinal cortex layer II, a region displaying important Alzheimer's disease pathology, in which expression changes have been reported for a wide variety of genes, has been investigated by evaluating the immunoreativity of two markers for methylation and eight methylation maintenance factors (43).

Hypermethylation of specific genes in autoimmune and other diseases was investigated in the following studies: heavy methylation (and histone deacetylation) of the FLIl gene in scleroderma fibroblasts and skin biopsy specimens shows that epigenetic mechanisms may take part in the fibrotic appearance of scleroderma (44); the killer-cell immunoglobulin-like receptor (KIR) genes expressed on natural killer (NK) cells - a elements of the innate immunity and the first line of defense against viral infections and malignancies - and on the "senescent" CD28-T cells, both involved in cardiovascular diseases, are suppressed by DNA methylation in most T cells, and DNA demethylation promotes KIR expression (45); in the same way, the KIR genes on the human NK cells derived from cell line NK-92MI exhibited epigenetic repression in consequence of the largely methylated promoter regions. According to Gao X. et al. study, treatment with 5-azacytidine apparently increased expression of KIRs, which leads to strong suppression of the NK cytolytic activity. This finding reveals the fact that aberrant methylation patterns of the KIR genes during NK-cell differentiation and maturation may have importance for their abnormal function (46)

1.3.2 Histone Modification

Genetic Unusual changes of the activity of chromatin modifiers were investigated in the following studies: global histone H3/H4 hypoacetylation in active CD4+ T cells and global H3K9 hypomethylation in both active and inactive CD4+ T cells of patients with SLE when compared with the controls was investigated, but there was no difference among global levels of H3K4 methylation between the patients and controls. Additionally, the metabolic NAD-dependent protein/histone deacetylase (SIRTl) mRNA level was apparently raised up in active lupus CD4+ T cells in comparison with controls (SIRTl is an anti-aging and anti-inflammatory protein that regulates pro-inflammatory mediators by deacetylating histone and non-histone proteins). Afterwards, it was discovered that mRNA levels of CREBBP, P300, HDAC2, HDAC7, SUV39H2, and EZH2 were significantly down-regulated in patients with active lupus (47); the HDAC gene expression was fallen in CD4+ T cells of patients with type 1 diabetes (TlDM), which may underlie the abnormal immune response of CD4+ T cells resulting in destruction of the insulin-producing pancreatic beta cells (48); changes in trimethylation of H3K4 and to a lesser degree in H3K9 have been identified in the human left ventricular tissue with retained or damaged function, pointing out global epigenetic changes in cardiac myocytes associated with heart failure (49).

Histone modifications within the specific genes were brought out in the following studies: a subset of epigenetically modified genes as result of a significant increase in genome-wide H3K9 dimethylation was found in lymphocytes, however not in monocytes from TlDM patients versus healthy control subjects, showing that histone methylation within the identified network might have effect on the etiology of TlDM and its complications. The analyzed genes included CLTA4, nevertheless many genes associated with autoimmune and inflammation-related pathways as well such as transforming growth factor ß, nuclear factor kB, p38 mitogen-activated protein kinase, toll-like receptor, and interleukin 6 (50); the active transcriptional state of the NFicB-p65 gene connected with the ambient or prior hyperglycaemia in diabetic patients and related to the phenomenon of "hyperglycaemia memory" is associated with remaining epigenetic marks such as enhanced methylation but not di- or trimethylation of H3K4 and reduced di- and trimethylation of H3K9 (51); the levels of SIRTl were reduced in macrophages and lungs of smokers and patients with

chronic obstructive pulmonary disease (COPD) because of its post-translational modifications by cigarette smoke-derived reactive components leading to increased acetylation oîRelAlp65. Thus, SIRTl plays a crucial role in the regulation of NF-kB dependent proinflammatory mediators in lungs of smokers and patients with COPD (52); acetylation of histone H4 associated with cyclooxygenase-2 (Cox-2) gene promoter that plays a critical role in the inflammatory response, as well as degradation of HDACl was induced by exposure to diesel exhaust particulate matters (DEP), which has been revealed to trigger pulmonary inflammation and exacerbate asthma and chronic obstructive pulmonary disease. DEP exposure induced recruitment of HAT P300 to the promoter of the Cox-2 gene, suggesting that along with HDAC 1 that plays a crucial role in mediating transcriptional activation of the Cox-2 gene, acetylation is also of importance in the regulation of its expression (53).

Histone acetylation reflects a balance of the histone acetyltransferases and the histone deacetylases, and is thus vulnerable to environmental effects that alter these reactions, potentially altering gene expression (54).

1.3.3 Epigenetics and Twins Studies

MZ twins establish an excellent example of how genetically identical individuals can exhibit differences and as a consequence provides a unique model to study the contribution role of epigenetic modifications in the appearance of the phenotype. MZ twins share a common genotype. Nonetheless, great majority of monozygotic twin pairs are not identical; several types of phenotypic discordance may be emerged, such as differences in susceptibilities to disease and a wide range of anthropomorphic aspects. There are several possible explanations for these observations; however, one is the existence of epigenetic differences. In order to address this case, the global and locus-specific differences in DNA methylation and histone acetylation of a large cohort of monozygotic twins have been tested thoroughly. Although twins are epigenetically impossible to tell apart during the early years of life, older monozygotic twins exhibited remarkable alteration in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation, influencing their gene-expression portrait. These findings reveal how an appreciation of epigenetics is missing from apprehension of how different phenotypes can be originated from the same genotype (55). In a study conducted by

Mario F. Fraga et al. in 2005, older twin pairs presented 2.5 times as many DNA methylation differences in the CpG islands of single-copy genes as did the younger twin-pairs. The RNA of the two most well-defined pairs of twins was extracted (the 3- and 50-year-old pairs) and gene expression microarray analysis was performed afterwards. According to the study, from a quantitative standpoint, there were four times as many differentially expressed genes in the older twin pair as in the younger twin pair (55). In autoimmune diseases, environmentally driven epigenetic changes are presumed to take part in their etiology. Javierre et al. reported the first high-throughput and candidate sequence analyses of DNA methylation to investigate discordance for autoimmune disease in twins. They used a cohort of MZ twins discordant for three diseases whose clinical signs often overlap: SLE, rheumatoid arthritis, and dermatomyositis. Only MZ twins discordant for SLE characterized widespread changes in the DNA methylation status of a significant number of genes. Gene ontology analysis revealed enrichment in categories associated with immune function. Individual analysis admitted the presence of DNA methylation and expression alteration in genes relevant to SLE pathogenesis. These changes happened in parallel with a global fall in the 5-methylcytosine content that was continuously accompanied with alteration in DNA methylation and expression levels of ribosomal RNA genes, even though no alteration in repetitive sequences were found. According to Javierre et.al.'s researches, not only identify potentially relevant DNA methylation markers for the clinical characterization of SLE patients but also support the notion that epigenetic changes may be critical in the clinical manifestations of autoimmune disease (56).

Specific methylated cytosine residues in DNA of many eukaryotes are in interaction with gene regulation. According to the recent evidences, environment–host interaction in some forms of autoimmunity can be seen as a consequence of the presence of the environmentally-induced epigenetic changes, particularly altered patterns of DNA methylation. A failure to maintain epigenetic homeostasis, because of the environmental impact, can cause abnormal gene expression in specific cells which leads to loss of tolerance, and the modified cells then take part to the manifestation of autoimmunity in genetically predisposed individuals (56). Mutations in genes encoding the DNA methyltransferase (DNMT) and methyl-binding proteins may change the pattern of gene methylation; this can result in cancer and congenital diseases. Modification of lysine 27 and lysine 9 of histone H3 (H3K27 and H3K9)

which occurs as a consequence of the alteration in the level of methyltransferase, correlate with changes in Rb signaling and disruption of the cell cycle in cancer cells (57).

Epigenetic mechanisms determine the phenotype without changes in the genotype. Epigenetic information, without encoding the information, is transferred from one generation to the next either at the cellular level or at the level of the whole organism. Epigenetic mechanisms function not only at the transcriptional and post-transcriptional level of gene activity but also at the level of protein translation and post-translational modifications. Cell differentiation, morphogenesis, variability and adaptability of an organism are processes in which they take part and may be affected by both genetic and environmental factors.

Disruption of the primary epigenetic pathways can cause silencing or inappropriate expression of specific genes; this can lead to initiation of newly categorized epigenetic diseases (27). Number of human diseases which have been found to be associated with aberrant epigenetic regulation have risen recently (58). Previously unrecognized molecular signatures of disease latency, onset and progression, mechanisms underlying disease pathogenesis, and responses to new and evolving therapeutic modalities are identified and characterized by the application of epigenetic principles (59). For instance, many skin diseases such as common skin cancer result from aberrant methylation of tumour suppressor gene promoter; the main cause of many autoimmune diseases is known to be hypomethylation (27).

Long-term changes in chronic disease susceptibility can be explained by epigenetic mechanism and the way it can be influenced by environmental factors. To look closer to the environmental effect on epigenetics, and as a consequence on development of diseases, an example can be mentioned here; prenatal under-nutrition or stresses may be one of two developmental pathways that may generate obesity causing that individuals develop with central or peripheral changes increasing their sensitivity to an obesogenic environment (60).

According to Grolleau-Julius et al.'s study, epigenetic changes can explicate the relationship between aging and autoimmunity. The fall in immunocompetence with age is co-occur with the raise in the incidence of autoimmune diseases. Aging of the immune system, or immunosenescence, is characterized by a fall of both T and B cell

function, and contradictory the existance of low-grade chronic inflammation. Viewing evidence indicates a key role for epigenetics in human pathologies, including inflammatory and neoplastic disorders (37). Although some functions of the immune system in the eldery are reduced, others remain unchanged or even increased (61). A number of hypotheses have been suggested to elucidate the relationship between aging and the development of autoimmunity (37). An important characteristic of epigenetic feature is that, while they are both stable and heritable, they are at the same time alterable by the environment (37).

1.4 High Performance Liquid Chromatography

HPLC is the main method used to separate nucleosides in this study. There are different kinds of HPLC with specific characteristics. The method used in this research is Isocariotic Cation Exchange HPLC.

1.4.1 Basic Principles of HPLC

Classic High performance liquid chromatography is fundamentally a highly improved form of column chromatography. Instead of a solvent which should be dripped through a column under gravity, it is forced through under high pressures of up to 400 atmospheres; therefore HPLC is a faster method.

One of the advantages of this method is that it allows the researcher to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. Accordingly, this allows a much better separation of the components of the mixture.

There is another major improvement over column chromatography which concerns the detection methods that can be used. These methods are highly automated and extremely sensitive (62).

1.4.2 Normal and Reverse Phase HPLC

In normal phase HPLC, the column is filled with tiny silica particles, and the solvent is non-polar. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

Non-polar compounds will pass more quickly through the column since the polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar ones will; on the other hand reverse phase HPLC uses non-polar stationary phase and aqueous, ands moderately polar mobile phase (62).

1.4.3 Isocariotic Cation Exchange HPLC

Nucleotides are positively charge in acidic solutions, enabling their separation by cation exchange chromatography. Among all nucleosides 5-mdC and dC have the highest affinity to benzenesulfonic acid-modified silica phase (64). To this end, isocariotic cation exchange HPLC is used to separate the nucleosides so that dC and 5-mdC peaks appeare as the last peaks.

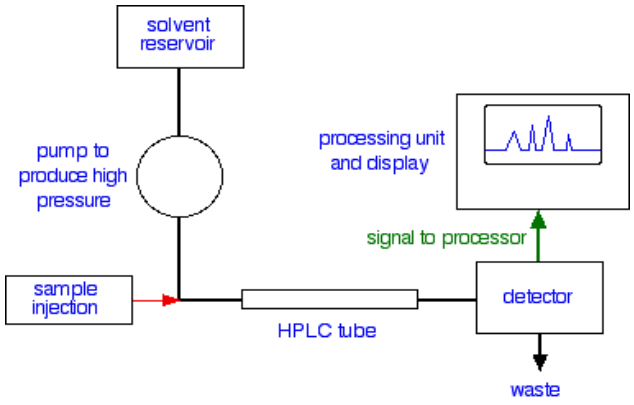

Fig 1.1: A flow scheme for HPLC

1.4.4 Injection of the Samples

Unlike gas chromatography pressure is involved in the injection of the sample. Injection of the samples is completely automated (62).

1.4.5 Retention Time

Retention time can be defined as the time taken for a particular compound to travel through the column to the detector. Calculating the time is done from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. The retention time will vary for a particular compound depending on:

- the pressure used (because that affects the flow rate of the solvent)
- he nature of the stationary phase (the material it is made of and particle size)
- the solvent's exact composition
- the column temperature

That means that conditions have to be carefully controlled retention times are being used as a way of identifying compounds (62).

1.4.6 The Detector

There are several ways of detecting when a substance has passed through the column. A common uses ultra-violet absorption.

Many organic compounds absorb UV light of various wavelengths. A beam of UV light emits light through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream results in a direct reading of how much of the light is absorbed (62).

The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.

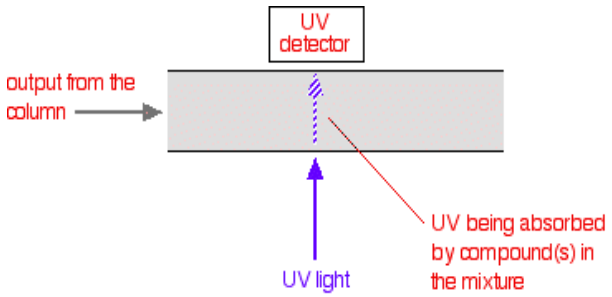

Fig. 1.2:.UV detector of HPLC

Since different compounds absorb most strongly in different parts of the UV spectrum, the absorbance of UV light by solvents do not interfere in the experiments.

Methanol, for example, absorbs at wavelengths below 205 nm, and water below 190 nm. If methanol-water mixture is being used as the solvent, wavelength greater than 205 nm must be used to avoid false readings from the solvent (62).

1.4.7 Interpreting the Output from the Detector

The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector and absorbing UV light. As long as the conditions on the column are under control, the retention times is being used to help to identify the compounds present – provided that pure samples of the various compounds have already been measured under those identical conditions (Standards, which are authentic nucleoside in this experiments) (62).

The peaks are also used as a measure of the quantities of the compounds present. Here "X" is a particular compound of interest.

If a solution containing a known amount of X gets injected into the machine, not only its retention time can be recorded, but it would be possible as well to relate the amount of X to the peak that was formed.
The area under the peak is proportional to the amount of X which has passed the detector, and this area can be calculated automatically by the computer linked to the display. The area it would measure is shown in green in the simplified diagram below (62).

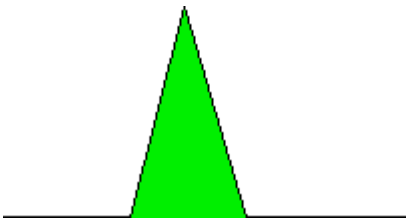

Fig. 1.3: Simplified HPLC diagram (61)

If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:

Fig. 1.4: Less concentration of injected compound can be interpreted by less area under the chromatogram (63)

This means that it is possible to calibrate the machine so that it can be used to find how much of a substance is present - even in very small quantities.

It is not possible to say anything about the relative amount of two different substances in the mixture (Xand Y) if *UV* absorption as your detection method is being used (62).

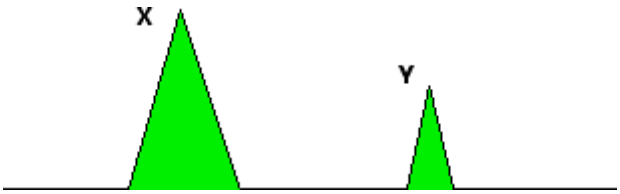

Fig. 1.5: two different diagrams belong to two different compounds (61)

In the diagram, the area under the peak for Y is less than that for X. That may be because there is less Y than X, but it could equally well be because Y absorbs UV light at the wavelength less than X does. There might be large quantities of Y present, but if it only absorbed weakly, it would only give a small peak (62).

1.5 The Aim of the Study

Retention In this study, it was aimed to investigate possible meaningful differences between methylation profile of monozygotic and dizygotic twins in which both of the twins are BS; additionally the same procedure were applied on the twins in whom one of them is healthy and the other one is BS. A final comparison was done between healthy control groups- with corresponding age and gender- and monozygotic and dizygotic twins with BS. We intended to show that difference in methylation profile of the individuals with the same genetic pattern (MZ twins), could be a risk factor for BS. Following the investigation of global methylation profile, locus specific methylation studies would enable us to investigate the effect of methylation pattern of the specific gene on the occurrence of the disease.

2. MATERIAL AND METHODS

2.1 Materials and Laboratory Equipment

2.1.1 Used Equipments

Lorem Appendiz A displays the equipments used in this project.

2.1.2 Used Chemicals, Enzymes, Markers, and Buffers

Chemicals, enzymes, and their related suppliers are given in Appendix B. Appendix C displays the preparation and the composition of buffers and solutions.

2.2 Collection and Storage of Blood Samples

Blood samples are collected from 8 Behçet syndrome twins and control group consist of 8 healthy twins with corresponding sex and age in Cerrahpaşa medical faculty Rheumatology polyclinic. Two 10 milliliter tubes were obtained from each person. A questionnaire, shown in Appendix D, is given to the subjects before the collection of the samples. According to the selection criteria, the subjects have to give their consent (shown in Appendix D) for the study. Blood samples are collected in EDTA tubes and kept in 0°C.

2.3 DNA Isolation from Blood Samples

The EDTA tubes contain a substance which mixes with the blood once has an anti-coagulating effect. Before the purification step, the sample has to be incubated in room temperature.

For each blood sample to be processed, 30 ml Red Blood Cell Lysis Buffer is added to a sterile 50 ml centrifuge tube. Centrifuge tube that will withstand a minimum of 900 × g and accommodate a total volume of 40 ml was used.

10 ml mammalian blood to each tube is added. The tube was capped, and mixed gently by inversion. Centrifuge tube was placed on a rocking platform or gyratory shaker for 10 min. Tubes were Centrifuged at 875× g for 10 min.

Carefully the clear, red supernatant was poured off and properly disposed (indicative of complete red cell lysis). Some residual liquid should remain with the white cell pellet.

The white pellet visible at the bottom of the tube in the residual supernatant was vortexed thoroughly.

5 ml White Cell Lysis Buffer was added, the tube was capped, and mixed thoroughly by vortexing.

Samples were transferred to a sterile 17 × 100 mm tub.

2.6 ml Protein Precipitation Solution was added to each sample.

Samples were centrifuged at 12,000 × g for 10 min.

The supernatant was poured, which contains the DNA, into a new sterile 50 ml centrifuge tube. Properly dispose of the protein pellet. Ethanol at this stage precipitates the DNA.

3 ml cold 70% ethanol was added to the DNA pellet in the tube, and samples were mixed the several times by gentle inversion. Samples were centrifuged at 875 × g for 5 minute. Supernatant was discarded afterwards.

DNA pellet was dried by placing the sample under vacuum without heat for a few minutes (until the ethanol was no longer visible).

To resuspend the DNA pellet, 1 ml TE Buffer, pH 8.0, or desired buffer was added and vortexed thoroughly. Samples were placed at 65°C for 30–60 min to aid in resuspension; samples were periodically being vortexed.

Samples were stored in +4°C.

2.4 Measuring DNA Amount

The amount of nucleic acid in the purified samples was calculated with NANODROP spectrophotometer which is shown in table 3.1.

2.5 Fully- Methylated DNA as a Marker for 5-mdC

Fully methylating DNA samples is done by applying CpG methylase kit to trace the rise in the area below the peak which belongs to 5-mdc.

2.5.1 CpG Methylase

CpG Methylase (M.Sssl) kit was obtained from Zymo Research.

CpG Methylase from Zymo Research completely methylates all cytosine residues (C^5) in double-stranded, nonmethylated and hemi-methylated DNA having the dinucleotide sequence 5′…CpG…3′. The recombinant CpG Methylase is isolated from an E. coli strain that expresses the Methylase gene from Spiroplasma sp. strain MQ1. The reaction conditions are optimized to maximize the processivity of the enzyme to ensure rapid, complete, and reproducible methylation of DNA for accurate DNA methylation analysis. This method was applied to fully methylate DNA samples.

2.5.2 Methylation Setup

The setup (below) was applied to DNA sample:

2 µl 10X CpG Reaction Buffer

1 µl 20X SAM (S-adenosylmethionine), 12 mM

4 µl DNA at 100-250 ng/µl

1 µl CpGMethylase (4 units/µl)

12 µl Water

Incubate at 30 °C for 2 hours

In order to completely methylate all the dinucleotides, CpG methylase was added after 2-4 hours of initial incubation which takes 4 h to overnight.

2.6 DNA Digestion

DNA digestion was applied for blood samples in two steps which were explained further.

2.6.1 Cutting DNA into Nucleotides

DNA digestion is applied during two steps. During the first step, in which DNA is cut into nucleotides (adenine, guanine, cytosine, and thymine).1 to 10 µg of DNA dissolved in 43 µl of water; 0.01unit Nuclease P1 is added to DNA samples and incubated in 65°C for 10 minutes (63); samples then was mixed with 5 µl of 10X digestion buffer; digestion buffer is consisted of the following chemicals: 200 mM acetic acid, 200 mM glycine, 50 mM $MgCl_2$,5 mM $ZnCl_2$, and 2 mM $CaCl_2$to adjusted the pH of the buffer to 5.3.Afterwards, 1 µl of nuclease mix was added. Nuclease mix is consisted of 80 units DNase I (bovine pancreas) and 1.2 units RNase per 10 µl solution.At the end of this stage, this mixture was incubated at 37 °C overnight (64).

2.6.2 Converting Nucleotides to Nucleosides

5 µl of 100 mM NaOH and 1 µl of calf intestine alkaline phosphatase (1 U/µl) were added. The mixture was incubated at 37 °C for a further 12 to 24 h (64).

2.6.3 Injecting Nucleosides into the HPLC Machine

The samples which had been digested and converted into nucleotide were diluted with 44 µl of 12 mM HCl prior to injection and transferred into HPLC vials (64).

2.6.4 HPLC System

The HPLC system included SCL-10A system controller, LC-10AD liquid chromatograph, DGU-14A Degasser, SPD-10A UV-VIS Detector, SIL-10AD Auto

injector, and CTO-10A Column oven. The system was equipped with a NUCLEOSIL 100SA, 10 µm, 25X0.46 column.

2.7 Mobile Phase

The mobile phase consisted of 60 mM acetic acid dissolved in 15% acetonitrile and was set to pH 4.8 with NaOH. A constant flow rate was maintained at 1 ml/min, and UV detection was performed at 277 nm with a bandwidth of 10 nm (64, 65).

2.8 Documentation and Interpretation

The chromatograms were documented by software equipment connected to HPLC machine. All of the chromatograms were documented as original reports in which retention time, peaks, and the area below the dC and 5-mdC peaks were recorded.

The amount of dC and 5-mdC were then calculated according to the calibration data obtained from the authentic dC and 5-mdC nucleosides peaks' area. Calibration data has been shown in table 3.3 and figure 3.4 and 3.5.

3. RESULTS

3.1 DNA Isolation Results

The DNA of BS samples were extracted by DNA Isolation Kit for Mammalian Blood Kit (Roche).

In Table 3.1 measured DNA concentrations of all the samples were illustrated.

Table 3.1 DNA concentration of the samples

Sample	DNA concentration (ng/uL)	Sample	DNA concentration (ng/uL)
Control		**BS**	
C-01-1	1157.3	BS-01-1	484.7
C-01-2	950.2	BS-01-2	559.7
C-02-1	1386.7	BS-02-1	772
C-02-2	573.9	BS-02-2	940.8
C-03-1	1107.8	BS-03-1	887.6
C-03-2	1358.4	BS-03-2	1210.9
C-04-1	1259	BS-04-1	670
C-04-2	622	BS-04-2	1895.7
C-05-1	1208.7	BS-05-1	741
C-05-2	1677.2	BS-05-2	270.6
C-06-1	1132.2	BS-06-1	350.4
C-06-2	443.4	BS-06-2	253
C-07-1	514.8	BS-07-1	486.9
C-07-2	440.2	BS-07-2	511.4
C-08-1	736.4	BS-08-1	1064.1
C-08-2	1488.5	BS-08-2	858.1

Measured DNA concentrations were used to determine the volume of DNA samples to be added to digestion mix in order to obtain the same amount of DNA for each HPLC injection (5 µg).

Authentic dc and 5-mdc is used as standard for the whole experiment. Since the concentrations of the standard nucleosides are known, the dc and 5-mdc in the DNA samples of BS and controls can be traced by their retention time.

In order to indicate dC and 5-mdC peaks in chromatograms, one of the DNA samples were digested and converted into nucleoside. Digested sample was injected into the HPLC machine and nucleoside peaks were obtained. This chromatogram was then compared with the chromatogram of the authentic dC and 5-mdC standards. As it is shown in the figures below, the retention time of the dC and 5-mdC nucleosides are approximately in the 7th and 9th minutes respectively.

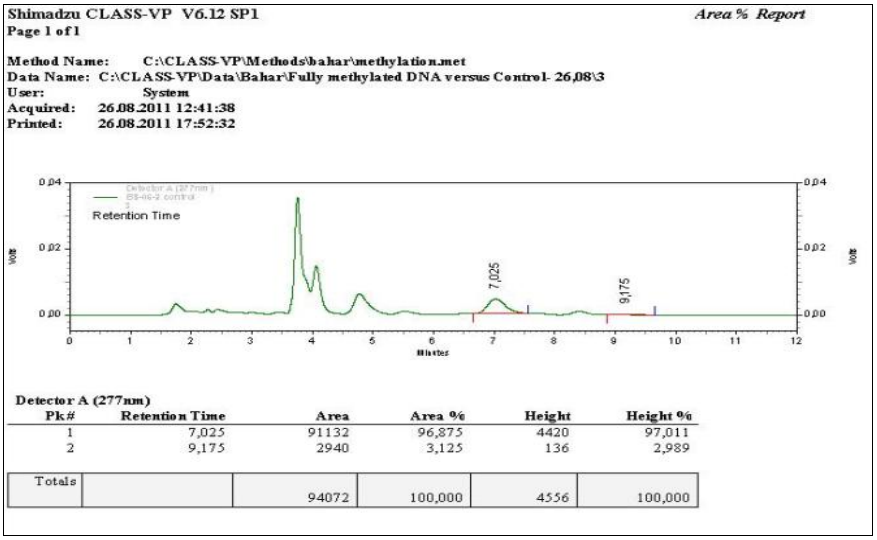

Figure 3.1: Chromatogram of digested DNA sample; peaks belong to dC and 5-mdC are appeared in 7th and 9th minute respectively

In the figure 3.1, dC and 5-mdC peaks have been introduced to the software and the area below the peak has been calculated for the further studies.

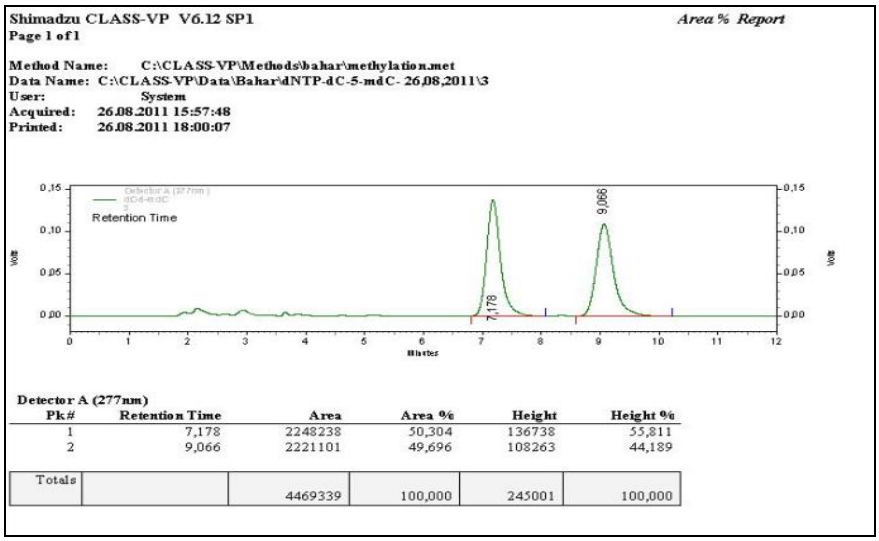

Figure 3.2: Chromatogram of injected authentic dC and 5-mdC as standards. The exact concentration of dC and 5-mdC were calculated according to the area below the nucleosides of interest

In order to confirm the retention time of 5-mdC, DNA sample were treated with Methylase enzyme and it is demonstrated that 5-mdC peak area was increased as expected.

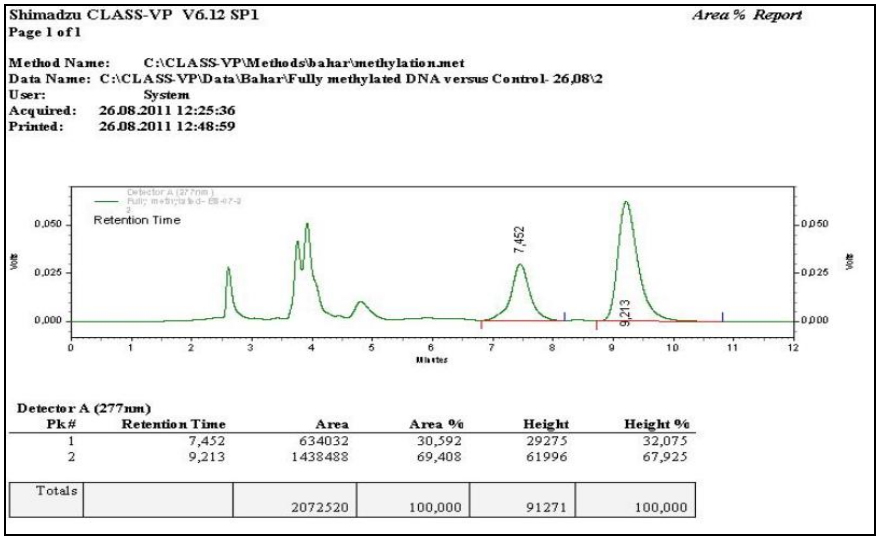

Figure 3.3: Chromatogram of digested DNA sample treated with methylase enzyme; fullymethylated sample confirmed the 5-mdC peak to be the last peak on chromatograms, which appears after dC in all the samples.

3.2 Behçet Syndrome Patients

The The data of the eight Behçet Syndrome twins whose DNA samples were used in the study is shown in Table 3.2.

Table 3.2 List of BS twins

ID	BS or Healthy (H)	Age	Gender M/F	MZ/DZ Concordant/discordant
BSİ-03-1	**BS**	**46**	**M**	**MZ Concordant**
BSİ-03-2	**BS**	**46**	**M**	
BSİ-02-1	**BS**	**23**	**M**	**MZ Concordant**
BSİ-02-2	**BS**	**23**	**M**	
BSİ-05-1	**BS**	**54**	**M**	**MZ Discordant**
BSİ-05-2	**H**	**54**	**M**	
BSİ-07-1	**BS**	**38**	**M**	**MZ Discordant**
BSİ-07-2	**H**	**38**	**M**	
BSİ-01-1	**BS**	**36**	**M**	**DZ Concordant**
BSİ-01-2	**BS**	**36**	**M**	
BSİ-06-1	**BS**	**30**	**M**	**DZ Discordant**
BSİ-06-2	**H**	**30**	**M**	
BSİ-04-1	**BS**	**35**	**F**	**DZ Discordant**
BSİ-04-2	**H**	**35**	**F**	
BSİ-08-1	**BS**	**41**	**M**	**DZ Discordant**
BSİ-08-2	**H**	**41**	**M**	

3.3 Calibration of dC and 5-mdC

The The calibration was done by injecting various concentrations of dC and 5-mdC authentic nucleosides and calculating the area below their peak. Concentrations of 0.2, 0.4 and 0.6 μg/mL were used to obtain the calibration curves that are demonstrated in Figure 3.4 and 3.5 (data shown in Table 3.3) illustrate the detected areas for the given concentrations of dC and 5-mdC controls.

Since the concentation of dC and 5-mdC was in very small amounts, the selected concentartions to obtain the calibration curves and the equation afterwards, were in small amounts (0.2, 0.4, and 0.6).

Table 3.3: Area calculated for various concentrations of dC and 5-mdC

dC		**5-mdC**	
Concentration of dC (µg/mL)	Area	Concentration of 5-mdC (µg/mL)	Area
0.2	170511	0.2	146332
0.4	319655	0.4	324993
0.6	467353	0.6	489954

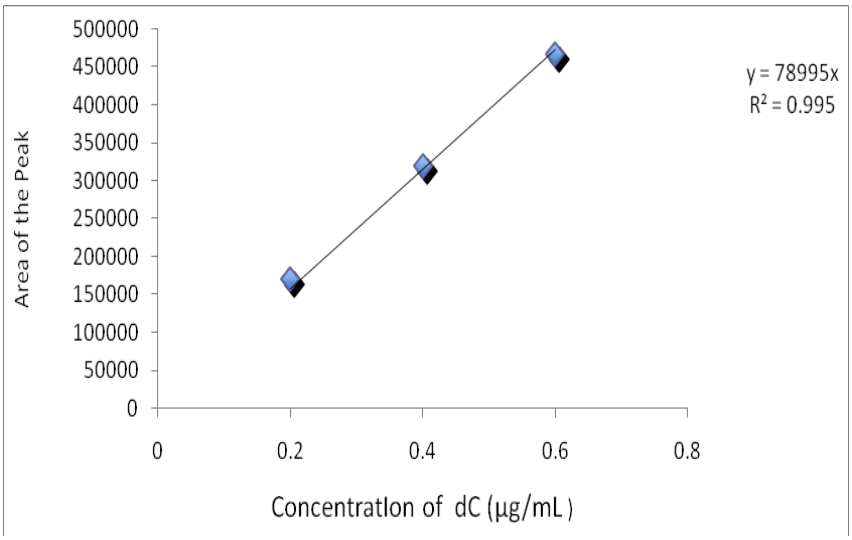

Figure 3.4: Calibration curve for dC nucleoside

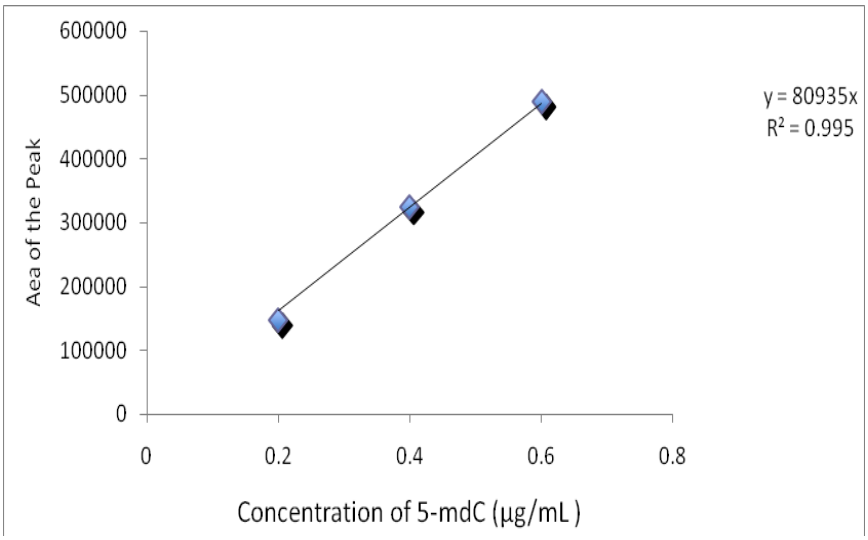

Figure 3.5: Calibration curve for 5-mdC nucleoside

The relation between the areas of the peaks and the concentration of the nucleosides were shown with two different equations. For the dC; y =78995x (R^2=0.995) and for 5-mdC; y =80935x (R^2=0.995) which were found and used for the calculations.

3.4 Chromatogram of BS versus Healthy Control

Cluster Figures 3.6- 21 in APPENDIX D demonstrate the chromatograms of one of the repeats of the HPLC measurements (4 BS twins and 4 healthy twins (n= 16), representative reports for HPLC analyses). The areas obtained under the peaks were used to calculate the amount of dC and 5-mdC in BS twins and healthy controls. These values are then used to determine and compare dC: 5-mdC ratios of BS twins and healthy control twins.

Data from 8 BS twins versus 8 healthy control groups were gathered and compared in the tables 3.4; the equations that were obtained from calibration curves of dC and 5-mdC authentic nucleosides were used to calculate the nucleoside concentration in the injected samples.

Table 3.4 demonstrates the average of the calculated ratios of 5-mdC: dC in the injected samples for both BS twins and healthy control twins. Injections were performed in triplicates and a separate ratio was calculated using the different areas on the chromotograms for each of them. Difference of the 5m-dC: dC ratios in-between the twins were also indicated on the Table 3.4, and this data was used for statistical analysis.

In order to highlight the difference in methylation pattern of BS and Healthy Controls in between twins, difference of the ratios were converted into “Fold Difference”. This enabled us to show the differences significantly in the charts:

- Average area for dC and 5-mdC and 5-mdC:dC ratio in each of the samples (BS versus healthy) were given as figures under each peak automatically (Class VP software)
- real amount of dC and 5-mdC were calculated for each of the repeats (Calibration equation)
- Average of three repeats were calculated and 5-mdC: dC was obtained

Table 3.4: Average area for dC and 5-mdC and 5-mdC:dC ratio in each of the samples (BS versus healthy); data of the twins (MZ/DZ and BS/H) are shown in this table. Differences of the calculated ratios were given as well which clarifies the difference in methylation profile of the twins in BS twins versus Healthy controls.

Sample	CalculatedC Average	Calculated 5-mdC Average	Ratio 5-mdC:dC Average	MZ/DZ BS/H	Fold Difference	Differences of Ratios of Twins
Control						
C-01-1	0.82620	0.02638	**0.0309±0.005**	MZ	0.094	0.00307
C-01-2	0.82305	0.02898	**0.0340±0.004**	MZ		
C-02-1	0.73438	0.02688	**0.0353±0.002**	MZ	0.099	0.00336
C-02-2	0.79630	0.02629	**0.0319±0.005**	MZ		
C-03-1	0.79000	0.02906	**0.0355±0.002**	MZ	0.0035	0.00128
C-03-2	0.90863	0.03468	**0.0368±0.002**	MZ		
C-04-1	0.83022	0.03190	**0.0370±0.002**	DZ	0.63	0.00228
C-04-2	0.78934	0.02839	**0.0347±0.0008**	DZ		
C-05-1	1.29752	0.05012	**0.0372±0.001**	DZ	0.0052	0.00020
C-05-2	0.79806	0.03100	**0.0374±0.001**	DZ		
C-06-1	0.68084	0.02617	**0.0370±0.002**	MZ	0.04	0.00167
C-06-2	2.68699	0.09845	**0.0353±0.001**	MZ		
C-07-1	0.57442	0.01946	**0.0328±0.002**	DZ	0.044	0.00148
C-07-2	0.70035	0.02484	**0.0342±0.001**	DZ		
C-08-1	0.71521	0.03332	**0.0445±0.001**	DZ	0.071	0.00332
C-08-2	0.66931	0.03362	**0.0478±0.003**	DZ		
BS						
BS-01-1	0.68491	0.02391	**0.0337±0.002**	DZ-BS	0.115	0.00414
BS-01-2	0.72178	0.02841	**0.0379±0.003**	DZ-BS		
BS-02-1	0.57829	0.02124	**0.0354±0.002**	MZ-BS	0.244	0.00987
BS-02-2	0.82467	0.03913	**0.0453±0.0003**	MZ-BS		
BS-03-1	0.79305	0.02962	**0.0360±0.001**	MZ-BS	0.048	0.00177
BS-03-2	0.78959	0.03100	**0.0378±0.001**	MZ-BS		
BS-04-1	0.89124	0.03267	**0.0354±0.002**	DZ-BS	0.053	0.00191
BS-04-2	0.65311	0.02529	**0.0373±0.001**	DZ-H		
BS-05-1	0.60788	0.02375	**0.0376±0.001**	MZ-BS	0.22	0.00762
BS-05-2	0.43199	0.01335	**0.0300±0.003**	MZ-H		
BS-06-1	0.67055	0.03084	**0.0440±0.004**	DZ-BS	0.09	0.00385
BS-06-2	0.67936	0.02839	**0.0401±0.003**	DZ-H		
BS-07-1	0.59575	0.02412	**0.0390±0.0008**	MZ-BS	0.09	0.00402
BS-07-2	0.76379	0.03426	**0.0430±0.005**	MZ-H		
BS-08-1	0.86000	0.03703	**0.0413±0.005**	DZ-BS	0.103	0.00405
BS-08-2	0.74890	0.02896	**0.0372±0.0008**	DZ-H		

It can be seen in the Table 3.4 that the ratio of 5-mdC: dC in all the samples was between 0.02- 0.05, which confirms the previous studies (66).

Table 3.5: Three repeats of each sample, variance, and standard deviation

Sample	Ratio 5-mdC:dC 1	Ratio 5-mdC:dC 2	Ratio 5-mdC:dC 3	Variance for each three repeat	Ratio 5-mdC:dC Average	Ratio 5-mdC:dC Standard Deviation
Control						
C-01-1	0.028648	0.026945	0.03664	2.68E-05	0.03094	0.005176
C-01-2	0.029424	0.035648	0.036376	1.46E-05	0.03401	0.003821
C-02-1	0.032441	0.036131	0.036527	5.08E-06	0.03532	0.002253
C-02-2	0.026407	0.034529	0.035326	2.44E-05	0.03196	0.004935
C-03-1	0.032658	0.036894	0.037171	6.4E-06	0.03548	0.002529
C-03-2	0.033931	0.038423	0.037776	5.9E-06	0.03677	0.002428
C-04-1	0.035335	0.038528	0.037425	2.63E-06	0.037	0.001622
C-04-2	0.033825	0.035572	0.034657	7.64E-07	0.03472	0.000874
C-05-1	0.035568	0.038184	0.037915	2.07E-06	0.03719	0.001439
C-05-2	0.035601	0.038235	0.037904	2.06E-06	0.03739	0.001435
C-06-1	0.03515	0.038711	0.038038	3.58E-06	0.03702	0.001892
C-06-2	0.034465	0.036522	0.0352	1.09E-06	0.03535	0.001042
C-07-1	0.031324	0.03444	0.032006	2.68E-06	0.03277	0.001638
C-07-2	0.032828	0.034861	0.035038	1.51E-06	0.03425	0.001228
C-08-1	0.042451	0.038474	0.05623	8.68E-05	0.04451	0.009318
C-08-2	0.045076	0.040565	0.058936	9.17E-05	0.04783	0.009574
BS						
BS-01-1	0.031438	0.035327	0.034197	4E-06	0.03373	0.002
BS-01-2	0.03628	0.042181	0.036526	1.11E-05	0.03787	0.003338
BS-02-1	0.033627	0.037245	0.037106	4.2E-06	0.03543	0.00205
BS-02-2	0.045225	0.045068	0.045591	7.2E-08	0.0453	0.000268
BS-03-1	0.035556	0.034721	0.03724	1.65E-06	0.03601	0.001283
BS-03-2	0.0361	0.038758	0.038864	2.45E-06	0.03778	0.001566
BS-04-1	0.03301	0.037446	0.036589	5.54E-06	0.03537	0.002353
BS-04-2	0.035891	0.037813	0.037998	1.36E-06	0.03728	0.001167
BS-05-1	0.035933	0.039022	0.038154	2.54E-06	0.0376	0.00159
BS-05-2	0.028731	0.032924	0.027804	7.44E-06	0.02998	0.002728
BS-06-1	0.042985	0.040449	0.047681	1.35E-05	0.04397	0.003669
BS-06-2	0.0396	0.044244	0.037619	1.16E-05	0.04012	0.0034
BS-07-1	0.039876	0.038317	0.038603	6.88E-07	0.03891	0.00083
BS-07-2	0.048937	0.041334	0.039327	2.57E-05	0.04293	0.00507
BS-08-1	0.03726	0.039283	0.04829	3.45E-05	0.04128	0.005872
BS-08-2	0.037415	0.037951	0.036355	6.6E-07	0.03723	0.000812

In Figures 3.6- 3.7, 5-mdC: dC fold differences were used to enable comparison between BS twins and Healthy Controls in between the twins; standard deviation for each of the values were shown above the related column. It can be seen that the values for Healthy Controls are smaller than those for BS twins which confirms the larger methylation pattern difference in BS twins compared to healthy controls. Figures 3.8- 3.10 illustrate fold differences for MZ and DZ in separate charts. A stronger significant relationship for MZ twins was identified compared with DZ twins.

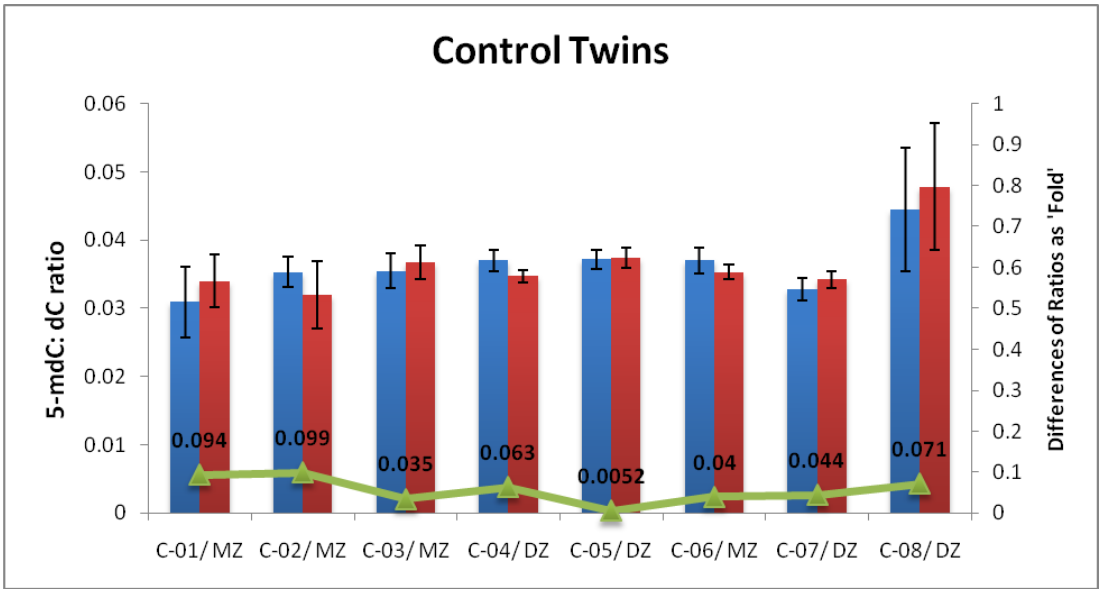

Figure 3.6: Fold difference chart for Healthy Controls

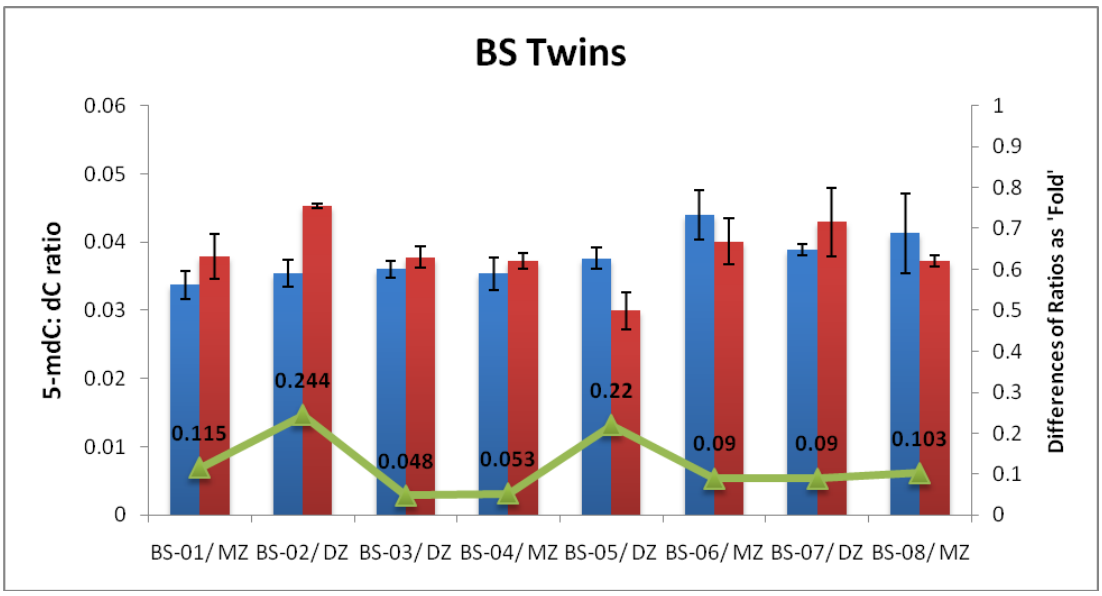

Figure 3.7: Fold difference chart for MZ BS

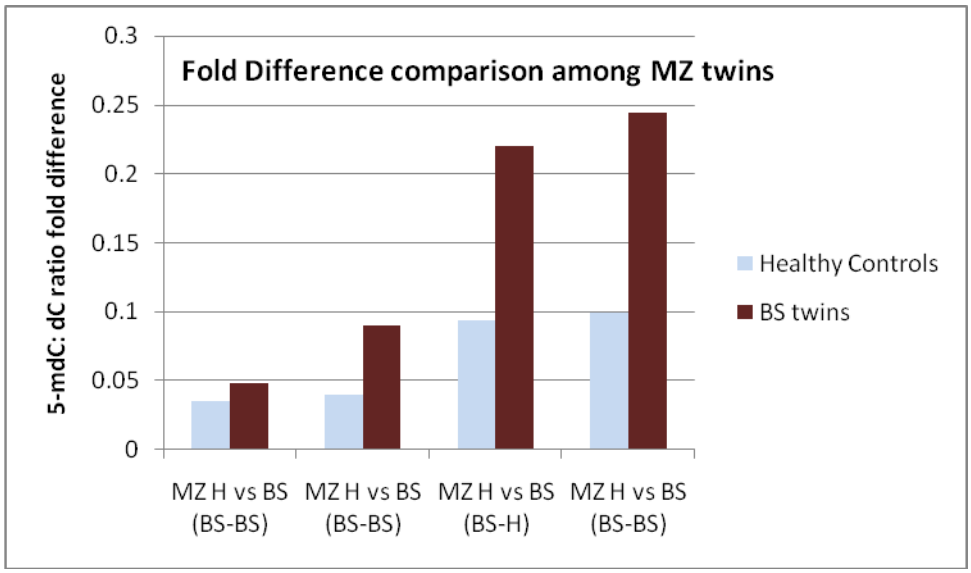

Figure 3.8: Fold difference chart for MZ Healthy Controls versus BS twins (P= 0.041)

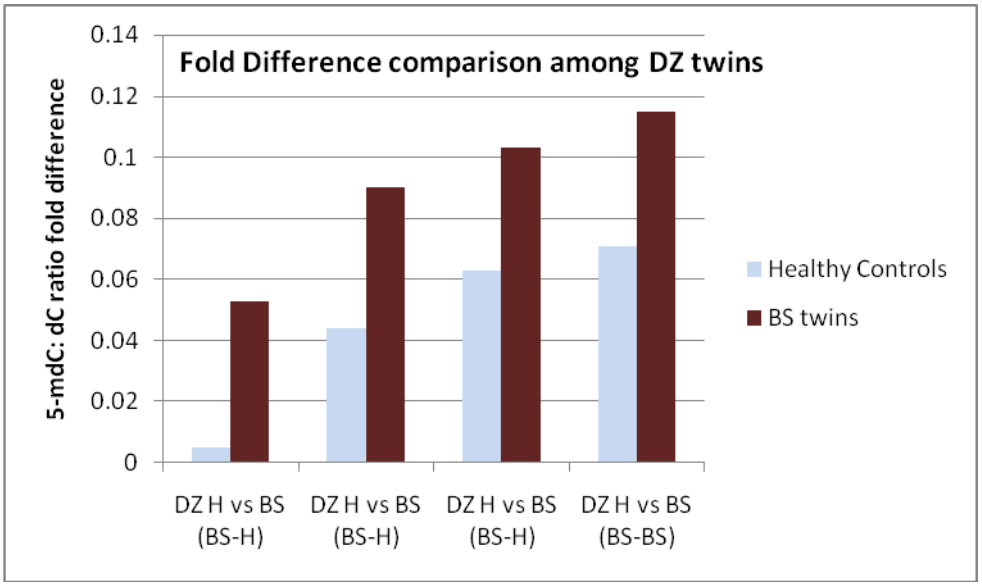

Figure 3.9: Fold difference chart for DZ Healthy Controls versus BS twins (P= 0.095)

In Figure 3.10 the 5-mdC: dC ratio difference among BS twins is compared with Healthy Controls; it is apparently illustrated that the values in BS twins group is larger than those in Healthy Control Groups.

According to the Student T- test, P value for this group was obtained as 0.0288 which indicated statistically significant relationship between BS twins and Healthy Controls.

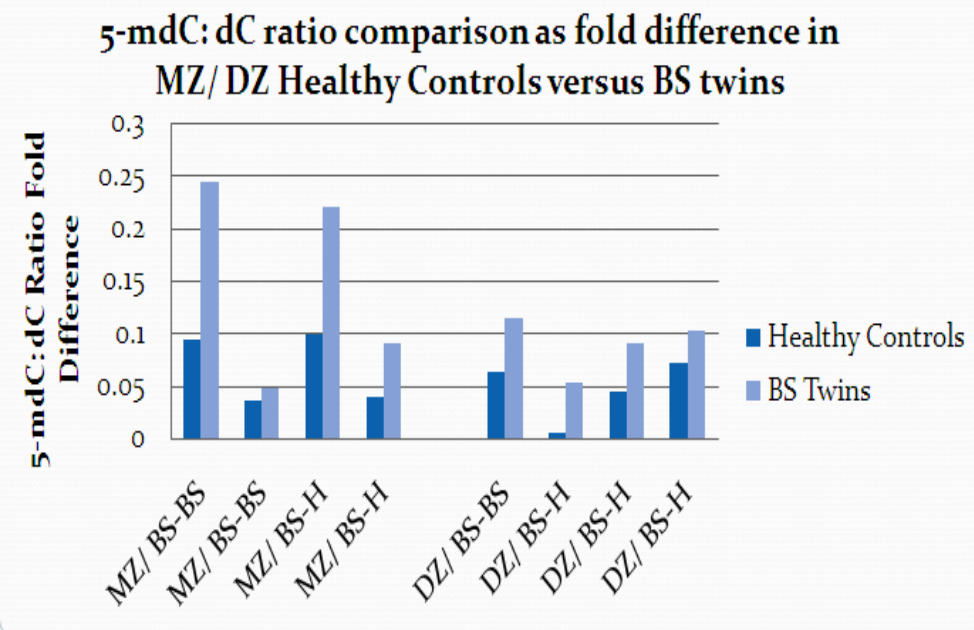

Figure 3.10: Fold difference in Healthy Controls versus BS twins (P= 0.0288)

3.5 Statistical Analyses

Comparison of the twins' parameters were done using student t-test between BS versus healthy twins, MZ BS twins versus MZ healthy twins, DZ BS twins versus DZ healthy twins, and between BS patients in which one of the twins is healthy and the other one is BS versus healthy twins (MZ/DZ).

According to the student t-test analyses, it was concluded that there is a smaller difference between methylation pattern of the twins in healthy control group compared to the twins in which one of them is healthy and the other one is BS (P= 0.02847).

There is statistically significant difference between the ratio of 5-mdC: dC in MZ/DZ twins with BS (both or one of them BS) versus MZ/DZ twins in healthy controls (P= 0.02876) assuming no significant relationship between methylation profile differences among twins and predisposition to BS as the null hypothesis. Another student analysis were done among healthy MZ and BS MZ twins and also healthy DZ and BS DZ twins in different groups; significant difference were found among MZ twins (P= 0.04133), whereas the null hypothesis could not be rejected at this significant level for DZ twins (P_2= 0.09551).

4. CONCLUSION AND DISCUSSION

The results obtained from this study confirm the literature on separation of deoxynucleosides in ten minutes under isocratic conditions. All 5 deoxynucleosides that are present in human DNA (Deoxyadenosine, Deoxyguanosine, Thymidine, and Deoxycytidine) were successfully separated using HPLC based methodology between 4^{th} and 10^{th} minutes. The last two peaks in the chromatograms which represented dC and 5-mdC enabled the global methylation patterns to be analyzed.

The analyses that were performed on 8 BS twins and 8 healthy control twins demonstrated that there is a possible relation between methylation profile and the Behçet Syndrome. According to the student t-test findings, it is hypothesized that the difference between the 5-mdC: dC ratio in healthy twins is smaller than the difference between the 5-mdC: dC ratio in BS twins. This can lead to the conclusion that there might be an environmental effect which can change the epigenetic pattern and influence the expression of the specific genes that may be responsible for the appearance of the disease.

Four separate analyses were performed on the 5-mdC: dC ratio of all the samples (BS versus Healthy Controls). The null hypothesis was that there is no statistically significant relationship between methylation profile and susceptibility to the disease. According to the results of three repeated HPLC analyses for each of the samples, it was hypothesized that the discrepancy between methylation profile (which is the most important feature of epigenetic pattern) between twins can be used to show the critical role of epigenetics on the appearance of complex diseases such as BS. It was thought that some factors (such as environmental effects) can influence the epigenetic pattern of the individuals that can lead to any change in the expression of specific genes. In this twins study, methylation profile of 8 BS twins (n= 16) and 8 healthy twins (n= 16) were analyzed to look for the effect of epigenetics on the susceptibility of the disease. In the first group, BS twins (MZ/DZ) in which both or one of the twins were BS, were compared with the healthy twins (MZ/DZ);

calculated P value showed that there might be a meaningful relationship between the epigenetic discrepancies and appearance of the disease (P= 0.02876); the difference between 5-mdC: dC ratio of each twins in healthy groups was smaller than the difference between 5-mdC: dC ratio in BS twins. This results were confirmed with student t-test when the comparison were applied between healthy control twins (MZ/DZ) versus BS twins (MZ/DZ) in which only one of the twins were BS (P= 0.02847). The latter comparison were applied to eliminate the BS twins in which both were BS so that to be able to investigate the role of epigenetics more specifically. The last two analyses were performed between BS twins (MZ) versus Healthy twins (MZ) in order to compare individuals with same genome. P value for this group of study were statistically meaningful (P= 0.04133) which lead to the conclusion that there might be epigenetic risk factors that can increase the susceptibility of BS in individual with the same genome (MZ twins); as the difference between twins with BS was larger than healthy twins. The last comparison between MZ BS twins and healthy MZ twins showed no significant relationship between 5-mdC: dC ratio difference and predisposition to BS (P= 0.09551).

5-mdC: dC ratios for BS twins were more than 0.003, whereas the ratios for Healthy Control groups were less than 0.0035. This points out the lager difference in methylation pattern among BS twins once more.

This hypothesis can be supported by the future experiments which are aimed to investigate the methylation profile of the specific genes which are thought to have a strong association with BS (HLA-B 51). To this end, findings of global methylation profile differences between twins in this project can be thought as a preliminary study for the further epigenetic researches.

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APPENDICES

APPENDIX A : Laboratory Equipment
APPENDIX B : Chemicals
APPENDIX C : Buffers
APPENDIX D : BS twins and Healthy Control Chromatograms
APPENDIX E :Questionnarie

APPENDIX A

Laboratory Equipment

Balances	Precisa 620C SCS
	Precisa BJ 610C
Centrifuges	Sigma 1-13 B. Braun International
	Allegra 25R Centrifuge Beckman Coulter
Pipettes	Gilson Pipetman 20 µL 200 µl, 1000 µl
pH meter	Mettler Toledo MP220
Vortex	HerdolphReax top
System Contriller	SCL-10A
Liquid Chromatograph	LC-10AD
Degasser	DGU-14A
UV-VIS Detector	SPD-10A
Auto Injector	SIL-10AD
Column Oven	CTO-10AC
HPLC Column	TEKNOKROMA- Nucleosil 100SA
Plastic Pachaging	Parafilm-PECHINEY
Heat Block	Dri-Block® DB-2D- Bibby Scientific Ltd
Serological Pipette	5, 10, 25 ml Greiner bio-one CELLSTAR®
Spectrophotometer	NANODROP- Thermo Scientific
EDTA Blood Tube	VACUTEST- ARZERGRANDE
Examination Gloves	AKO-MED
Distilled water machine	TKA WASSER- AUFBEREITUNGS
Magnetic Stirrer	Dragon Lab MS-H-S
Ultrasonic Bath	Transsonic TP 690

APPENDIX B

Chemicals

Ethanol	Riedel-de Haën
$MgCl_2$	Fermentas
NaOH	Riedel-de Haën
HPLC Grade Water	Merck
HPLC Grade Methanol	J.T. Baker
HPLC Grade Acetonitrile	Merck
Acetic Acid	Riedel-de Haën
Glysin	Applichem
$ZnCl_2$	Riedel-de Haën
$CaCl_2$	Merck
HCl	Merck
Nuclease Mix (DNase bovine pancrease and RNase)	GE Healthcare
Alkaline Phosphatase Calf Intestinal (CIP)	FINNZYMES

APPENDIX C

Buffers

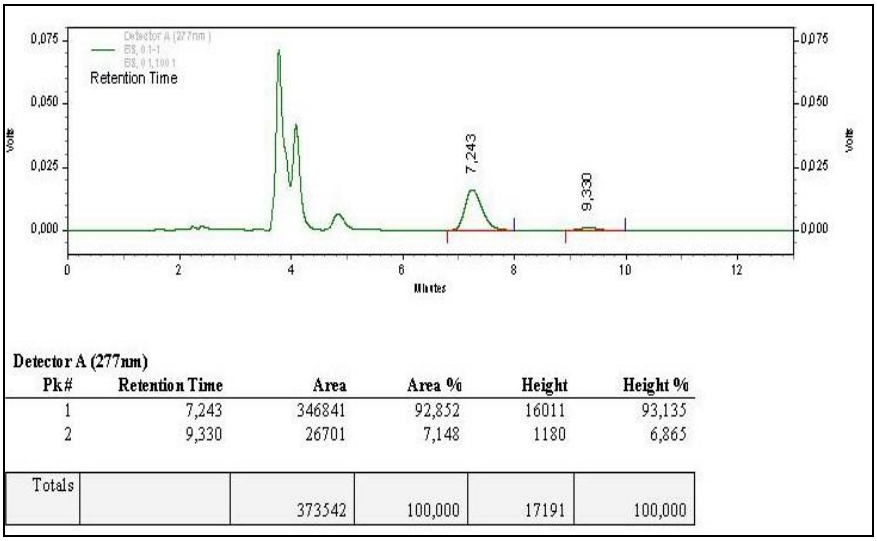
Digestion Buffer

Acetic acid	200 mM
Glycine	200 mM
$MgCl_2$	50 mM
$ZnCl_2$	5 mM
$CaCl_2$	2 mM

adjusted with **NaOH** to pH 5.3
pH to 8.0

APPENDIX D

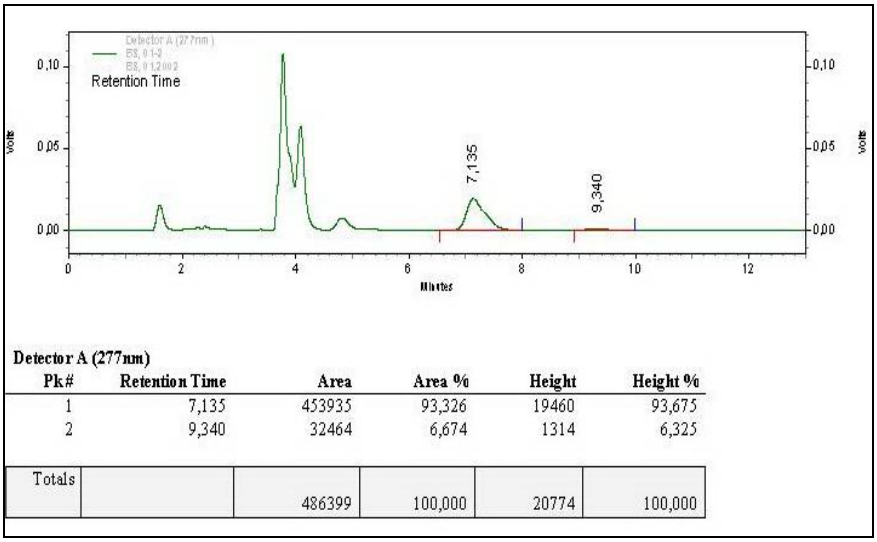
BS twins and Healthy Control Chromatograms



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,243	346841	92,852	16011	93,135
2	9,330	26701	7,148	1180	6,865
Totals		373542	100,000	17191	100,000

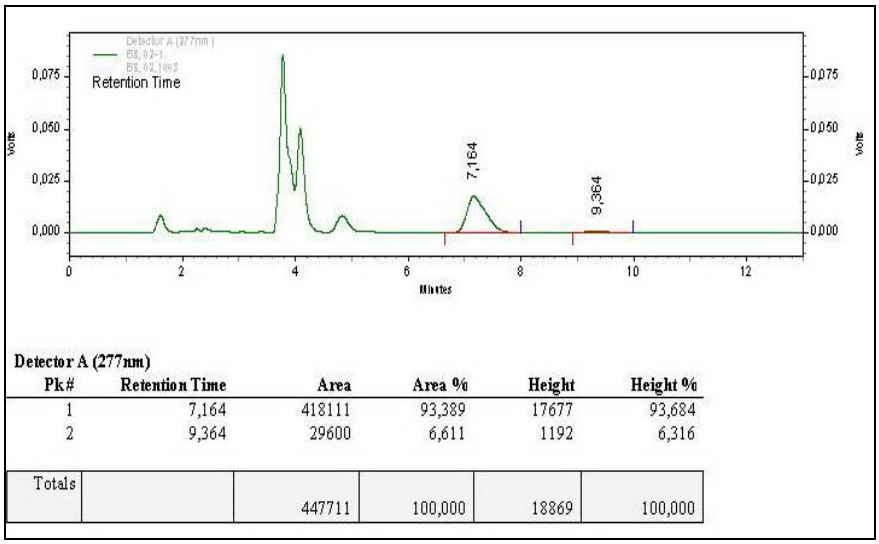
Figure D.1: BS-01-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,135	453935	93,326	19460	93,675
2	9,340	32464	6,674	1314	6,325
Totals		486399	100,000	20774	100,000

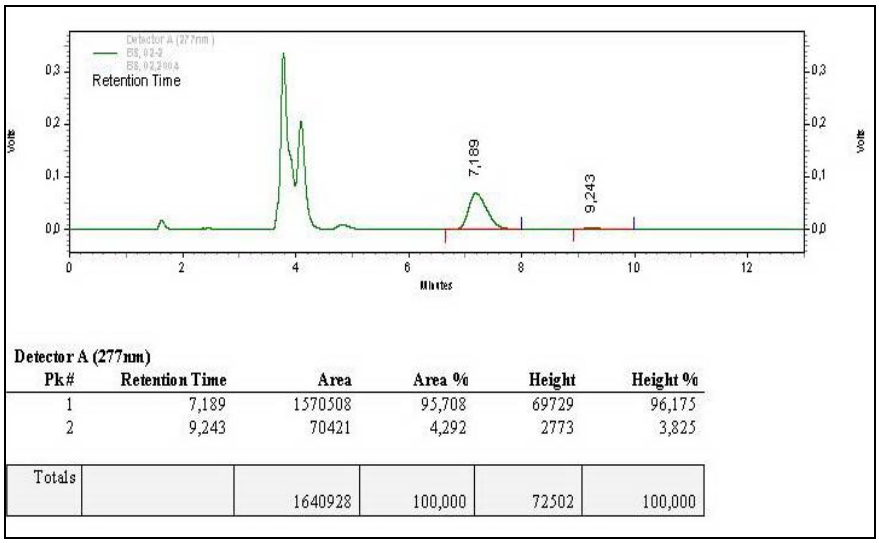
Figure D.2: BS-01-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,164	418111	93,389	17677	93,684
2	9,364	29600	6,611	1192	6,316
Totals		447711	100,000	18869	100,000

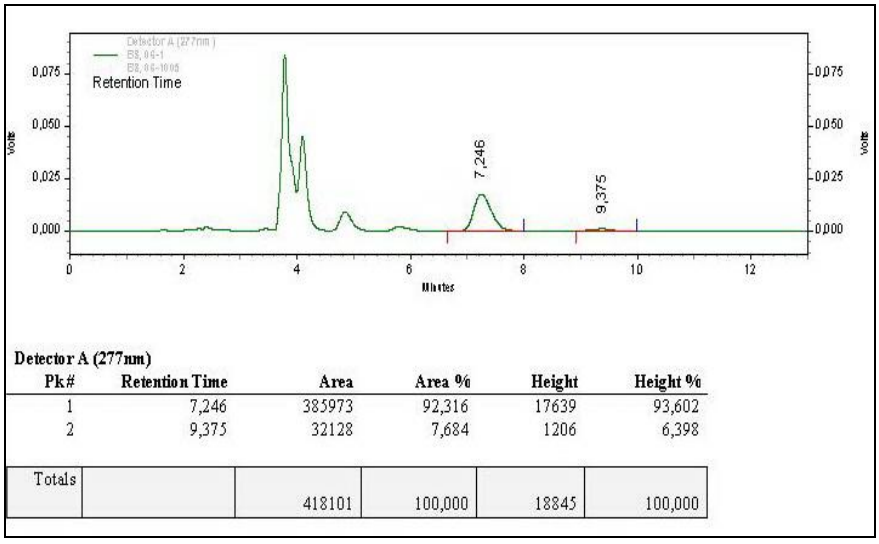
Figure D.3: BS-02-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,189	1570508	95,708	69729	96,175
2	9,243	70421	4,292	2773	3,825
Totals		1640928	100,000	72502	100,000

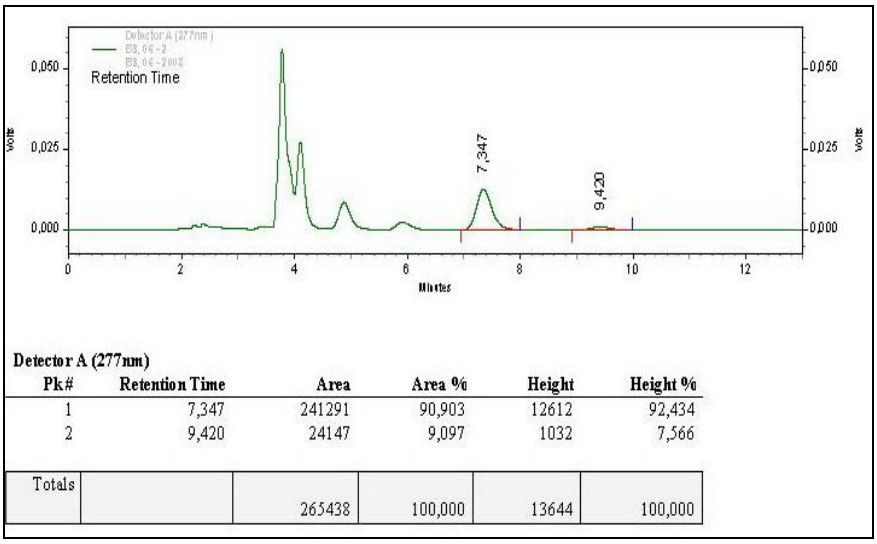
Figure D.4: BS-02-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,246	385973	92,316	17639	93,602
2	9,375	32128	7,684	1206	6,398
Totals		418101	100,000	18845	100,000

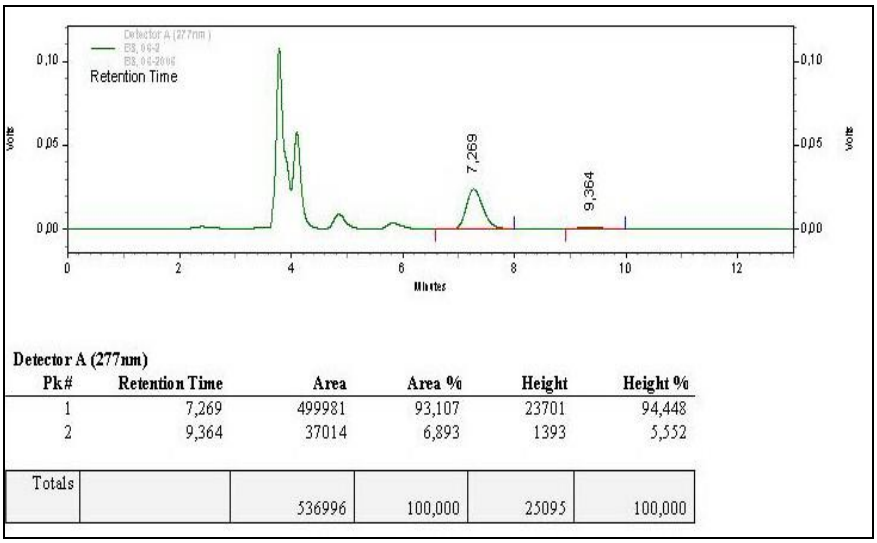
Figure D.5: BS-06-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,347	241291	90,903	12612	92,434
2	9,420	24147	9,097	1032	7,566
Totals		265438	100,000	13644	100,000

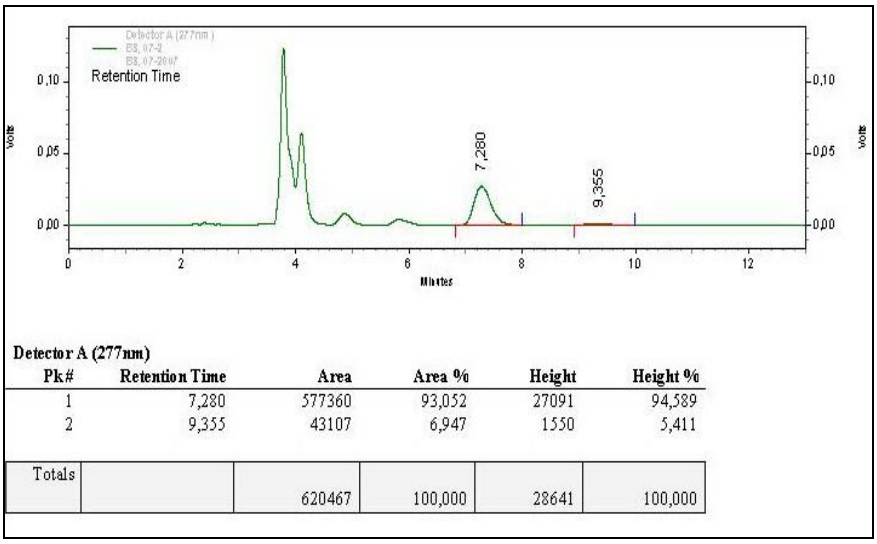
Figure D.6: BS-06-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,269	499981	93,107	23701	94,448
2	9,364	37014	6,893	1393	5,552
Totals		536996	100,000	25095	100,000

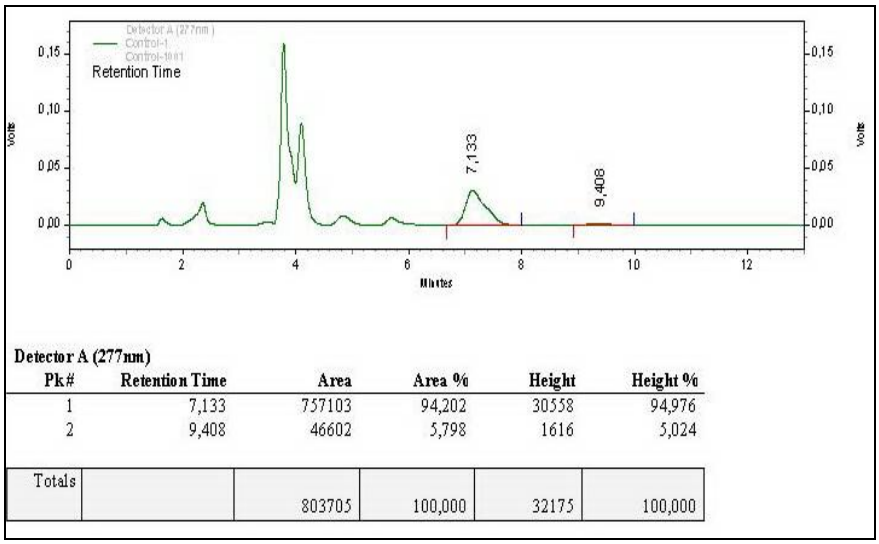
Figure D.7: BS-07-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,280	577360	93,052	27091	94,589
2	9,355	43107	6,947	1550	5,411
Totals		620467	100,000	28641	100,000

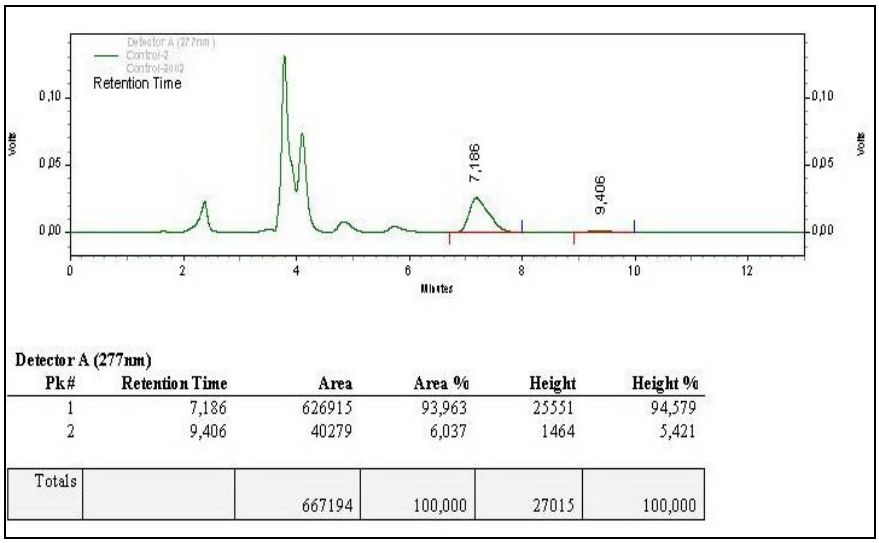
Figure D.8: BS-07-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,133	757103	94,202	30558	94,976
2	9,408	46602	5,798	1616	5,024
Totals		803705	100,000	32175	100,000

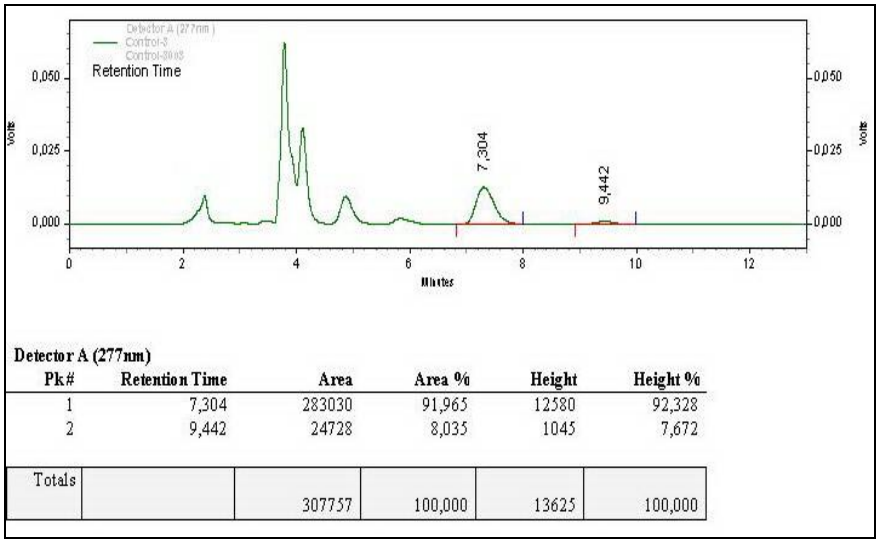
Figure D.9: C-01-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,186	626915	93,963	25551	94,579
2	9,406	40279	6,037	1464	5,421
Totals		667194	100,000	27015	100,000

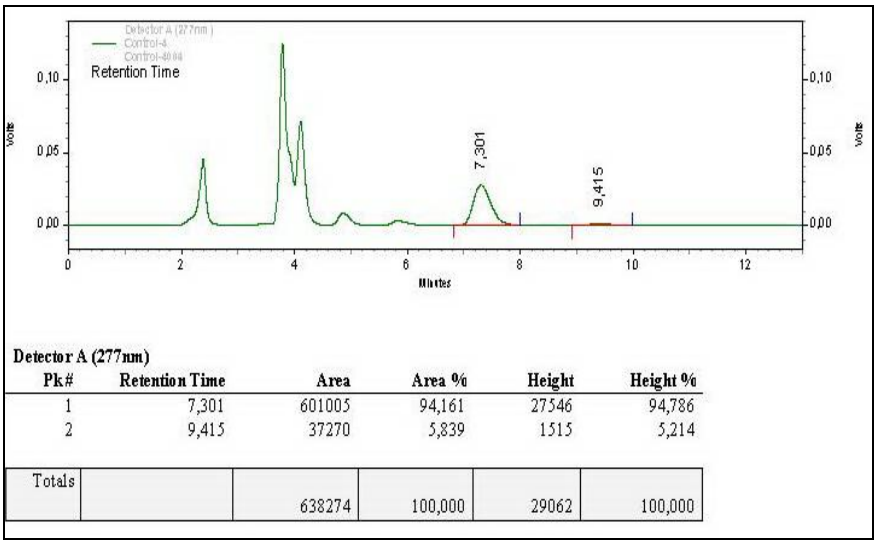
Figure D.10: C-01-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,304	283030	91,965	12580	92,328
2	9,442	24728	8,035	1045	7,672
Totals		307757	100,000	13625	100,000

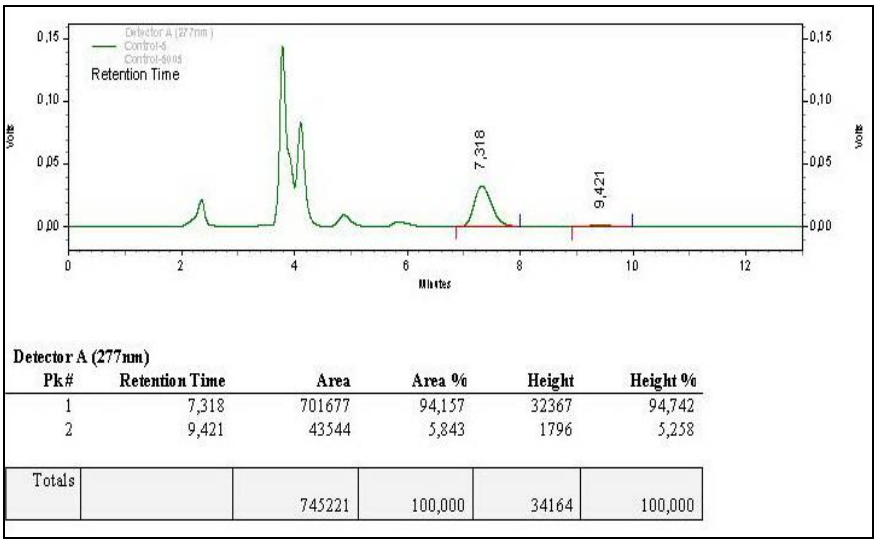
Figure D.11: C-02-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,301	601005	94,161	27546	94,786
2	9,415	37270	5,839	1515	5,214
Totals		638274	100,000	29062	100,000

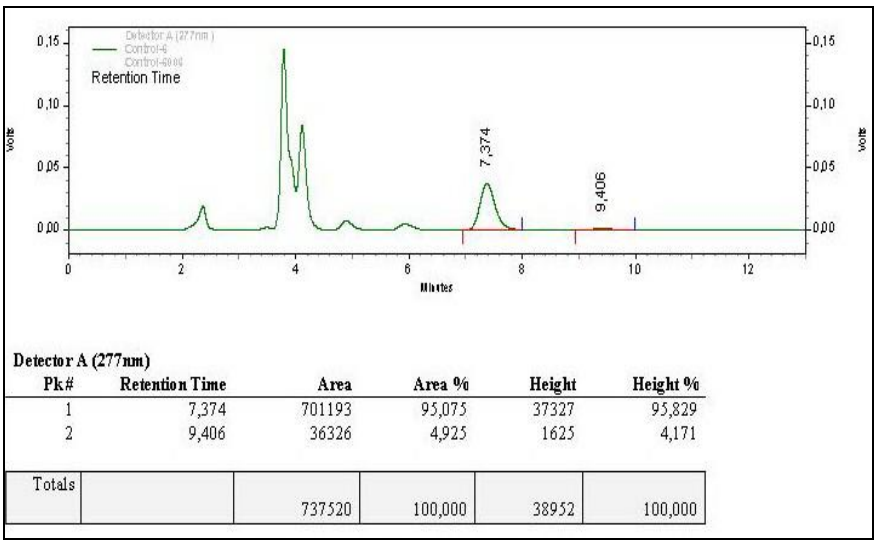
Figure D.12: C-02-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,318	701677	94,157	32367	94,742
2	9,421	43544	5,843	1796	5,258
Totals		745221	100,000	34164	100,000

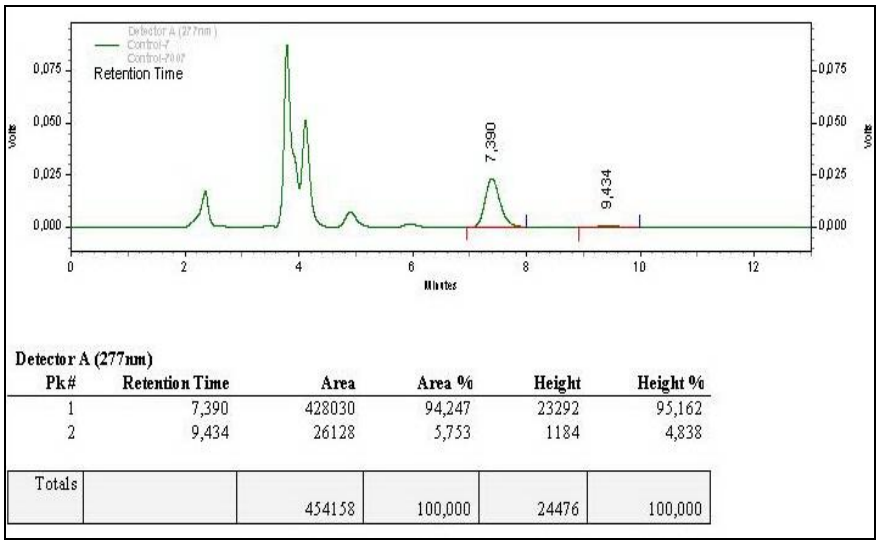
Figure D.13: C-03-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,374	701193	95,075	37327	95,829
2	9,406	36326	4,925	1625	4,171
Totals		737520	100,000	38952	100,000

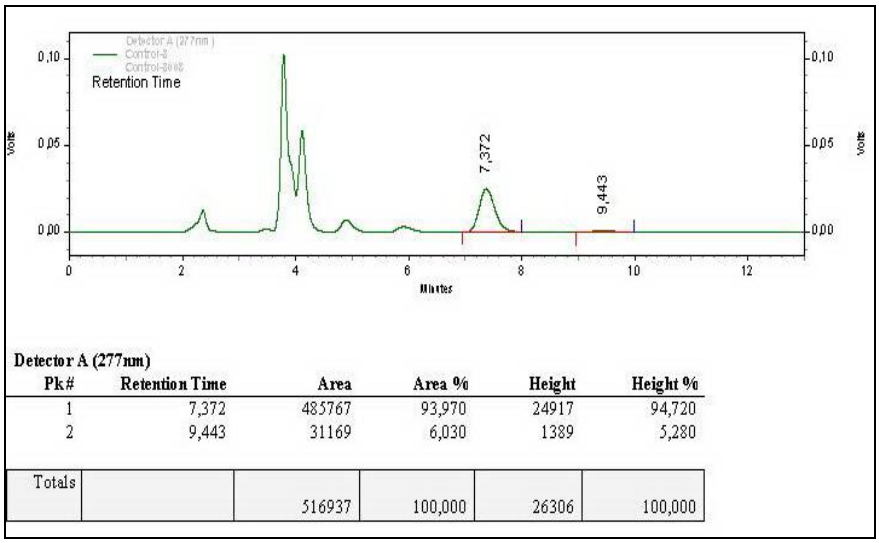
Figure D.14: C-03-2 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,390	428030	94,247	23292	95,162
2	9,434	26128	5,753	1184	4,838
Totals		454158	100,000	24476	100,000

Figure D.15: C-04-1 dC compared with 5-mdC



Detector A (277nm)

Pk#	Retention Time	Area	Area %	Height	Height %
1	7,372	485767	93,970	24917	94,720
2	9,443	31169	6,030	1389	5,280
Totals		516937	100,000	26306	100,000

Figure D.16: C-04-2 dC compared with 5-mdC

APPENDIX E

Questionnaire

BEHÇET SENDROMLU İKİZLERDE EPİGENETİK DEĞİŞİKLERİN ARAŞTIRILMASI ANKET FORMU

Hasta / Hasta Yakını :
Adı- Soyadı :
Formun doldurulduğu tarih :
Yaş :
Cinsiyet :
İzleyen hekim :

A) DEMOGRAFİK VERİLER

Doğum tarihi:
Doğum yeri:
15 yaşına kadar en çok yaşadığı yer
Medeni durumu
Meslek
Çalışma durumu
Sosyo ekonomik düzey
Öğrenim düzeyi
Toplam eğitim yılı

B) ÖZ VE SOYGEÇMİŞ

Özgeçmiş (BS var/yok; başkahastalıkvar/yok)
Soygeçmiş (Ailede BS var/yok, şüpheli, diğer)
Ailede var ise kimde?
Ailede bilinen otoimmün hastalık var/yok? (kimde)

C) KLİNİK BİLGİ

a) Başlangıç tarihi
b) Başlangıç şekli
c) Semptomlar
d) İlaç /İlaçyanıtı/Süresi/

D) LABORATUVAR BULGULAR

a) Romatoid Faktör
b) HsCRP
c) HLA genotipi
d) Diğer genetic markırlar (TNF, MICA..)
e) DNA fingerprint

HASTA ve HASTA YAKINI BİLGİLENDİRME VE OLUR FORMU

Bir klinik çalışmaya katılmak için davet edilmiş bulunmaktasınız. Aşağıdaki bilgileri okuduktan sonra çalışmaya katılmak isterseniz, bu formu imzalayınız. Formu imzalamanız çalışmanın kapsamı ve riskleri hakkında bilgilendirildiğinizi ve kararınızı serbestçe verdiğinizi belirtmektedir. Bu rıza formunun bir kopyası size verilecektir. Bu formda anlamadığınız ifadeler varsa çalışmadaki doktorlara sorarak bilgi edininiz.

BEHÇET SENDROMLU İKİZLERDE EPİGENETİK DEĞİŞİKLERİN ARAŞTIRILMASI

Ülkemizde sık görülen Behçet sendromu, ortaya çıkma nedeni bilinmeyen, her çapta damarları etkileyen sistemik bir damar iltihabı hastalığıdır. Bu çalışmada İstanbul Üniversitesi Cerrahpaşa Tıp Fakültesi Romatoloji Bilim Dalı ve İstanbul Teknik Üniversitesi Moleküler Biyoloji ve Genetik bölümü ortak olarak Behçet Hastalığı olan ikizler ve bu bireylerle yaş ve cinsiyet açısından eşleştirilmiş bireylerin dahil edildiği genetik bir araştırma yürütülmektedir. Bu kapsamda bireylerin genetik materyali (DNA) üzerinde yapılan incelemelerin hastalığın ortaya çıkma, gelişme süreci üzerinde yatkınlığı arttıran etkenler hakkındaki mevcut bilgileri geliştirmesi hedeflenmektedir.

Yukarıda gönüllü kişiye araştırmaya katılmadan önce verilmesi gereken bilgileri gösteren metni okudum. Bunlar hakkında bana yazılı ve sözlü açıklamalar yapıldı. Bu koşullarla söz konusu araştırmaya kendi rızamla, hiçbir baskı ve zorlama olmaksızın katılmayı kabul ediyorum.

Tarih:

Gönüllünün Adı-Soyadı:
İmza:
Adresi:
Açıklamayı yapan araştırıcının
Adı-Soyadı:
İmza:

CURRICULUM VITAE

Candidate's full name: Bahar Shamloo

Place and date of birth: Tabriz- Iran 01/05/1986

Permanent Address: Mahmut şevket paşa caddesi, 45/6 Çeliktepe, Istanbul, Turkey

Universities and Colleges attended:

B.Sc. of Microbiology (full-time system) in Azad University (North-Tehran Branch) in Iran, since 09/2004 to 19/02/2008 with G.P.A of 16.42/20.

Masters degree of Molecular Biology and Genetics in Istanbul Technical University since 2009 with G.P.A of 3.44/4 in Turkey.

TOEFL (iBT) score is 91

GRE score is: Verbal: 460 Quantitative: 710