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"Mutation Analysis for Five Palestinian Families Affected by Isolated Congenital Methylmalonic Acidemia"

By

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In Partial Fulfillment of the Requirements for the Degree
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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

"Mutation Analysis for Five Palestinian Families Affected by Isolated Congenital Methylmalonic Acidemia"

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Mutation Analysis for Five Palestinian Families Affected by Isolated Congenital Methylmalonic Acidemia

Abstract:

- **Background:**

  Isolated Methylmalonic acidemia (MMA) is a heterogeneous autosomal-recessive metabolic disorder caused 60% by mutations in the vitamin B_{12}-dependent enzyme methylmalonyl-CoA mutase (MUT). Shortage of this apoenzyme causes MMA. It is an organic acid metabolism aberration that affects systemic metabolic homeostasis, and might cause mental retardation, or even lead to neonatal death. To date, nearly 250 different mutations have been identified in MUT gene.

- **Materials & Methods:**

  Using 250K Nsp Affymetrix SNP arrays, we finely mapped the MUT gene as candidate in the four consanguineous families. Exons and exon-intron boundaries of the MUT gene were analyzed by polymerase chain reaction and direct Sanger sequencing. Co-segregation analysis was performed to confirm the mutation's pathogenicity in all families. Haplotype analysis was done in order to determine the origin of the Missense mutation, **N219Y**. mRNA splicing analysis was done to determine the influence of the splicing mutation **IVS8+3 a > g** on the mRNA level. A screening was taken place in Al-Ubeidiya and Wad-Rahal villages.

- **Results:**

  A homozyogous missense mutation, **N219Y**, in the MUT gene was identified in three unrelated families from Al-Ubeidiya village, and **IVS8+3 a > g** was identified in two families from Wad Rahal. Haplotype analysis revealed that the spread of the **N219Y** among the three families has a founder effect. The mRNA splicing analysis confirmed that exon8 is totally skipped. We further investigated the carrier frequency of **N219Y** mutation in Al-Ubeidiya village.

- **Conclusion:**

  This is the first Palestinian study to carry mutation analysis of the gene responsible for MMA. This will provide a molecular diagnostic aid for differential diagnosis of MMA and could be applied for carrier detection and prenatal diagnosis among the Palestinian families at risk of MMA. The definitive diagnosis allows a specific treatment.

- **Keywords**:

  Isolated Methylmalonic academia, MUT gene, Heterogeneous, Haplotype analysis.

"فحص جيني لخمس عوائل فلسطينية مصابة بمرض حموضة الدم الوراثي"

مُلخّص:

- **خلفية:** حموضة الدم الوراثي هو مرض أيضي متنحي، 60% منه يحدث نتيجة حدوث طفرة في الأنزيم المسمى methylmalonyl-CoA mutase اللازم لإتمام تحطيم بعض أنواع الأحماض الأمينية والدهون وهذا الأنزيم بحاجة لفيتامين B12 كعامل مساعد. هذا المرض يسبب مشاكل في التوازن الأيضي في الجسم، ويمكن أن يسبب التخلف العقلي، أو حتى تؤدي إلى موت الأطفال حديثي الولادة .حتى الآن، قد تم تحديد ما يقرب من 250طفرات مختلفة في الجين المسؤول عن ترجمة وإنتاج هذا الإنزيم (MUT).

- **الطرق المستخدمة:** في هذه الدراسة تم الكشف عن الجين (MUT) كسبب مشترك بين الأفراد المصابين بمرض حموضة الدم الوراثي في العائلات الفلسطينية بإستخدام تقنية 250K Nsp Affymetrix SNP arrays. وبإستخدام polymerase chain reaction ثم Sanger sequencing تم البحث في الأسباب الجينية المتسببة في هذا الخلل الوراثي. ثم تم أكتشاف كيفية انتقال الطفرات المسؤولة عن المرض من جيل إلى آخر في العائلات المشاركة. وكمحاولة للكشف عن أصل إحدى الطفرات المكتشفة N219Y، تم القيام بفحص Haplotype بين العوائل الفلسطينية والعوائل الفرنسية. وقد تم فحص تأثير الطفرة الثانية IVS8+3 a > g على معالجة RNA خلال عمل mRNA splicing analysis. وتم القيام بمسح لمنطقة العبيدية وواد رحال المعروفتان بإنتشار مرض حموضة الدم بين سكانها.

- **النتائج:** تم الكشف عن وجود طفرتين معروفتين من قبل، الاولى N219Y وجدت في ثلاث عائلات فلسطينيه والتي أنتجت تغير في الأنزيم إذ تم إستبدال الحمض الأميني أسبرجين في الموقع 219 الى الحمض الأميني تايروزين. والطفرة الثانية IVS8+3 a > g و التي أدت الى حذف (exon 8) كليا في عملية معالجة RNA وهذا أثر بشكل مباشر على شكل ووظيفة الإنزيم. لقد وجد أن الطفرة الأولى N219Y قد نتجت من أصل واحد بين العائلات الفلسطينية. وكشف المسح عن ما نسبته 4.95% من حاملي الطفرة في منطقة العبيدية.

- **الأستنتاج:** تعتبر هذه الدراسة أول دراسة من نوعها تتناول الفحص الجيني لمرض حموضة الدم الوراثي في فلسطين. قدمت هذه الدراسة وسيلة فحص جينية للكشف عن حاملي المرض و عن الأجنة لدى النساء الحوامل.

- **الكلمات الدالة:** Isolated Methylmalonic acidemia, MUT gene, Heterogeneous, Haplotype analysis.

Declaration

I declare that the Master Thesis entitled " Mutation Analysis for Five Palestinian Families Affected by Isolated Congenital Methylmalonic Acidemia" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Dedication

I would dedicate this thesis first to my beloved parents who encourage me with a constant support, love and patience all the time; especially my mother soul the source of inspiration and who has been always there for me whenever I needed. I dedicate my thesis to my Raed who has been a supportive and proud husband. To my two little sons Mohammad and Tayma', I wish you would always look up to me.

Finally, I dedicate this thesis and give a special thanks to my father-in-law who has supported me throughout the entire master program and he believed in me.

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Abbreviations

MMA	Methylmalonic acidemia
MUT	Name of Gene
BCOA	Branched chain organic aciduria
MSUD	Maple syrup urine
IVA	Isovaleric aciduria
PA	Propionic aciduria
PCC	Propionyl CoA carboxylase enzyme
MCE	Methylmalonyl CoA epimerase
AdoCbl	Adenosylcobalamine
MCM	Methylmalonyl CoA mutase
MCA	Methylcitrate
GC/MS	Gas chromatography with mass spectrometry
HPLC	High performance liquid Chromatography
MS/Ms	Tandem mass spectrometry
PGD	Preimplantation Genetic Diagnosis
PCR	Polymerase chain reaction
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeats
TD	Touch down
EDTA	Ethylene diamine tetra-acetic acid
NTC	Negative Template Control
WT	Wild Type
NBS	Newborn Screening
HC	Homocystinuria
ATR	ATP-adenosyltransferase
IEM	Inborn Error of Metabolism

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CHAPTER 01: Introduction

Methylmalonic aciduria (MMA) is a metabolic disorder and is one of the most frequently encountered branched-chain organic acidurias (BCOA), which include maple syrup urine disease (MSUD), isovaleric aciduria (IVA), propionic aciduria (PA) and methylmalonic aciduria (MMA). BCOA are group of severe inborn error of metabolic disorders where a single particular enzymatic function is blocked resulting in abnormal metabolic pathway. (Fenton et al., 2001)

The impaired metabolic pathway leads to an abnormal accumulation of substrates known as organic acids which could be precursor metabolites or intermediates of different alternative pathways. This consequently increases toxicity and causes serious health problems due to widespread disruptions of metabolic homeostasis. In addition, it causes defects in energy production and utilization due to deficiency of certain substrates needed for Krebs cycle. (Baumgartner et al., 2014 and Matsuiet et al., 1983).

In particular, Methylmalonic aciduria (MMA) is a group of inherited autosomal recessive disorders of propionate catabolism. In human, propionyl CO-A is an intermediate metabolite that arises from catabolism of several propiogenic essential branched amino acids (isoleucine, valine, threonine, and methionine) and to a lesser extent from cholesterol and odd-chain length fatty acids. In addition, a gut probionobacteria is considered to be another source for propionyl CO-A. (Barshop et al., 1991, Fenton et al., 2001 and Baumgartner et al., 2014)

1.1 Pathogenesis of MMA:

As shown in figure (1.1),The major pathway of Propionyl CO-A metabolism begins with its carboxylation in mitochondrial matrix to form (D)-methylmalonyl CO-A through a reversible reaction catalyzed by biotin-dependent propionyl-CoA carboxylase enzyme (PCC). The (D)-methylmalonyl CO-A, is racemised by Methylmalonyl-CoA epimerase (MCE) to its L-enantiomer. This branched carbon chain is isomerized by adenosylcobalamine (AdoCbl)-dependent-L-methylmalonyl-CoA mutase (MCM) to the linear carbon chain of succinyl-CoA; a 4-carbon intermediate in Krebs Cycle. (Chandler and Venditti, 2005, Barshop et al., 1991 and Fenton et al., 2001)

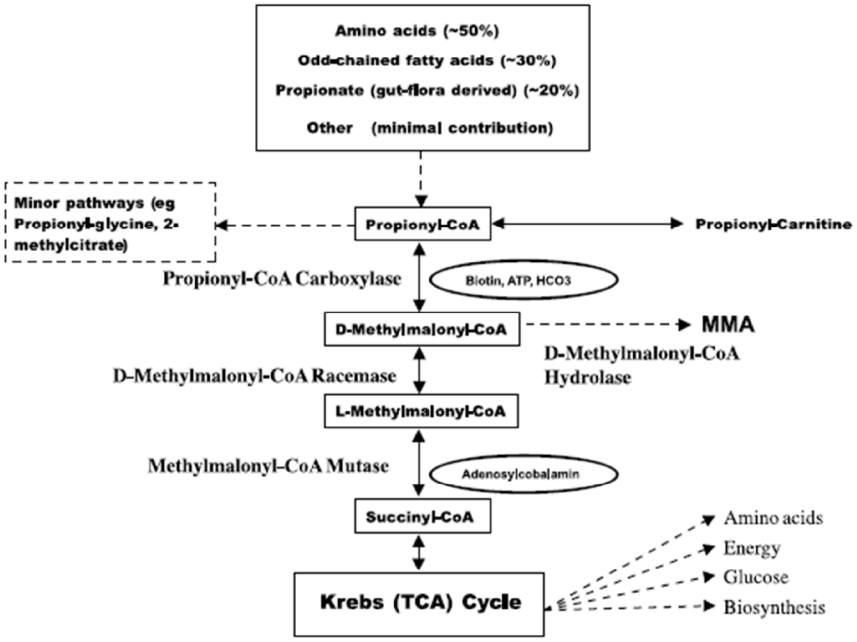


Figure 1.1: Major pathway of propionyl CoA metabolism. (Chandler & Venditti, 2005)

MMA is genetically a heterogeneous disorder caused by mutations at many different loci encoding series of proteins involved at the most crucial step in human propionate catabolism pathway. The conversion of methylmalonic CO-A to succinyl CO-A as seen in figure 1.1, is catalyzed by a mitochondrial matrix-localized enzyme; methylmalonyl-CoA mutase (MCM). This enzyme is encoded by MUT gene, and requires an essential cofactor adenosylcobalamin (AdoCbl) which is an activated form of vitamin B_{12} (cobalamin). Inherited MMA is caused either as a result of defect in activity of MCM apoenzyme and it is called MMA-MUT type or B12 unresponsive type. Or rarely as a result of defect in the transport or metabolism of AdoCbl cofactor and this is called MMA-CBL or B12 responsive type and consists of MMAA, MMAB, and MMADHC. MMA could be classified based on the type of accumulation metabolites; either being combined or isolated type, where the former is characterized by accumulation of Homocystine and methylmalonic acid and this results from defects at the early steps of B12 biosynthesis while the later one characterized by accumulation of methylmalonic acid only and this yielded as a result of defect in the later steps of B12 biosynthesis. Mut^{o} & Mut^{-} (Chandler and Venditti, 2005 and Manoli and Venditti, 2010) See Table 1.1

Table 1.1: Summary of the causative genes in isolated MMA.

Gene symbol	Location	Encoded protein	Locus name MIM number	Proportion in isolated MMA cases	OMIM number	References
Isolated MMA Cobalamin types:						
MMAA	4q31.21	MMAA metallochaperone G-protein	MMAA (607481)	~25%	251100	(Dobson, et al., 2002)
MMAB	12q24.11	cob(I)alamin adenosyltransferase (ATR)	MMAB (607568)	12%	251110	(Dobson et al., 2002)
MMADHC variant 2	2q23.2		C2orf25 (611935)	unknown	277410	(Coelho et al., 2008)
Isolated MMA Mut type:						
MUT	6p12.3	Methylmalonyl CoA mutase (MCM)	MUT (609058)	60% (78% mut^0 enzymatic subtype, 22% mut^- enzymatic subtype)	251000	(Ledley et al., 1990)

Data about genes, protein name, locus name, chromosomal location, and gene symbol are collected from databases: OMIM.

1.2 Pathophysiology of MMA:

Blocking the major metabolism pathway of propionyl CoA leads to accumulation of toxic disease-related metabolites in urine, blood and body tissues especially kidney and brain. The most important metabolites are methylmalonic acid (MMA) and propionic acid (PA) besides to its alternative products such as; 2-methylcitrate (2-MCA), 3-hydroxypropionic acid, propionylcarnitine and propionylglycine. Figure: 1.2. These metabolites are likely to be more significant in methylmalonic acidemia than in propionic acidemia where they display a serious metabolic instability producing ketoacidosis, hypoglycemia, hyperglycinemia, and hyperammonemia. These metbolites lead to multi-systemic complications, and high

mortality. (Chandler and Venditti, Forny et al., 2014, Kolker et al., 2003 and Horster and Hoffmann, 2004).

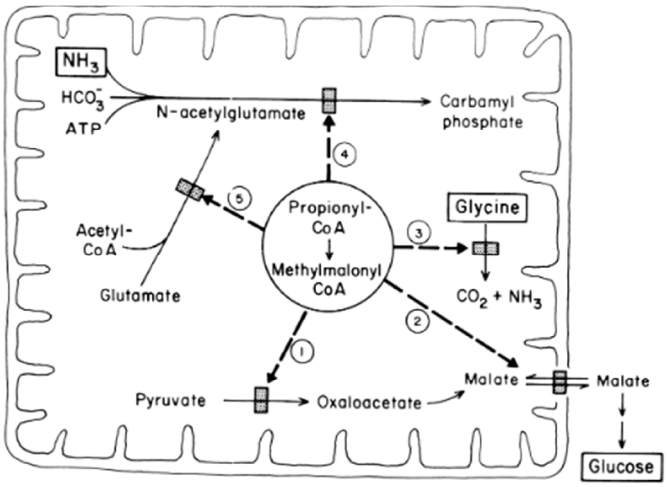


Figure 1.2: Mechanisms of metabolites in MMA disorder

Fig 1.2: The proposed mechanisms of metabolites in the pathopysiology of MMA disorder. Inhibitory effects of the enlarged intramitochondrial pools of acyl CoA esters such as propionyl CoA) or their respective free acids on selected mitochondrial functions are shown by the numbered dashed lines corresponding to the following enzymatic or shuttle-mediated reactions:1) pyruvate carboxylase; 2) the trans mitochondrial malate shuttle; 3) the glycine cleavage enzyme; 4) carbamyl phosphate synthetase I; and 5) N-acetylglutamate synthetase. (Fenton et al., 2001)

The most important metabolite is Methylmalonate, it inhibits gluconeogenesis through direct Pyruvate Carboxylase inhibition which stimulates fat mobilization subsequently increases acidosis and ketone bodies formation (ketosis) (Tuchman et al., 2007 and Oberholzer et al., 1967). In addition it is found that methylmalonate induces neuron apoptosis through activation of the related signaling pathways. (Han et al., 2015)

1.3 Clinical features:

The onset of clinical manifestations varies from severe and poor outcome in neonatal form (the first days of life) to milder later infancy form (during the first year of life) and rarely delayed to adulthood period. (Manoli and Venditti, 2010)

Although the etiology of MMA is heterogeneous, the clinical symptoms are quite similar. The major manifestations include poor feeding, vomiting, fever, muscular hypotonia, andfailure to thrive. Usually MMA patients are dominated by a risk of relapses including life-threatening episodes of an acute metabolic decompensation provoked by Protein food intake.

These relapses require an immediate intervention. Many patients develop long- term complications such as severe organ failure especially renal failure and neurological signs (extrapyramidal movement disorder, developmental delay, intellectual disability and basal ganglia stroke. (Horster and Hoffmann, 2004, Chandler and Venditti, 2005 and Manoli and Venditti, 2010)

1.4 Diagnosis and Treatment:

In most cases of MMA, early accurate diagnosis and appropriate management reduce or eliminate the associated complications, improve prognosis and may prevent death especially premature death (Horster and Hoffmann, 2004).

1.4.1 Diagnosis of MMA consists of four parts (Baumgartner et al., 2014 and Horster and Hoffmann, 2004):

- **Family History**: MMA is a congenital autosomal recessive disorder
- **Physical examination**: It includes dysmorphic or coarse facial, cataracts, hepatosplenomegaly, myopathy etc.
- **Initial screening tests**: It includes urine and blood analysis to investigate complete Blood count; neutropenia and thrombocytopenia, glucose level, blood gases and blood electrolytes, Plasma ammonia, blood arterial lactate, vitamin B_{12} concentration, total plasma homocysteine, creatinine and Urine ketones (acetoacetate)
- **Advanced screening tests**: These tests are keys to exclude any other type of IEM. It includes:
  - Urine organic acid analysis using gas chromatography with mass spectrometry (GC/MS).
  - Plasma amino acid analysis using a quantitative method such as column chromatography, high-performance liquid chromatography (HPLC), or GC/MS.
  - Plasma or serum acylcarnitine (C2-C) and propionyl carnitine (C3-C) profiles using tandem mass spectrometry (MS/MS) which may reveal high C3/C2 ratio. Those are the target compound for newborn screening.
  - Molecular genetic testing to identify the location and the mutation profile in the genes may cause MMA. See Table 1.1

- **Specific enzymatic subtype of methylmalonic acidemia to determine the exact type of MMA:**
    - B_{12} responsiveness test to differentiate between Mut and Cbl types.
    - Enzyme activity assay: This involves cultured skin fibroblasts, from affected individuals, incubated with ^{14}C-labeled propionic acid and measuring the incorporation of ^{14}C radiolabeled in the precursor intermediate.
    - Complementation analysis. This is a powerful technique for identification specific genes through their expression in fibroblast heterokaryons, generated by fusion of fibroblast from an affected individual that has been mixed with a panel of established cell lines of known status (e.g., mut^o, cblA) where they are tested for restoration of function.

1.4.2 Treatment of MMA; three goals should be entertained (Horster and Hoffmann, 2004 and Baumgartner et al., 2014):

- Prevent the acute episodes of metabolic decompensation by observing the patients carefully and provide them with an adequate balance of protein, energy and other nutrients that promotes growth. In severe cases, liver transplantation appears to protect against acute metabolic decompensation, and progressive renal and neurologic complications.
- Reduce the production of toxic metabolites through catabolism manipulation induced by low protein diet, oral antibiotic therapy (metronidazole). In B_{12}-responsive patients and some mut^- patients, an intramuscular injection of hydroxycobalamin can be a very efficient treatment (Forny et al., 2014).
- Prevent accumulation of toxic metabolites by enhancing their excretion. Metabolic pathways through L-carnitine (given orally or IV in urgent cases) will bind to organic acids and enhance their eliminations. Alkali supplements (orally or IV urgent) such as sodium citrate, sodium benzoate or potassium acetate are useful in treating acidosis and hyperammonemia, sometimes dialysis helps in rapid removal of ammonia. (Fenton et al., 2001).

1.5 Problem statements and objectives

MMA is often an early in life fatal disorder (Wikoff et al., 2007), and if not is a long life disorder. When providing a suitable treatment, the affected patients remain at risk for intermittent metabolic decompensation, growth and food problems, neurological impairments, metabolic infarction of the basal ganglia, renal disease and premature death (Venditti et al. 2009). MMA patients require special education and social care.

The age of onset and clinical presentation, response to cobalamin supplement and long-term outcome in affected patients depend on the nature and the severity of the enzymatic defect underling the disorder; in mut^o class, it seems that the patients presented signs and symptoms earlier in infancy than any other class. Mut complementation group does not responsed to cbl supplements while others cblA, cblB and cblD do, though some patients of cblB complementation group are unresponsive while cblA uniformly responses well. The mut^o group has the poorest prognosis on the contrast to cblA which has the best long-term outcomes. Most cblA, cblB, and mut^- patients live longer, whereas most mut^o patients die during the first few months of life (Baumgartner et al., 2014). In spite of that, 60% of MMA patients have mut complementation group that involve partial or total deficiency in the functional of mutase enzyme. (Manoli and Venditti, 2010)

Therefore, it is very important to determine the class of methylmalonic acidemias mutation profile which may provide a diagnostic, prognostic and therapeutic tool, Mutation study will also provide the basis for genetic counselling and management of the disease incident (Baumgartner et al., 2014).

MMA is a rare disorder. Incidence is difficult to be estimated because many MMA patients die in the first decade of life with poor diagnosis (Fenton et al., 2001). In western populations MMA incident is 1:48,000 to 1:61,000 births (Baumgartner et al., 2014). In some populations such as Asian and specially the Middle East, MMA is the most common organic acidemias and it is believed to be more prevalent because of consanguineous marriages (Sheikhmoonesi et al,. 2013). MMA is found among Palestinian families which also known to have high rate of consanguineous marriages. Due to the increasing incidence of MMA cases attend at Caritas Baby Hospital clinics, we decided to investigate the underlying genetic causes of MMA in a cohort of patients and families at risk for MMA.

In this study; we started with four Palestinian families from Bethlehem and Hebron regions, which were diagnosed as unresponsive B_{12} methylmalonic acidemia.

More specifically the study aims to:

- Identify candidate region and gene/s inherited with the MMA phenotypes using Homozygosity mapping and bioinformatic tools.
- Sequencing MUT as a causative mutant gene in MMA Palestinian families.
- Genotyping of 200 healthy unaffected Palestinian controls and the unaffected individuals in each family to detect the inheritance pattern of each mutation.
- Confirm and validate the causative mutations (missense and splicing site mutations).
- Perform mRNA expression assays for the splicing site mutation to confirm how it participates in the pathogenesis of MMA disorder.
- Trying to define the haplotype block in genetic disequilibrium with the define mutation for PGD "Preimplantation Genetic Diagnosis" utilization in high risk families. Identify the carrier frequency of the missense mutation in Al-Ubeidiya population.
- Provide a rapid and simple MMA diagnostic tool through a buccal swap.

CHAPTER 2: Literature Review

2.1 METHYLMALONIC ACIDEMIA HISTORY:

Over the past five decades, great progress has taken place in order to understand the fundamental aspects of Methylmalonic acidemia disorder and to provide an accurate rapid diagnosis and the proper treatment. (Chandler and Venditti, 2005 and Venditti et al., 2007)

MMA was firstly described as hereditary disease by Oberholzer and colleagues and Stokke and colleagues. (Venditti et al., 2007) Oberholzer et al. (1967) described two unrelated MMA patients, the first one was initially suspected to have renal tubular acidosis, suffered from dehydration, hyperglycinemia and persistent acidosis, died after episodes of acute metabolic acidosis at age 2 years in 1959. After 7 years of death he was demonstrated as MMA case by examining his stored plasma. The second case was born in 1960, she was found also to have renal tubular acidosis, problems with growth and motor skills at early stage of life, excreted a very acid urine although treating with alkaline solution. Investigation of urinary organic acids revealed large amounts of unknown acids that had been isolated and proved to be Methylmalonic Acid. (Oberholzer et al 1967)

In the same year, Stokke et al 1967 studied the third patients that did not respond to cobalamin administration but displayed clinical improvement and a significant decrease in concentration of urinary methylmalonic acid with hyperalimentation containing elementary amino acids, glucose and little of fats but unfortunately he died of septicemia infection. This indicated the importance of vitamin B12 in MMA (Stokke et al 1967)

Oberholzer and his colleagues noticed a marked deficiency in the conversion of propionyl CoA to succinyl CoA meanwhile incubation of white cell homogenates with propionyl CoA, and ^{14}C-labelled Na_2CO_3; they demonstrated a possible defect in the methylmalonyl CoA mutase activity. (Oberholzer et al 1967)

In the next year, Rosenberg and colleagues reported a patient with ketotic hyperglycinemia and severe acidosis and responded well to vitamin B_{12} and this proved a role for the vitamin in human propionyl CoA metabolism and MMA pathogenesis. In addition, they indicated that primary methylmalonic acidemia and ketotic hyperglycinemia were different disorders. (Rosenberg et al., 1968)

Two years later, Morrow and colleagues recognized two variants of MMA; one is dimethyl-benzimidazolyl- cobamide coenzyme (DBCC) unresponsive where the defect appears to be in the structure protein of the mutase while the other form is cobalamin responsive that involves defect either in the DBCC binding to the mutase apoenzyme or in the metabolism of itself. (Morrow et al., 1969)

In 1972, Ellen Song Kang and his colleagues described a patient with hyperammonemia, acidosis, and coma. They suggested a defect in methylmalonyl-CoA racemase (EC 5.1.99.1), the enzyme which catalyzes conversion of methylmalonyl-CoA (form a) to its optical isomer, methylmalonyl-CoA (form b) but later it was showed to have a lesion at mutase locus. (Kang et al., 1972)

Besides to previous studies, a series of reports were published and descried many MMA patients lead to better understanding of clinical and the biochemical foundation of MMA and to explain the metabolic perturbations induced by MMA. (Venditti et al., 2007)

By the middle of 1970s, it was clear that all of the previous afforts augmented that the site of the metabolic block of MMA is in the conversion of methylmalonyl CoA to succinyl CoA; a step in which the cobamide coenzyme form of vitamin B12 (DBCC) is required, and methylmalonylCoA is an intermediate. Based on that, two forms of MMA have been distinguished; mut-type MMA and cobalamin type.

In 1975, Gravel and his colleagues by applying complementation analysis of fibroblast heterokaryons, they assigned patient cell lines to four distinct biochemically and genetically complementation groups; the mut-type MMA, and the cobalamin type MMA that comprises CblA, CblB and CblC, this indicates that those phenotypes arise from mutations of distinct gene products (Gravel et al., 1975). In 1978, Willard and his colleagues presented evidences for a new class of human cobalamin mutant based on complementation study for patient cells that showed deficiency in cobalamin content and N_5-methyltetrahydrofolate-homocysteine S-methyltransferase and methylmalonyl CoA mutase and they designated it as cbl D (Willard et al., 1978).

Two years later, Willard and Rosenberg examined the biochemical features of cell lines with a defect in the mutase apoenzyme and under saturating concentrations of AdoCbl then applying MUT enzyme activity assay and testing the ability of propionate incorporation,

demonstrated that mut class are categorized clinically and biochemically into two subtypes and arise from allelic mutations at a single locus ; one without detectable residual mutase activity, and is designated mut^{o} mutants and the other displayed some ability to incorporate metabolisim of [1–^{14}C] propionyl CoA into trichloroacetic acid perceptible material and increased by cobalamin administration which is designated mut^{-}. (Willard and Rosenberg, 1980)

In 1986, Watkins and Rosenblatt identified another new class CblF by applying the same previous used complementation analysis of fibroblast heterokaryon and testing [1–^{14}C] propionate incorporation. This has been found in fibroblasts from a patient present an elevation in methylamalonic acid and homocysteine.

2.2 Types of MMA disorders:

By the end of 1980s, several dependent and related researches provided evidence that methylamalonic acidemia disorders can result from defect in one of the following as shown in figure 2.2 (Fenton et al., 2001):

- Methylmalonyl coenzyme A mutase (MCM; EC 5.4.99.2) apoenzyme activity and this type is designated as Mut-type, in which it can be distinguished into either mut^{o} where there is no detectable enzyme activity (completely deficiency in the functional mutase enzyme) or mut^{-} which has a partial deficiency in the functional mutase apoenzyme, this type is also known as B_{12} -unresponsive form and produced isolated MMA which characterized lonely by methylmalonic acid elevation.

- Metabolism of vitamin B12 which is called Cbl-type or B12- responsive type, this type initially was distinguished into four complementation groups, but later and over the years, it contains at least eight types cblA–cblH that block the production or utilization of adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl) or both cofactors where they are associated respectively with isolated MMA, isolated homocystenuria (HC) or combined MMA and HC based on the type of accumulated metabolites (methylmalonic acid, homocystine or both respectively). (Froese and Gravel, 2010)

2.2.1 COBALAMIN CBL-MMA TYPES:

- **COBALAMIN STRUCTURE:** In mammals, Cobalamin is known as vitamin B_{12} which is an essential vitamin used for two crucial enzymes; N_5-methyltetrahydrofolate-homocysteine S-methyltransferase that converts homocysteine to methionine, and uses vitamin B_{12} as methylcobalamin, and methylmalonyl CoA mutase which uses it as adenosylcobalamin.

  In 1926, Cobalamin was discovered by Minot and Murphy through using oral liver extract as a treatment for pernicious anemia, and in 1948 Smith and Ricks isolated the responsible Crystalline compound for that effect from the liver, later in 1956, Hodgkin elucidated the three dimensional structure of Cobalamin by using X-ray crystallography then 5 years later, the organometallic nature of cobalamin is determined by Lenhert and Hodgkin. (Fenton et al., 2001, Froese and Gravel, 2010 and Takahashi-Iniguez et al., 2012)

  As shown in figure 2.1. Cobalamin is a complex vitamin and it is a large organometallic molecule, 1300–1500 Da in size, contains a naturally occurring metal–carbon bonds. It is composed of central cobalt (CO) atom surrounded by four pyrrole rings that donated their nitrogen ligands to form an equatorial corrin ring. Additionally, Co atom binds to two extra ligands; one extending below the corrin ring called the α-axial ligand, commonly corrinoids have 5,6-dimethylbenzimidazole (DMB) phosphoribosyl moiety as a nucleotide base, and are known as cobamides, the nitrogen of 5,6-dimethylbenzimidazole (DMB) phosphoribosyl moiety through one of its propionamide side chains attacks back to the corrin ring and thus it coordinates the cobalt in the free cobalamin (Takahashi-Iniguez et al., 2012). The other ligand is the upper or β-axial ligand which varies based on the modification state or the substitutions binding to cobalamin (R- group). In mammalian tissue only four functional molecules have been isolated (Fenton et al., 2001); glutathionylcobalamin (GSCbl), a cyano group (CNCbl), methyl (MeCbl) Methylcobalamin or 5′-deoxyadenosyl (AdoCbl) groups and hydroxyl group (OHCbl). (Takahashi-Iniguez et al., 2012)

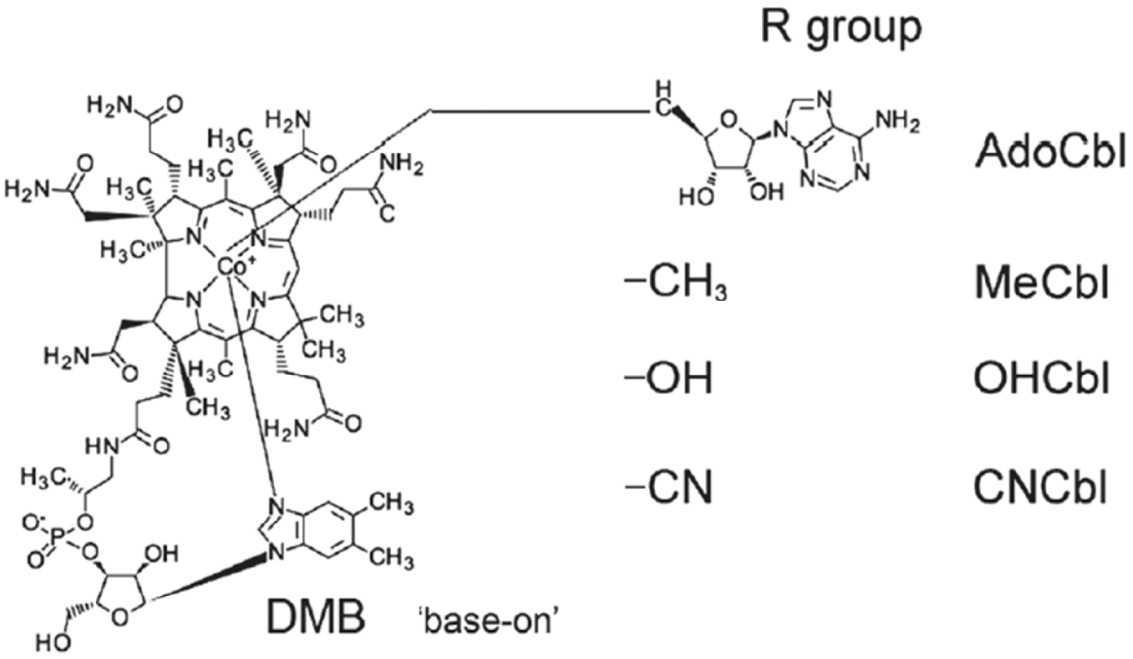


Figure 2.1: Chemical formula of Cobalamin and its derivatives

Fig 2.1: Chemical formula of Cobalamin and its derivatives shown in right side; R group represent the β or upper axial ligand and DMB (dimethylbenzimidazole phosphoribosyl) represent the α or lower axial ligand. (Froese and Gravel, 2010)

- **METABOLISM OF COBALAMIN TO ADOCBL:** Cobalamin vitamin is synthesized in small quantities by a few microorganisms found in soil, water, or intestine of animals. Thus it is essential to be acquired through dietary uptake in human. Viatmin B_{12} is found mostly in milk, eggs, fish and meat. (Fenton et al., 2001)

  In humans, the absorption, transport and cellular uptake of cobalamin is complex in which series of processing steps are required to convert cobalamin vitamin to its coenzyme forms (Froese and Gravel, 2010)

  In human, cobalamin vitamin has a unique and specialized mechanism of absorption depends on the presence of an independent transport system (intrinsic factor). The high concentration of hydrochloric acid present in the stomach help to release Food-bound cobalamin (R-Cbl) which subsequently bound to R-binders (or haptocorrins HC) (previously named transcobalamin I); glycoproteins secreted by the stomach and salivary glands to protect cobalamin vitamin from denaturation in the stomach. Then by pancreatic protease in the small intestine, cobalamin is release from HC and bound to intrinsic factor to prevent any intracellular degraded, The IF–Cbl complex is absorbed by specific ileal receptors through phagocytotosis. (Froese and Gravel, 2010 and Gherasim et al., 2013)

Cbl in ileal enterocyte is released from IF and binding to transcobalamin (TC) (previously named transcobalamin II) which facilitate the uptake of cobalamin from bloodstream by cells as a complex of Cbl–TC bound to the TC receptor (TCblR) of the lysosome. Once Cbl is released as OH-Cbl form by assistants of cblF protein (LMBRD1 locus product) to the cyrosol from TC, as shown in fig 2.2, cblC protein (MMACHC locus product) accepts it, to initiate the immediate processing reduction of trivalent cobalt (CoIII) to divalent (CoII) by efficient removal of the upper axial ligand attached to the central cobalt, CblD protein (MMADHC locus product), which is thought to interact with MMACHC as part of a chaperone role, presents cobalamin to the cytosolic proteins (by variant 1) or to the mitochondrial matrix (variants 2) pathways. To synthesis AdoCbl cofactor; cblB protein accept the cbl where the next reduction step to monovalent (CoI) and adenosylation by adding Ado-group takes place, then present the cofactor to MCM enzyme which is already has been found as a complex with cblA protein that works as a gatekeeper for both the enzyme and the reaction. While in MeCbl synthesis; which reduced (CoII) to (CoI), add methyl-group and facilitate the methyltransferase reaction. (Froese and Gravel, 2010 and Gherasim et al., 2013)

Somatic cell complementation analysis is used to classify inborn errors of cobalamin metabolism; up to date eight complementation groups have been distinguished (cblA-cblH), the naming was done based on the order in which they were discovered. Of these, the cblF(OMIM 277380), cblC (OMIM 277400) and cblD1 (OMIM 277410) affect the earlier steps in cobalamin metabolism (outside the mitochondrial matrix) and those steps are common for both AdoCbl and MeCbl coenzymes synthesis, thus they cause elevation in methylmalonic acid in addition to Homocystine level and this type called methylmalonic aciduria and homocystinuria (combined MMA/HC). The cblE (OMIM 236270) and cblG (OMIM 250940) affect the synthesis of MeCbl alone where Homocystine is accumulated only; this is called Homocystineuria (HC). While the cblA(OMIM 251100), cblB (OMIM 251110) and cblD var2 (cblH) (OMIM 277410) affect the late steps of cobalamin metabolism in mitochondrial matrix which blocked uniquely AdoCbl synthesis and this is called Isolated Methylmalonic acidemia and characterized by accumulation of methylmalonic acid lonely . (Gherasim et al., 2013, Quadros, 2010 and Garcia et al., 2013)

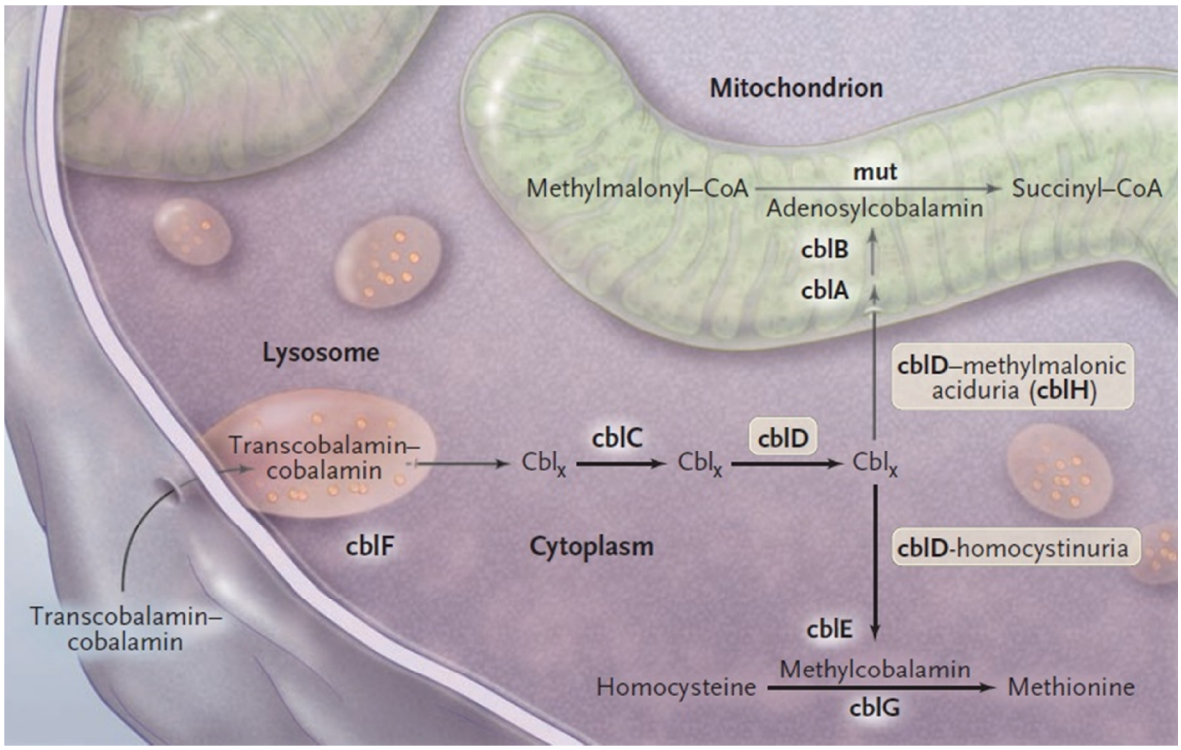


Figure 2.2: Intracellular processing of vitamin B12

Fig 2.2: Intracellular processing of vitamin B12 showing sites of defects in complementation groups; the exact form of cobalamin at this stage is unclear (as indicated by "Cblx"). (Coelho et al., 2008)

2.2.2 ISOLATED MMA DISORDERS:

Isolated MMA consists of four complementation groups; cblA type OMIM 251100, cblB type OMIM 251110 cblD-variant 2 (cblH type) OMIM 277410 and mut type (Mut^- and Mut^0) (OMIM 251000) **Table1.1**, 60% of isolated MMA cases has mutations in mut gene. (Moreno-Garcia et al., 2012 and Manoli and Venditti, 2010)

2.2.2.1 ISOLATED MMA CBL- TYPE DISORDERS:

Consists of cblA type OMIM 251100, cblB type OMIM 251110 and cblD-variant 2 (cblH type) OMIM 277410

- **MMAA GENE; CBLA TYPE:** MMA CblA type results from mutation in MMAA gene with its protein product described as a G-protein metallochaperone involved in the adenosylcobalamin-dependent methylmalonyl CoA mutase (MCM) assembly. (Jost et al., 2015) Previously it was thought that CblA patients suffer from a defect in a mitochondrial, NADPH-dependent aquacobalamin reductase of cob (III) to cob(II). (Rosenblatt and Cooper, 1990) Then it was speculated that it could play a function in mitochondrial pores. (Dobson et al., 2002)

In 2001, two genes were described clustered in proximity to MCM in Methylbacterium extorquens AM1, one of them named MeaB and it was investigated to have accessory role to MCM function. (Bobik and Rasche, 2001 and Korotkova, et al., 2002)

By examination of the bacterial gene MeaB and based on bioinformatics analysis and orthology relationships, the human gene responsible for CblA was identified. It is located on chromosome 4q31.1-q31.2. It contains 7 exons and spans about 17.1 kb and encodes a predicted protein of 418 amino acids. Amino acid sequence analysis demonstrates the presence of P-loop (Walker A), Mg^{2+}-binding aspartate residue, Walker B motif, and GTP-binding motif. It was suggested that MeaB play roles in MCM assembly and protection from oxidative inactivation. (Dobson et al., 2002, Korotkova and Lidstrom, 2004 and Bobik and Rasche, 2001)

Banerjee and colleagues worked extensively on MeaB, the MMAA orthologue from Methylobacterium extorquens which shows 46% identity and 67% similarity. They solved the MeaB structure; in both nucleotide- free form and in the presence of GDP/GTP. They showed that MeaB is a homodimer, each subunit consists of three crucial regions. The first region is the GTPase active site located in central G domain which includes 7 parallel stranded B-sheet (P-loop GTPase). The second region is the C and N termini which consist of alpha-helical extensions; the former is essential for homodimer formation while the latter is important in interaction with MCM. The third region is the switch loop regions (I and II) which are found buried at the dimer interface and they undergo a large conformation followed GTP hydrolysis needed to communicate signals subsequently. (Hubbard et al., 2007 and Forese et al., 2010)

Moreover, they demonstrated that MeaB association with MCM influence the docking of 5'-adenosylcobalamin into the active site of the mutase enzyme through GTP binding and hydrolysis where the state of MeaB regulates the affinity of mutase to bind its cofactor and to enable cofactor delivary from adenosyltransferase as a gate-keeper. In addition it works as an editor through discrimination of AdoCbl from any other cofactor derivative and subsequent protects MCM from oxidative inactivation. (Padovani et al., 2006, Padovani and Banerjee, 2006 and Lofgren et al., 2013)

Several trials had taken in order to determine the mechanism by which such metallochaperones performed functions. Recently, a trial to visualize molecular architecture for a G-protein metallochaperone together with mutase enzyme was described. (Jost et al., 2015) Jost and his colleagues provided the first crystal visualization of the juxtaposition of a G-protein: mutase complex with bound AdoCbl and GDP•Mg^{2+}

and they predicted the interaction takes place in human G-protein: MCM complexes. See figure 2.3.; (Valentin et al., 2015)

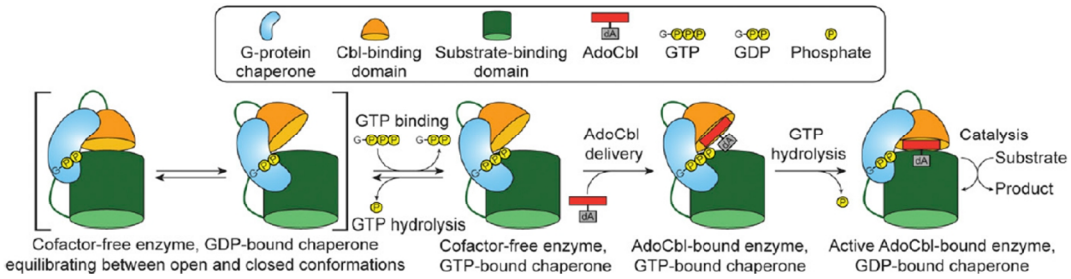


Figure 2.3 G-protein:enzyme complex. (Valentin et al., 2015)

G-protein:enzyme complex reveals that association of G-protein in GDP state stimulates Cbl domain motion equilibrating open and closed conformation of MCM enzyme. In the open state, cbl domain is slightly dissociated than substrate binding domain and His- loop is exposed to help in the cofactor docking and being ready to replace the Cbl dimethylbenzimidazole tail. The binding of GTP promotes the direct transfer of Adocbl from adenosyltransferase to its binding pocket. Now the GTP hydrolysis ejects the adenosyltransferase away and collapse the open state to obtain a closed catalytically active state to secure MCM from inactivation. It is clear that G- protein has a key role in loading AdoCbl and protects MCM from inactivation by assisting in conformational changes and in the sequestering of cofactor inside an enzyme active site Moreover, there is evidence that GTP binding stabilizes open state but it needs more elucidations in the future. (Valentin et al., 2015 and Gherasim et al., 2013)

Most of the Mutations in MMAA are corresponding to nonsense of framshifts, more than 30 mutations have been identified in cblA patients as shown in figure 2.4, CblA patients response uniformly to vitamin B12 supplements. (Froese and Gravel, 2010) Some mutations were mapped and characterized biochemically and it was demonstrated that some mutations may affects directly the robustness of the interaction with MUT or completely abolish complex formation such as homozygous G188R mutation which locates at Switch I motif in the G-domain, at the dimer interface (Froese et al., 2010). Other mutations have deleterious effects on its structure and stability. (Froese and Gravel, 2010)

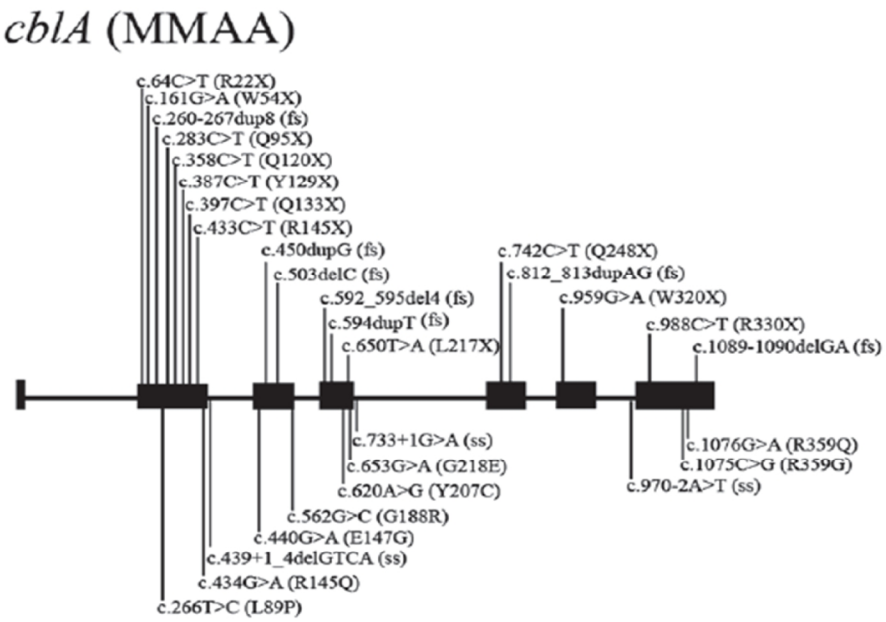


Figure 2.4: Known mutations in the CblA complementation group. (Froese and Gravel, 2010)

- **MMAB GENE; CBLB TYPE:** The cblB complementation group is caused as a result of a defect in a cblB locus or MMAB gene which encodes ATP-adenosyltransferase (ATR) enzyme. ATR is an enzyme that catalyzes the final step in synthesis (AdoCbl) from ATP and Cobalamin II in which ATR plays a bifunctionalroles; it is a tailor and a deliverer of AdoCbl; it means it catalyzes the reductive adenosylation of cobalamin and transfers adenosylcobalamin to MMAA-MCM complex in the mitochondria. (Padovani et al., 2008 and Gherasim et al., 2013)

  The gene of cblB was identified in the survey of MCM-containing gene clusters in microbial genomes and was named MMAB. MMAB gene is localized to chromosome12q24 consists of 9 exons. Human ATR includes an N-terminal 32 amino acid mitochondrial leader sequence. (Dobson et al., 2002 and Leal et al., 2003)

  Salmonella enteric, as a model, express three different structurally groups of ATR enzymes; CobA-type, EutT-type and PduO-type. The latter one (PduO-type enzyme) is found in human cells and it is the most widely distributed of these adenosyltransferases with homologues identified in species of archaeotes and prokaryotes, and in many eukaryotes ranging from yeast to humans. (Mera and Escalante-Semerena, 2010)

  Mutations in MMAB gene causes deficiency in the functional MCM and it corresponds to CblB complementation group of MMA. To date in the MMAB gene, 24 mutations have been identified as shown in figure 2.7. Mutations in CblB patients included mostly

missense mutations, and lesser duplications, deletion and splice-site mutations. Most of the mutations cluster in exon 7, which encodes the active sit. Many mutations have been mimicked in bacterial ATR in order to be studied structurally and functionally; such as nonsense Q234X mutation which suggests a weaker binding of AdoCbl. Mutations such as p.R186W, p.R190H, and p.E193K are presumed to affect ATR instability while p.R191W reduced affinity for the substrate and cofactor. (Lofgren and Banerjee, 2011, Schubert, H.L.; Hill, 2006 and Jorge-Finnigan et al., 2010)

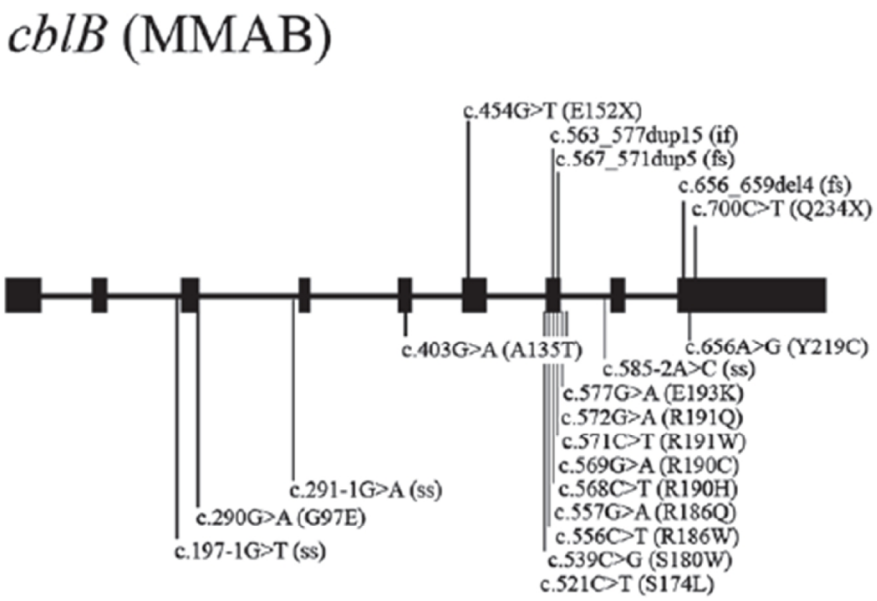


Figure 2.5 : Known mutations in the CblB complementation group (Froese and Gravel, 2010)

- **MMAD GENE; CBLD VARIANT2 OR CBLH:** This complementation group was described for the first time in 1978 (Willard et al., 1978), till 2004 it was thought that mutations in this type are associated with combined methylmalonic aciduria and homocystinuria (combined MMA/HC) but later Suormala and his colleagues demonstrated that some of cblD complementation group showed unexpected deficiency in AdoCbl synthesis without affecting MeCbl and this provides evidence for heterogeneity within the cblD mutant class thus they classified it under two variants; cblD varient1 that had MS and MCM deficiency and variant 2 had only MCM deficiency (Suormala et al., 2004). Four years later the same group cloned the cblD gene and named it as MMADHC (formerly C20rf25). It is localized to human chromosome 2q23.2 and the putative protein has 296. (Coelho et al., 2008)

Several studies have been conducted to investigate MMADHC protein. This protein contains various domains required for mitochondrial targeting and AdoCbl and MeCbl synthesis. Accordingly, the location and the nature of the mutations determine the type of the biochemical phenotype. The N-terminal domain contains a mitochondrial targeting sequence which is required for AdoCbl synthesis, while the C-terminal domain is required for MeCbl synthesis. In addition they indicated that CblD might play a role in the delivery of cobalamin from CblC to its. (Jusufi et al., 2014, Stucki et al., 2012 and Gherasim et al., 2013)

It is thought that the cblH which was identified as a novel complementation group in 2000 is considered as one type of cblD var2; therefore is officially called those days cblH. (Coelho et al., 2008)

The cblD disorder is rare with fewer cases; up to 2014, 18 patients were diagnosed as cblD complementation group; 5 with the classic form, combined type, 6 with isolated cblD-HC and 7 with cblD-MMA. Figure 2.9 shows the reported mutations, up to date, in cblD complementation group. Most of the mutations are Missense or truncated mutations (nonsense and frame shift). (Froese and Gravel, 2010)

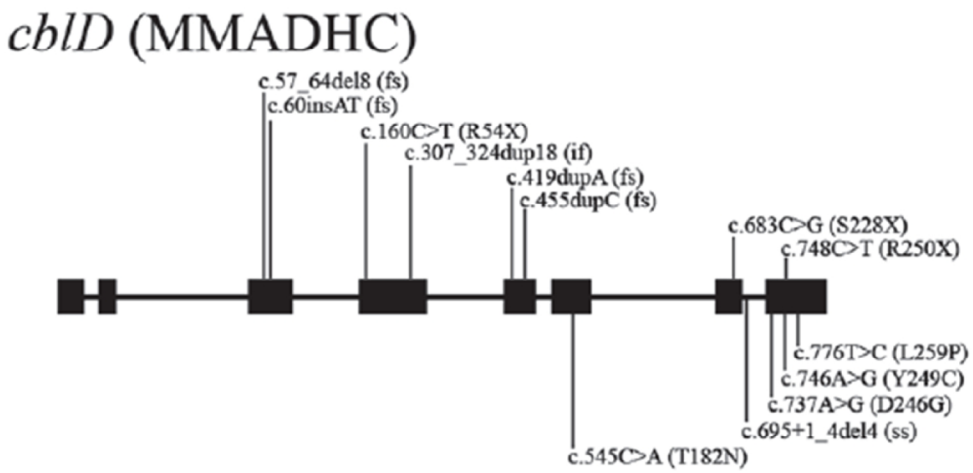


Figure 2.6: Mutations in the MMADHC gene underlying the defect of cblD complementation Group. (Froese and Gravel, 2010

The cblD complementation group represenst the most controversial and complex class of inherited cobalamin defects; several related cblD mechanistic basis is under investigation and requires more elaborations. (Gherasim et al., 2013)

2.2.2.2 MUT- MMA TYPE:

- **MUTASE ENZYME (MCM):** MCM is a mitochondrial matrix enzyme (EC 5.4.99.2) catalyzes the reversible isomerization of L-methylmalonyl-CoA to succinyl-CoA. It is one member of two known Cbl -dependent isomerases subfamily uses Cbl as 5'-deoxyadenosyl Cbl (AdoCbl) and it is the only known enzyme of carbon skeleton rearrangement class that is found in organisms ranging from humans to bacteria. It is well studied at the enzymatic and structural levels. (Takahashi-Iniguez et al., (2012)

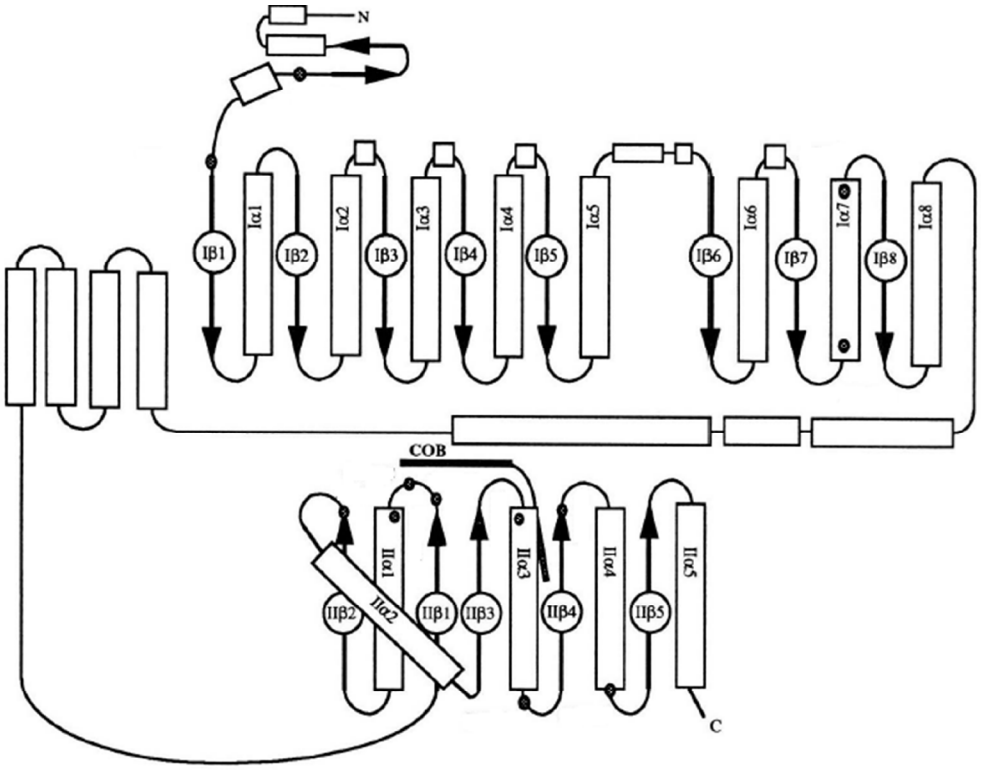
  Series of studies have been done from 1955 till 1963, by Ochoa and co-workers to investigate the metabolism of propionic acid in sheep kidney and liver and showed a strong evidences for isomeration by an enzyme that converts methylmalonate to succinate and utilizes 2 moles of tightly bound of cobalamin as a cofactor / 1 mole of enzyme. Cannata et al in 1965 succeeded in purifying methylmalonyl-CoA mutase and determined its properties; molecular weight 78 kDa, optimal PH and KM for AdoCbl of 4.7 nM.These findings had not been noticeable till 1967 when Oberholzer et.al and Stokke et al as said before described the congenital MMA patients who died as a result of defect in MCM enzyme. (Fenton et al., 2001, Mazumder et al., 1963 and Cannata et al., 1965)

  MCM was purified from many sources; from human placenta (Kolhouse et al; 1980) and from human liver Fenton in 1982 who indicated that the human holoenzyme MCM is a homodimer enzyme with identical subunits of 72,000 -77,000 Da MCM is a mitochondrial protein, encoded by a nuclear gene, synthesized in cytoplasm and exported to mitochondrial matrix losing 3000-4000 Da quickly converted into its mature form. (Takahashi-Iniguez et al., 2012) MCM cDNA was sequenced and cloned from human liver. Ledley and his colleagues recognized the gene product of the MUT locus which assigned to chromosome 6pl2-21.2 (Ledley et al., 1988) and showed that the open reading frame of MCM encodes 750 amino acids, 32 are mitochondria leader sequence, and the predicted molecular weight is 83,009 Da. MUT locus is large and consists of 13 exons expaning 35kb of DNA region, Exon 1 conatins the promoter region. (Ledley et al., 1990 and Gruber and Kratky, 2001)

  Mancia and his colleagues investigated several forms of P. shermanii MCM psMUT crystallographic 3D structure, (Mancia et al., 1996) and a homology model of human MCM was constructed using standard modeling software. (Thoma and Leadlay, 1996)

Each subunit of the human MCM contains two main functional domains: 1) a cobalamin binding domain (residues 578-750) showing a fold of (α/β)5 barrel with a large b -sheet containing five parallel strand forming a groove at the C-terminal end. 2) A coenzyme-A ester (substrate) binding pocket that threaded through the center of (TIM) barrel fold (α/β)8 (residues 86–423) at the N-terminal end which is preceded by an extended segment that may involve in dimerization (residues 33–85). The two domains are connected by a long spacer (residues 424–577) which enclosed the (α/β)8 barrel domain. The active site is formed from the interface of two domains which forms a crevice to accommodate the AdoCbl. (Thoma and Leadlay, 1996) Figure: 2.10 elucidate the functional domains and their corresponding exons.

A)



B)

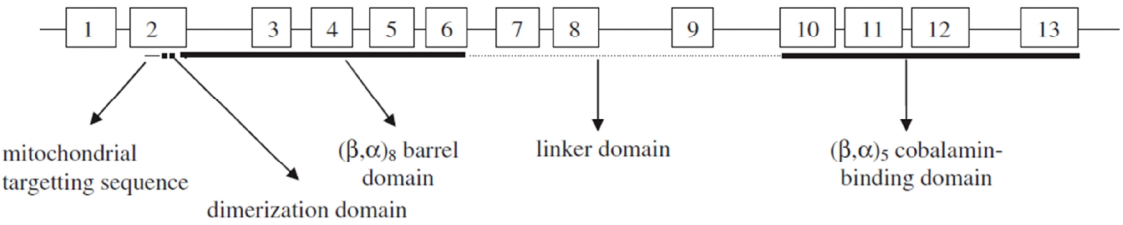


Figure 2.7: A) Topology diagram of human MCM enzyme. See the text. (Thoma and Leadlay, 1996) B) A graphic representation of MUT gene . The boxes represent exons and lines represent introns. (Acquaviva et al., 2005)

Mancia et al compared substrate-free and substrate-bound of P. shermanii MCM structures, they indicated that MCM is found in two conformations; open (substrate free) and closed (substrate bound). In the open conformation, a rigid body consist of four pairs of (α/β)8 move toward both the remaining four pairs and cbl-binding domain in order to allow TIM barrel open and permit the pantotheine chain to bind and access the active site. While in the closed configuration, the active site becomes completely buried and the TIM barrel domain is bonded and closed up with the substrate (Mancia et al., 1999, Mancia and Evan, 1997 and Mancia et al., 1996) this model contributed in understanding the MCM enzyme-substrate interaction; (Mancia and Evan, 1997)

- **MUTATIONS IN MUT GENE:**

Mutations in Mut gene cause methylmalonyl CoA mutase deficiency partially or completely and generate recessive autosomic methylmalonic academia disorder. (Gravel et al., 1975) Several known pathogenic mutations have been mapped to interpret the MMA mut type phenotypic characteristics and to interrogate mutations at the protein level. (Thoma and Leadlay, 1996, Acquaviva et al., 2005 and Forny et al., 2014)

Two hundred-fifty mutations have been identified spread throughout all MUT's introns and exons as seen in Figure: 2.12. Most of the mutations are missense changes (131 of 243, 54%), as shown Figure: 2.13. Most of mutations are private and rare and thus considered representative for a certain ethnic populations such as; African Americans (p.G717V and c.2150G>T), Caucasians (p.N219Y, c.655A>T; p.R369H, c.1106G>A; p.R694W, c.2080C>T) (Acquaviva et al., 2005) and Turkish Asians (p.P615T, c.1843C>A). Also most patients are compound heterozygotes. (Forny et al., 2014)

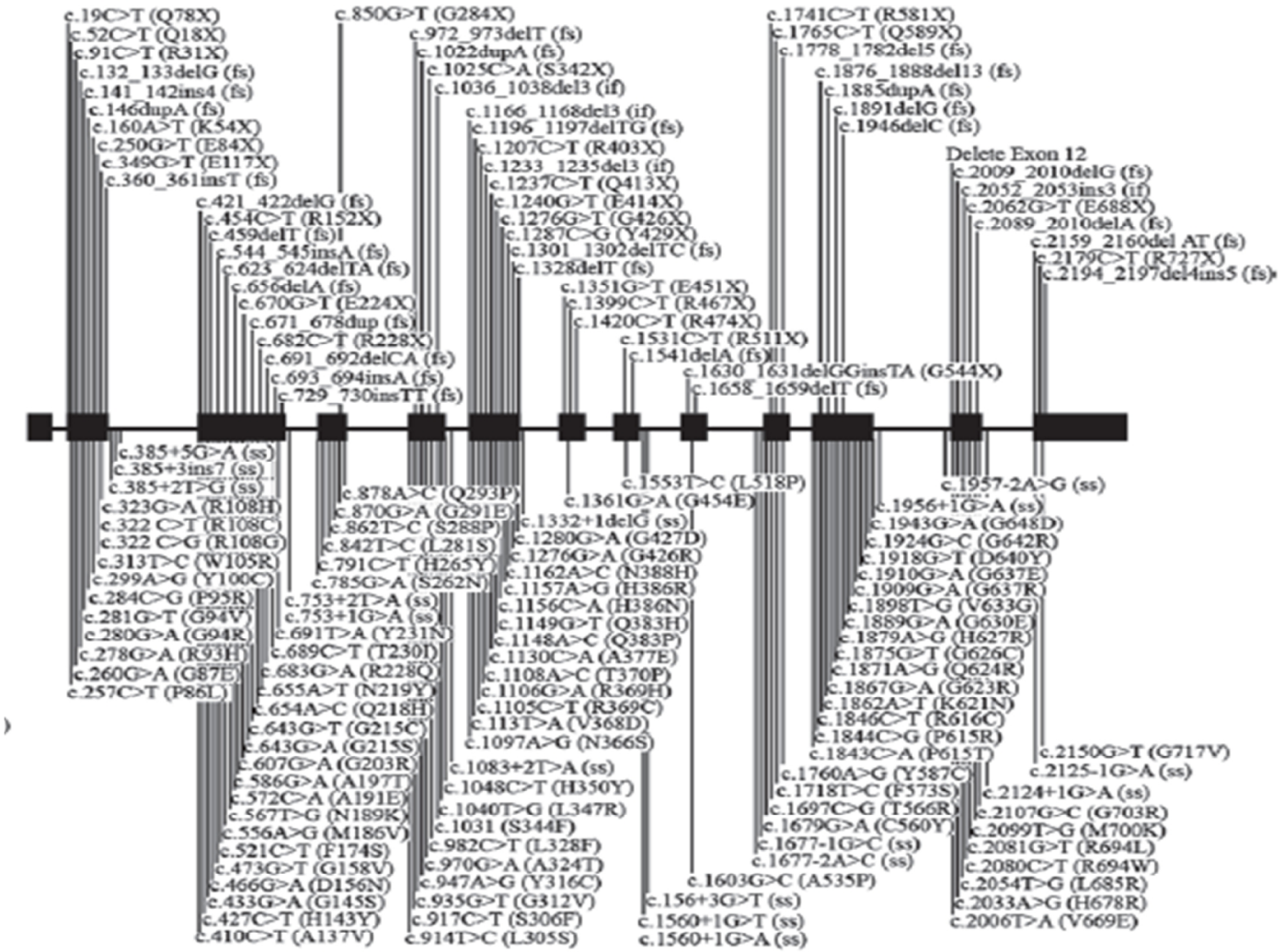


Figure 2.8: **Mutations in the MUT gene (Froese and Gravel, 2010)**

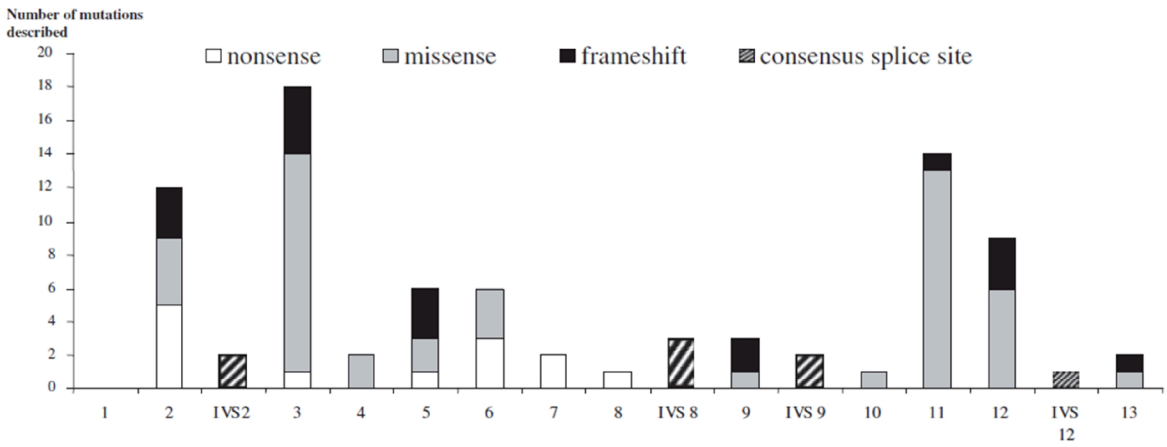


Figure 2.9: Mutations repartition within the MUT locus

Figure: 2.9 Mutations repartition within the MUT locus. Most of the Missense mutations are concentrated in exons 2 and 3 (of (b/a)8 barrel domain) which code for the first four beta sheets and their related helix plus the fifth helix and in exon 11 of the (b/a)5 cofactor-binding domain. (Acquaviva et al., 2005)

Missense Mutations participate in MMA pathogenesis in several ways; some showed decreased in enzyme activity due to reduced substrate or cofactor affinity, some may affect Protein destablization due to affecting its insolubility, another may reduce protein level due to misfolding or affect homo- dimerization. (Thoma and Leadlay, 1996 and Forny et al., 2014)

Most of the mut^{-} mutations involve residues located in the (b/a)5 cofactor-binding domain, and some may found in the (b/a)8 barrel domain especially at the surface of the cofactor crevice which may change its conformation or affect its contact with the cofactor-binding domain. Whereas most of residues which are involved in the mut^{o} mutations belong to the (b/a)8 barrel domain and if located in the cofactor-binding domain, it will affect either the enzyme folding or the mechanism of the reaction (Acquaviva et al., 2005 and Thoma and Leadlay, 1996)

Chapter Three: Materials and Methods

3.1 Family Asserting:

We have investigated four unrelated patients descending from consanguineous marriages of MERC-(T, I, AJ & BB) families. Caritas Baby Hospital diagnosed them as B12-unresponsive MMA. Clinical diagnosis was done for each patient after obtaining the informed consents in accordance with the guidelines of Bethlehem University IRB Committee. Figure (4.1) shows the pedigrees for those families; they span through three generations.

3.2 SNP Microarrays Homozygosity Mapping:

In order to define the shared genomic regions of homozygosity blocks in MMA patients which may harbor the disease/ phenotype causing gene, a whole genome scan using 250K Nsp Affymetrix SNP arrays was carried out. DNA samples from patients of families I and T, and a control sample were hybridized to GeneChip_Human Mapping 250K Nsp arrays (Affymetrix, Cat# 900768) and the experiment was performed according to protocols provided by the manufacturer.

The SNP results of the families were analyzed using the Genotyping Analysis Software v 4.0 (Affymetrix).

3.3 Bioinformatics Analysis

The regions of shared homozygosity were screened, and on chromosome 6; MUT gene was found to be the candidate gene for MMA.

3.4 Molecular Analysis for MUT gene:

3.4.1 ISOLATION OF DNA BY SALTING-OUT TECHNIQUE

Five to ten ml of blood for each patient and his/ her parents was collected in a sterile EDTA vacutainer tube. Red Blood Cell lysis buffer was added 4 times the volume of the blood and mixed gently. Tubes were kept on ice for 30 minutes till the blood becomes transparent and then centrifuged at 2000 rounds per minute (rpm) for 10 minutes at 4°C. The supernatant was carefully removed and the pellet was re-suspended in 3 ml Red Blood Cell lysis buffer and re-centrifuged at same conditions. After breaking the pellet, it was suspended in a mix of 3ml of 1X Lysis buffer ,100µl of 20% SDS (Amresoc-Cat#:M112) and 100µl of 5mg/ml Proteinase K (Amresco-Cat#E115) , followed by incubation at 37°C overnight. After incubation, 1ml of 6M NaCl was added to the lysate and vigorously vortexed until the solution get a foamy appearance. Then it was centrifuged at 3000 rpm for 20 minutes at room

temperature. The supernatant (the clear upper phase) was transferred gently into a 15ml-tube, avoiding the salt protein deposit. Cold 100% ethanol (EtOH) was added in twice the volume of the supernatant and gently mixed by inverting the tube till fine threads appear which the DNA is. DNA was removed with a glass Pasteur pipette and washed in 70% EtOH. After air-drying for a few minutes on the Pasteur pipette, DNA was dissolved in 0.02% Sodium Azide (Sigma-Aldrich- Cat#: S2002) (200-1000ml depending on the amount of DNA) and left at room temperature overnight.)

3.4.2 DETECTION OF MUTATION BY DIRECT SEQUENCING:

3.4.2.1 PRIMER design:

By using UCSC Genome Browser website; MUT gene contains 13 exons, specific primers were designed using Primer 3.0 (http://primer3.wi.mit.edu/) for each exon and exon boundries as shown in **Table 3.1**

Table 3.1: Primers used for amplification of MUT gene.

# of exon	Primer	Sequence	Length	Temp	GC%
1	Left (F)	AAACAGAGGAGACCCGGAAG	20	60.62	55.00
	Right	GGGTAGAGGAGGGACAGGAG	20	60.07	65.00
2	Left	CTATTTCCCACCCCCTCTTC	20	59.76	55.00
	Right	ACAGAGATTAACCCCCAAAAA	21	57.60	38.10
3	Left	CTGTTATGAGATGAATCAACATTTTT	26	57.83	26.92
	Right	TGTTGTAAAAATTCCTACATTCAAGG	26	59.74	30.77
4	Left	AATGTTTATGCTTAGAAAGTGGATTT	26	58.00	26.92
	Right	ATAAAATGGTCCTATGCATTTCTT	24	57.35	29.17
5	Left	AGACCTTGATTTTCTAGTGTGTGA	24	57.12	37.5
	Right	TTATGCTTCAGAATATTTATCATTTCA	27	57.63	22.22
6	Left	TTTGTCTGAATTTATTTGCTATTCTGA	27	59.64	25.93
	Right	TTTGCAAACATCGTTTAAATTATTA	25	57.20	20.00
7	Left	TGTTGCTTTAATTTTCTCCCAAG	23	59.67	34.78
	Right	TTTACCCAAGTTCCATTTTTCA	22	58.52	31.82
8	Left	CTCTCACACCCCCTTCTCAG	20	59.83	60.00
	Right	GAGCAAGTTTCTCAATGCCTTA	22	58.65	40.91

# of exon	Primer	Sequence	Length	Temp	GC%
9	Left	TGCATCAGGGTCTAATCTCTTG	22	59.34	45.45
	Right	TTCAAAATTCCTCAGCCACA	20	59.25	40.00
10	Left	GGCACACAGCTAATGTTGGA	20	59.72	50.00
	Right	TGAAATTCTGGCCTAAGAAACC	22	59.61	40.91
11	Left	AAACTTGAAAGATTTGCTGTGAA	23	58.11	30.43
	Right	CTTATGGGCAGGAATGGAGA	20	60.03	50.00
12	Left	TCCTTTTCAGTGCAGATGCTT	21	60.01	42.86
	Right	TGGTTTGCAAGGAGGTAGTTC	21	59.23	47.62
13	Left	ATACCAGTTGAGAAGGTTTTGG	22	57.29	40.91
	Right	AGCATGACACCAGGCCTATAA	21	59.61	47.62

3.4.2.2 PCR AMPLIFICATION PROTOCOL AND PTOGRAM:

All exons and exon-intron bounderies of MUT were screened for mutations. The first step was amplification by PCR using Thermal Cycler (Applied Biosystems, Gene Amp PCR System 9700) machine.

- **Reaction protocol**

Reagent	**volume in µl**
Taq enzyme	0.05ml
Primer F (10pml)	0.5ml
Primer R (10pml)	0.5ml
DNTPs	2ml
Buffer	2.5ml
$MgCl_2$	2ml
DNA (100ng/ µl)	1ml
DDH_2O	10.5ml

We multiply each fraction by the number of samples that we have, +1 (as control 'NTC').

- **PCR program (T.D 55 programs):**

Step1: initial denaturation	**95** ºC for 4 min.		
Step2 (3 cycles): denaturation	**94** ºC for 30 sec,	**63** ºC for 30 sec,	**72** ºC for 30 sec
Step3 (3 cycles):	**94** ºC for 30 sec,	**61** ºC for 30 sec,	**72** ºC for 30 sec
Step4 (3 cycles):	**94** ºC for 30 sec,	**59** ºC for 30 sec,	**72** ºC for 30 sec
Step5 (3 cycles):	**94** ºC for 30 sec,	**57** ºC for 30 sec,	**72** ºC for 30 sec
Step6 (35 cycles):	**94** ºC for 30 sec,	**55** ºC for 30 sec,	**72** ºC for 30 sec
Step7: final extension	**72** ºC for 5 min,	**4** ºC for 10 min	

*Annealing temperature depends on the melting temperature of the primers used for PCR amplification
** Extension time was determined according to the size of amplified product (each 1KB= 1min)

Amplification processes were performed by our PCR machine GeneAmp-PCR system 9700 from Applied Biosystem.

3.4.2.3 ELECTROPHORESIS OF PCR PRODUCTS USING AGAROSE GELS

The concentration of the used agarose gel was 1.5% which was determined based on the sizes of the DNA fragments being run. One percent agarose gel containing 0.01% ethidium bromide (Amresco-Cat # E406) was usually used; prepared using 1X TAE running buffer then poured into the electrophoresis tray till it becomes solid. Three µl of loading buffer was added to three µl of PCR product then loaded onto the gel along with DNA size ladder (Thermo Scientific-Cat# SM0241) and run in 1X TAE running buffer at 100V for 30 minutes. DNA fragments were observed and documented using BioRad ultraviolet imaging system.

3.4.2.4 CLEANING DNA FOLLOWING PCR

The PCR products should be cleaned from the remaining primers and the free nucleotides by using two enzymes: Exonuclease I (New England Biolabs, Cat# M0293L) and Antaractic Phosphatase (New England Biolabs, Cat#M0289L), where 0.25µl of each were added to 1.5µl nuclease free H2O with 5µl PCR product to get final volume of 7µl then put in PCR machine at the following program: 37oC for 30min, 80oC for 20min, and 4^{o}C until being used.

3.4.2.5 SEQUENCING OF THE PURIFIED PCR PRODUCT

Sanger sequencing reaction performed by preparing approximately 10ng per 100bp of the length of PCR fragment as a DNA sample to a total volume of 16µl where 1X reaction

prepared by 0.2µl of BigDye, 1.5µl of 5X buffer, 0.75µl of 64X buffer, 0.2µl of forward or reverse primer, 2.0µl of cleaned PCR product, and completed with 11.05µl of ddH_2O. The mix was run on ABI 3130 DNA Sequencer (Applied Biosystems, S/N: 20355-023) at 64X program:

96°C for 01min	
96°C for 10sec	
50°C for 05sec	**30 cycles**
60°C for 04min	
04°C for 10min	

3.4.2.6 CLEANING OF THE SEQUENCED PCR USING EDTA/ETHANOL PRECIPITATION METHOD

Cleaning up the PCR products from primers, excess dNTPs, and unincorporated dyes is the final step before running the PCR product on the sequencing machine. This is done by adding 100 ul of Absolute Ethanol and 5 µl of 125mM EDTA (Amresco-Cat # 0720) for each 16 µl of sequencing reactions to be cleaned and precipitated, followed by incubating the reactions at -20° for 30 minutes, then centrifugation for 30 minutes at 3800 RPM at 4°C. The supernatant was discarded and 60 µl of 70% Ethanol were added to each reaction, and centrifuged again for 20 minutes. Again the supernatant was discarded, and the samples were inverted on tissue for 1 minute at 500 rpm. Drying the samples was performed at 95°C for 5 minutes. Adding 16 µl of Hi-Di Formamid (Applied Biosystems, Cat # 4311320) and drying the samples again at 95°C for 2 minutes. At last, the reactions were placed on ice for 5 minutes before loaded on the 96 –well Optical Reaction Plate and run on sequencing machine; Illumina Genome Analyzer (Illumina, Illumina HiSeq 2000).

3.4.2.7 CHECKING THE SEQUENCES BY PEAK SCANNER SOFTWARE V1.0 AND IDENTIFYING MUTATIONS

3.4.3 VALIDATION:

To define whether the mutations are biologically and/or clinically relevant we performed several common and specific analyses:

- Sanger sequencing was done for both affected and unaffected individuals of all families to check the mutation segregation pattern.
- In addition, to ascertain mutation absence in controls, carrier rates were determined by testing a control group using direct sequencing, of at least 200 Palestinian individuals who were already available at the Hereditary lab as DNA samples.

v SPECIFIC VALIDATIONS:

3.4.3.1 Missense mutation Validation:

- By using UCSC browser tool to check the missense mutation not being SNP and whether being conserved or not.
- By using Polyphen-2 to predict the effects of the deleterious variants on the protein level

3.4.3.2 Splicing mutation Validation:

mRNA Splicing Analysis:

A new MMA family MERC-CA from Wad Rahal has recently joined our research and we checked its patient for our mutations. He is found homozygous for the missplicing mutation **IVS8+3A>G**. We did the mRNA expression study on five individuals from this family (the patient, his parents and two brothers).

i. Isolation of RNA from Blood:

The protocol involved RNA extraction by using Trizol:

- In EDTA tubes, five ml of blood was collected from each individual.
- 45 ml of RBC lysis buffer was added to each tube and kept on ice for 30 min then centrifugated at 2000 rpm and 4^{o} C for 10 min.
- The supernatant was carefully removed and the pellet was resuspended in 10ml RBC lysis buffer and centrifugation was repeated.
- The supernatants were discarded then adding1ml of Trizol (Invitrogen Cat# 15596-018) to each tube to re-suspend the pellets.

- Transferring the samples to 1.5 ml tubes and shaking them vigorously by hands for 1 min then they were incubated at room temperature for 5 min.
- Shaking well for 15sec after adding 200ul of chloroform (Biolabs Cat# 67-66-3) then incubated for 3 min.
- The samples were spun at full speed for 15 min at 4^{o} C.
- The colorless phase was transferred to new 1.5 ml tubes and 500ul of isorpoanol was added leaving them at room temperature for 10 min.
- The samples were spun at full speed for 10 min at 4^{o} C.
- Supernatants were discarded again and pellets were washed once with 75% ethanol.
- Again the samples were spun at full speed for 10 min at 4^{o} C.
- The supernatants were removed completely then leaving them open for 5 min to let the pellets dry.
- 25 ul of water was added to redissolve the pellets.

ii. Reverse Transcriptase PCR:

- By using the_qScript™ cDNA Synthesis Kit (Quanat Biosciences-Cat#95047-500) and according to the manufacturer's protocol and program the extracted RNA considered as a template for reverse transcriptase to be converted to cDNA.

iii. Sanger sequencing:

- By using bioinformatic tools; UCSC and Primer 3.0 (http://primer3.wi.mit.edu/) are used to design forward and reverse primers on exon 9 and exon 5 respectively.

Table 3.2: Primers used for amplification and sequencing of cDNA

Primer	Sequence	Start	Length	Temp	GC%
Left	CTTCTGGGGAATTGGAATGA	10	20	59.86	45.00
Right	GAGATGCATCCACTGCAAGA	774	20	59.95	50.00
SEQUENCE SIZE: 781					
INCLUDED REGION SIZE: 781					
PRODUCT SIZE: 765, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00					

- The same procedures of PCR amplification, gel electrophoresis and Sanger sequencing were applied as shown previously in mutation analysis Sanger sequencing part sections (3.4.2.2 -3.4.2.7), except the PCR protocol

Basic PCR touchdown protocol

v Reaction protocol

Reagent	**volume in µl**
2X PCR ReadyMix (Abgene _Cat# AB-0575-DC-LD)	12.5µl
Primer F (10pml)	0.5µl
Primer R (10pml)	0.5µl
DNA (100ng/ µl)	1µl
DDH_2O	10.5µl

We multiply each fraction by the number of samples that we are doing +1 (for control "NTC").

v PCR program (T.D 60 program)

Step1:	**95** ºC for 4 min.
Step2 (3 cycles):	**94** ºC for 30 sec, **68** ºC for 30 sec, **72** ºC for 30 sec
Step3 (3 cycles):	**94** ºC for 30 sec, **68** ºC for 30 sec, **72** ºC for 30 sec
Step4 (3 cycles):	**94** ºC for 30 sec, **64** ºC for 30 sec, **72** ºC for 30 sec
Step5 (3 cycles):	**94** ºC for 30 sec, **62** ºC for 30 sec, **72** ºC for 30 sec
Step6 (35 cycles):	**94** ºC for 30 sec, **60** ºC for 30 sec, **72** ºC for 30 sec
Step7:	**72** ºC for 5 min, **4** ºC for 10 min

3.4.4 HAPLOTYPING ANALYSIS:

The haplotype analysis was done as a trial to find the origin of the Missense mutation (N219Y) as it is common in Caucasian population. Based on that several correspondences had been done through Medical Genetics Institute, Shaare Zedek Medical Center to contact with Jean-FrancËois Benoist from Hopital Robert debré in Paris France who is the researcher in charge for the work that found the same mutation as a novel in 2005 and he with his colleagues published the relevant article (Acquaviva1. C et al., 2001)

Twenty six samples (French samples) have been received, the same used in that study; 6 out of 26 were homozygous for the same mutation (N219Y) while the others were heterozygous. Only the six homozygous samples plus T3, I3 and AJ3 samples shared in the haplotyping analysis.

i. Microsatellites:

Tow markers STR (Short Tandem Repeats) have been chosen on each side of MUT gene (upstream and downstream sides) on the short arm of the chromosome 6. We chose 4 markers that have been previously reported and have a high degree of heterozygosity among individuals.

Reverse and Forward primers were designed for each marker using Primer 3.0 (http://primer3.wi.mit.edu/) to amplify the regions for genotyping (see Table 3.3 for primer sequences)

Table 3.3: Primer sequences used for Haplotype analysis

Marker	Template	Primer sequences	Size
M1: D6S1632	DNA	F1: *GTGCAGAGCATCATGCGCCAGGCATATAGCATTTCTC R1: GGTATGAGGGCTCTGGTTC	137-167 bps
M3: D6S1669	DNA	F2: *GTGCAGAGCATCATGCTGTATCCACTGCCATCACTT R2: AGCACCAAATGACACAGAAC	243-257 bps
M4: D6S465	DNA	F1: *GTGCAGAGCATCATGCTAAAAATGGGCACTTTCACT R2: ACACTTCAGATCAGGTCAAC	183-198 bps
M2: D6S1714	DNA	F1: *GTGCAGAGCATCATGCACCCCCTATTTCCAGC R1: CCATTTTATGCCATTTCTC	117-133 ps

*F-primers has a tail sequence complement to the tail attached to the fluorescently labeled (FAM)

ii. Genotyping PCR program

The ninth (T3, I3, AJ3 and the sixth homozygous French) sequences of the DNA for genotyping were PCR amplified using the primers.

v Reaction protocol

Each specific primer couple was mixed with the other components of the PCR reaction as follows:

Reagent	**Volume/µl**
Buffer	2.5µl
DNTPs	2.0 µl
$MgCl_2$	1.0 µl
Forward primer (10pM)	0.05 µl
Reverse primer (10pM)	0.5 µl
FAM	0.5 µl
Q-Solution	2.0 µl
Sample DNA	1 µl (50-100 ng)
Taq polymerase	0.05 µl
DDH_2O	15.4 µl

Each fraction is multiplied by the number of all samples +1 (for a control "NTC"). The touchdown is 60 for 30 sec.

v PCR program

Step1:	**95** ºC for 4 min.
Step2 (3 cycles):	**94** ºC for 30 sec, **68** ºC for 30 sec, **72** ºC for 30 sec
Step3 (3 cycles):	**94** ºC for 30 sec, **68** ºC for 30 sec, **72** ºC for 30 sec
Step4 (3 cycles):	**94** ºC for 30 sec, **64** ºC for 30 sec, **72** ºC for 30 sec
Step5 (3 cycles):	**94** ºC for 30 sec, **62** ºC for 30 sec, **72** ºC for 30 sec
Step6 (35 cycles):	**94** ºC for 30 sec, **60** ºC for 30 sec, **72** ºC for 30 sec
Step7:	**72** ºC for 5 min, **4** ºC ∞

Annealing temperature depends on the melting temperature of the primer used.
On 1.5% agarose gel, the success of PCR amplification was checked up.

iii. Genotyping

The PCR product was diluted 1:10 (1 µl of PCR product + 10 µl of Nuclease free water (N.F). The next step is preparing the mix for gene scan, which was done according to the following procedure:

- For 1X reaction; 1 µl of PCR product was added to a mix of 0.3 µl ROX (400 X-Rhodamine) and 12 µl of HiDi (Highly deionized formeamide).
- Incubate the mixture on Hotplate (95°C) for 5 minutes to denature DNA strand
- Incubation on ice for another 5 minutes

Now the mixture is ready to be analyzed on Genemapper v4.0 (3130XL Genetic Analyzer from Applied Biosystem)

3.4.5 VILLAGE SCREENING

The screening for Al-Ubeidiya village is done to determine the carrier frequency, because it is noticed that the missense mutation (N219Y) happens to be frequent in that village.

Pre-sample collection phase:

In order to do that, a collaborative network was established between the following parties:

- Hereditary Research Lab in Bethlehem University.
- Ministry of Health.
- Outpatients from Caritas Baby Hospital
- Al-Ubeidiya Municipality
- Ministry of Education

After getting the approval from Ministry of Health and the agreement with the village elders in addition to the Al-Ubeidiya Municipality, a team consists of Dr.Nader Handal (Pediatric Doctor), Mrs Hiba Sady (Social worker) and Jimmy Zaidan (Outpatient clinic Headness) from Caritas Baby Hospital in Bethlehem and Amal Abu Rayan (Associate researcher) and me from Hereditary Research Laboratory in Bethlehem University arranged once a week visit since 11/6/2014 to collect samples from Al-Ubeidiya village.

Mrs Hiba started to inform the known families affected with MMA in the village to come to the village clinic to provide us with their samples.

Other families in Al-Ubeidiya were informed through the mosques and the village elders. Brochures as illustrated in appendices, which consists of a simple MMA definition and the purpose of the screening, were prepared and distributed throughout the village before and through the samples collection. Till this stage, 400 samples nearly were collected.

The tenth percent (the representative sample) of Al-Ubeidiya village population, which consists of 14,000, is 1400 ones. Thus we decided to collect samples from the secondary schools in the village. An approval was gotten from the ministry of education. The teachers and headmistresses informed their students and helped us in collecting samples smoothly. As a result, 888 samples were collected.

Sample Collection phase:

Consent forms as shown in appendices, were prepared to be signed by volunteers from the village.

The sample was taken from cheek cells using buccal brush or swap of Extracta™ DNA Prep for PCR (Quanta Biosciences; Cat # 95091-025/ -250) which then placed and twirl into 250 µl extraction reagent in 1.5ml microcentrifuge tube, and to ensure remaining and submerging the sample in the tube, the brush was cut inside.

Post-samples Collection Phase:

Later on, the samples were transferred to Bethlehem Hereditary lab, where they were heated to 95^{o} for 30 minutes. Then cooling to room temperature and adding an equal volume (250 µl) of stabilization buffer.

After this procedure, the samples would be ready to be amplified by PCR and preceded to the next sequencing steps as mentioned previously in sections (3.4.2.2 -3.4.2.7). But in this type of samples; 2.5 µl of extract is used up in a 25 µl PCR reaction.

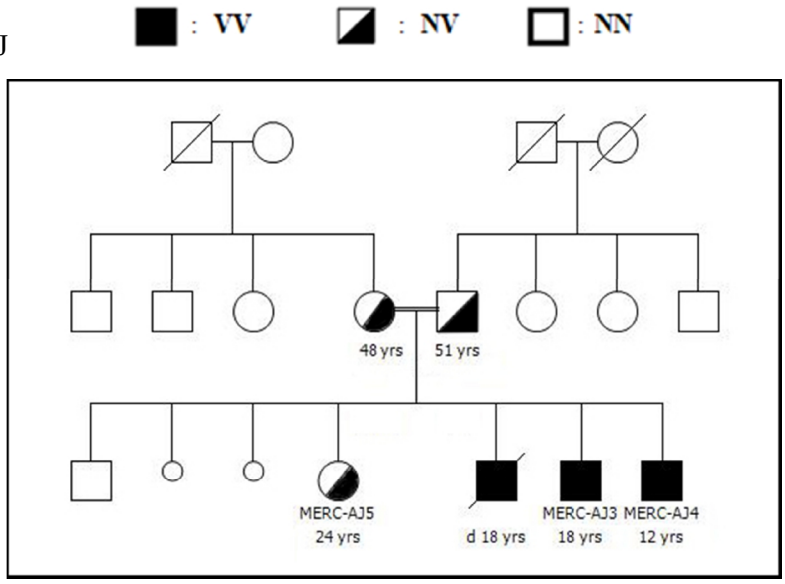
When the results being ready, a special report shown in appendices, had been prepared and submitted for each volunteer by himself.

Chapter Four: Result

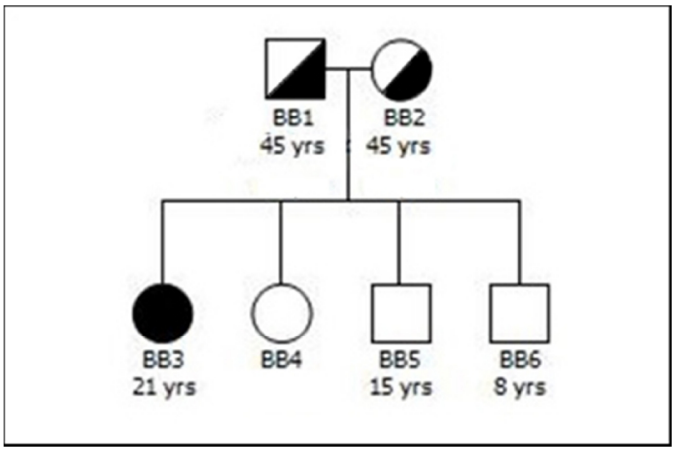
4.1 Clinical Data

Pedigrees of participated families were constructed as shown in Figure 4.1 Patients of Families MERC-I, T, AJ are from Al-Ubeidiya village while patient of family MERC-BB are from Wad Rahal. They were examined and confirmed to have B12-unresponsive MMA by Dr. Nader Handal at Caritas Baby Hospital. The pedigrees were constructed through family interviews. The pedigrees demonstrate the inheritance of MMA phenotypes in autosomal recessive pattern. Table 4.1 illustrates detailed description the age of onset, clinical manifestations and the outcomes for each patient.

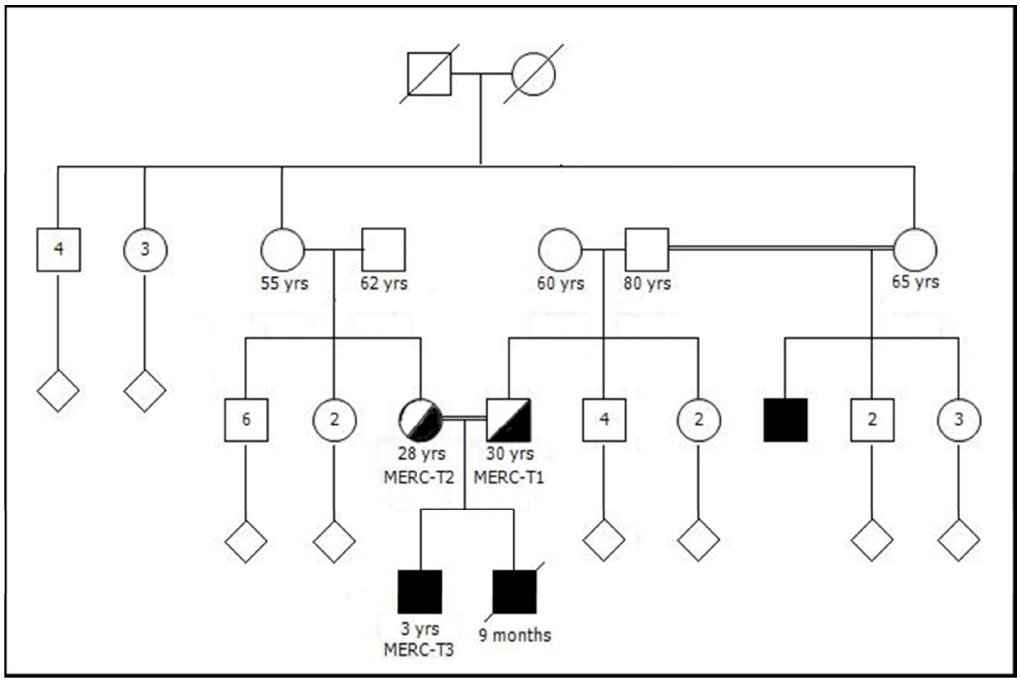
A) MERC- AJ



B) MERC- BB



C) MERC- T



D) MERC- I

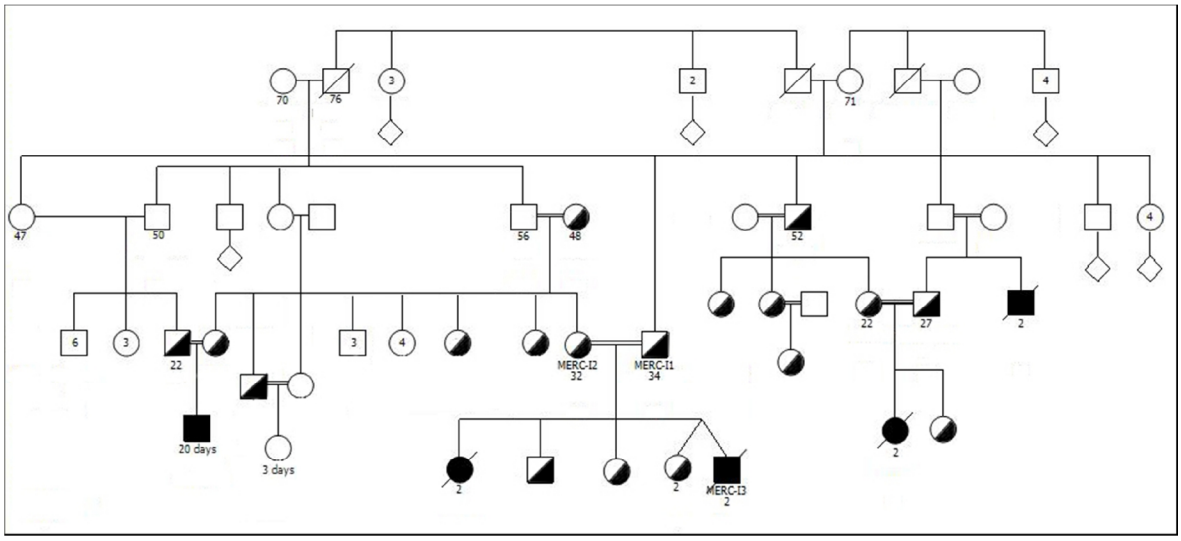


Figure 4.1 : Pedigrees of families with isolated methylmalonic acidemia

Fig. 4.1: Pedigrees of families with isolated methylmalonic acidemia A) MERC- AJ B) MERC-BB C) MERC-T D) MERC- I. Perfect segregation of the genotype with the phenotype in an autosomal recessive inheritance. : **VV** : **NV** : **NN**

Table 4.1: Clinical Data of Participated Patients

Patients Code	Age and symptoms at presentation	Outcome	Treatment/ extra comments	Genotype
T3	First week of life: Poor feeding Fever Hypo-activity Vomiting	Encephalopathy (Convulsion) Mental Retardation	Treatment: restricted protein intake, carnitin supplement	Missense c. 731 A>T p.N219Y
I3	First week of life: Poor feeding Fever Hypo-activity Vomiting	Encephalopathy (Convulsion) Mental Retardation	Comment: Died following sever metabolic acidosis and extrapyramidal symptoms at 3.5 years	Missense c. 731 A>T p.N219Y
AJ3 **AJ4**	3 months: Poor feeding Failure to thrive	**AJ3:** Renal failure Weak vision **AJ4:** Encephalopathy brain abnormalities Metabolic Stroke followed a metabolic decompensation Cannot walk easily	Treatment: Carnitine, restricted protein intake Comment: they have MMA Brother died at 16 years following severe renal failure	Missense c. 731 A>T p.N219Y
BB3	11 months: Fever, vomiting	Renal failure Decrease visual equity Cannot walk (wheel chair)	Treatment: Carnitine, restricted protein intake, Flagyl Calcium supplement She is on renal dialysis.	IVS8+3A>G (Splicing site)
*The data was collected based on Caritas Baby Hospital pediatric clinic diagnosis.				

4.2 SNP Microarrays Homozygosity Mapping Results:

As shown in **Table 4.2** which summaries the shared homozygous regions of the affected individuals (T3 & I3). We did screening for the candidate gene for MMA in the first two regions (in chromosome 9 and 16), nothing was found. While on the short arm of chromosome 6; MUT gene is found as a candidate gene for MMA disorder.

Table 4.2: The shared homozygous regions of the affected individuals (T3 & I3)

Chromosome	Block Name	Start Pos	End Pos	No of SNPs	PhysLength
9	Chr9.2	38739117	71156551	18	32417434
16	Chr16.6	34877723	48512981	78	13635258
6	Chr6.2	44452011	52051957	700	7599946
21	Chr21.1	9764385	14649798	5	4885413
18	Chr18.3	14602140	18681293	21	4079153
16	Chr16.4	2716633	5392358	129	2675725
14	Chr14.11	104978460	107139823	26	2161363

4.3 Sanger sequencing Results of the candidate gene (MUT):

Sanger sequencing was applied for each patient with his/her parents from all families MERC-(T, I, AJ, BB). This study revealed the presence of two distinct homozygous MUT mutations, one is missense mutation found in exon 3, c. 731 A>T p.N219Y, while the other is a splicing mutation found in the junction between exon 7 and intron 8 (IVS8+3a>g).

Both mutations have been previously reported in patients with enzymatically confirmed mut^{o} class MMA.

In families T, I and AJ; data revealed one shared variant among all the affected patients. This variant turned to be T>A missense substitution mutation in exon 3; patients were homozygous for the substitution whereas their parents were heterozygous as shown in figure (4.2) At the protein level, the 731 T>A, substitution resulted in the replacement of an asparagine residue by a tyrosine at position 219 (N219Y), Change from medium size and polar (N) residue to large size and aromatic one (Y). This substitution mutation has been previously reported twice in the same year 2001 in Caucasian population (Acquaviva, Benoist et al. 2001) and in Palestinian population (Berger, Shaag et al. 2001).

In family BB; the patient was homozygous for the splicing mutation (IVS8+3a>g) at the third nucleotide of the 5'-splice site of intron 8 as shown in figure (4.2). This mutation has been previously reported in Palestinian population (Berger, Shaag et al. 2001). Our data demonstrated that this mis-splicing mutation resulting in a rearranged transcript with skipping of exon 8.

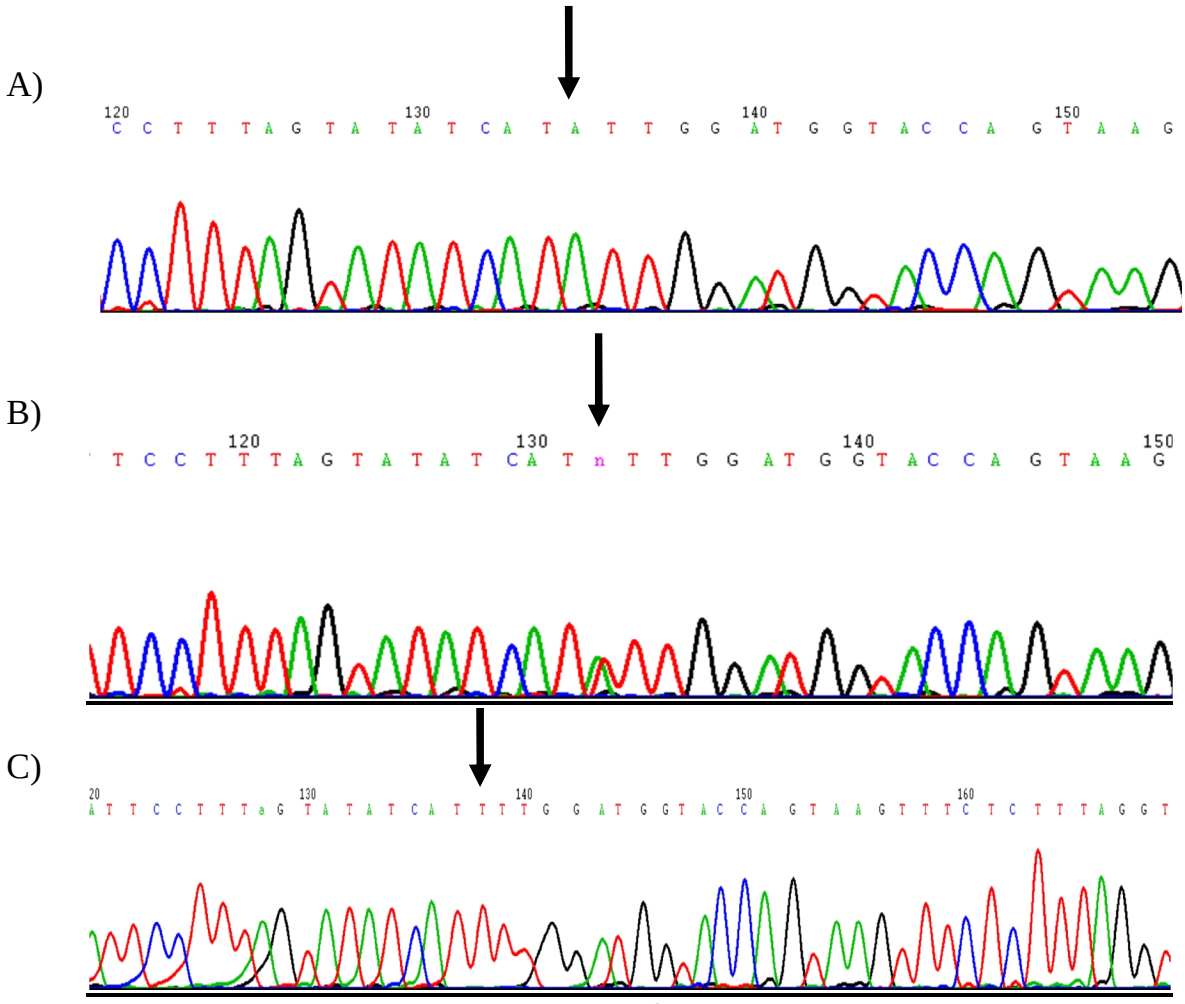
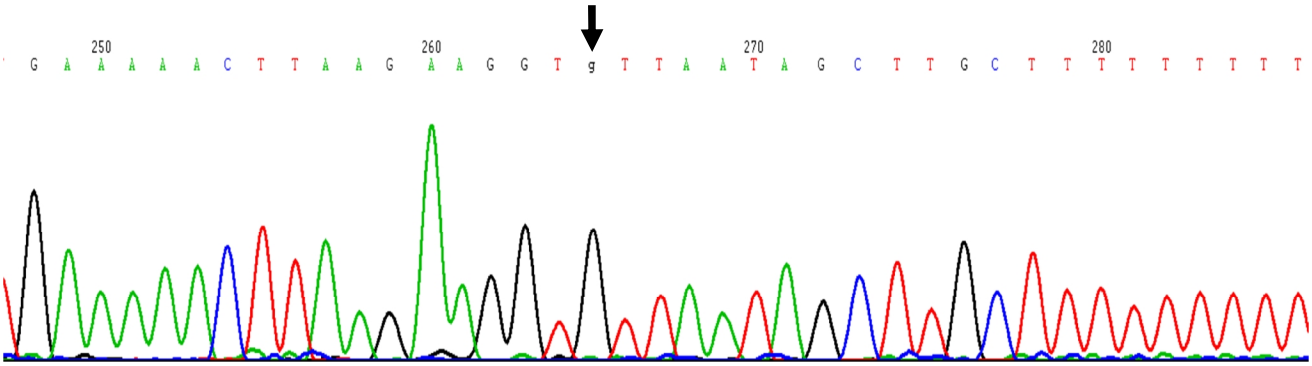


Figure 4.2: Sanger sequencing results for the Missense mutation

Fig 4.2: Sanger sequencing results for the Missense mutation N219Y; A) Sanger sequences for homozygous affected patients (T3, I3 and AJ3) B) heterozygous unaffected individual (all parents of affected individuals are heterozygous at this mutation site) and C) wild type genotype of unaffected healthy control. The arrow points to the site of the mutation.

Homozygous affected patient (BB_3)



Heterozygous unaffected parents (BB1& BB2).

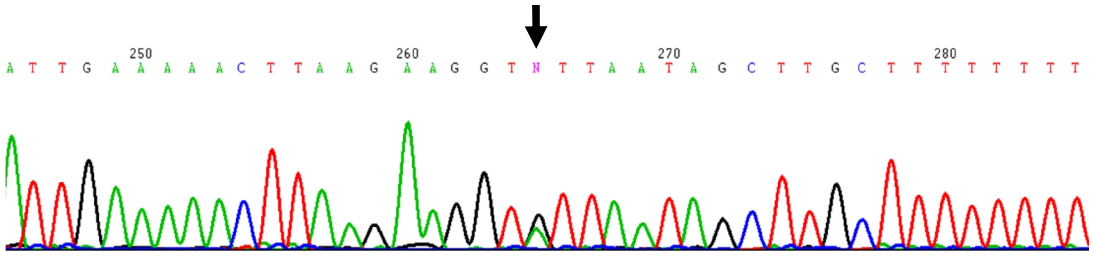


Figure 4.3: Sanger sequencing result for the Missplicing mutation

Fig 4.3: Sanger sequencing result for the Missplicing mutation IVS8+3A>G; A) Sanger sequences for homozygous affected patients (BB_3) vs heterozygous genotype of unaffected parents (BB_1& BB_2). The arrow points to the site of the mutation.

4.4 Validation:

Validation was done for the two mutations:

4.4.1 Missense mutation validation:

1- By using the UCSC genome browser Asparigine (Asn219) is located in an amino-acid stretch that shows high level of homology among the species from human to fish as illustrated in Figure (4.4, A). Consequently, any changes in this amino acid will probably lead to structural changes and abnormal function of the protein.

2- The variant c. T731A (p.N219Y) was run through the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) program. "A prediction tool for the impact of a particular amino acid substitution on structure and function of human protein". The effect of this substitution was predicted to be probably damaging on the stability and function of mutase enzyme with a score of 1.00 (sensitivity: 0. 00; specificity: **1.00**). Figure (4.4, B)

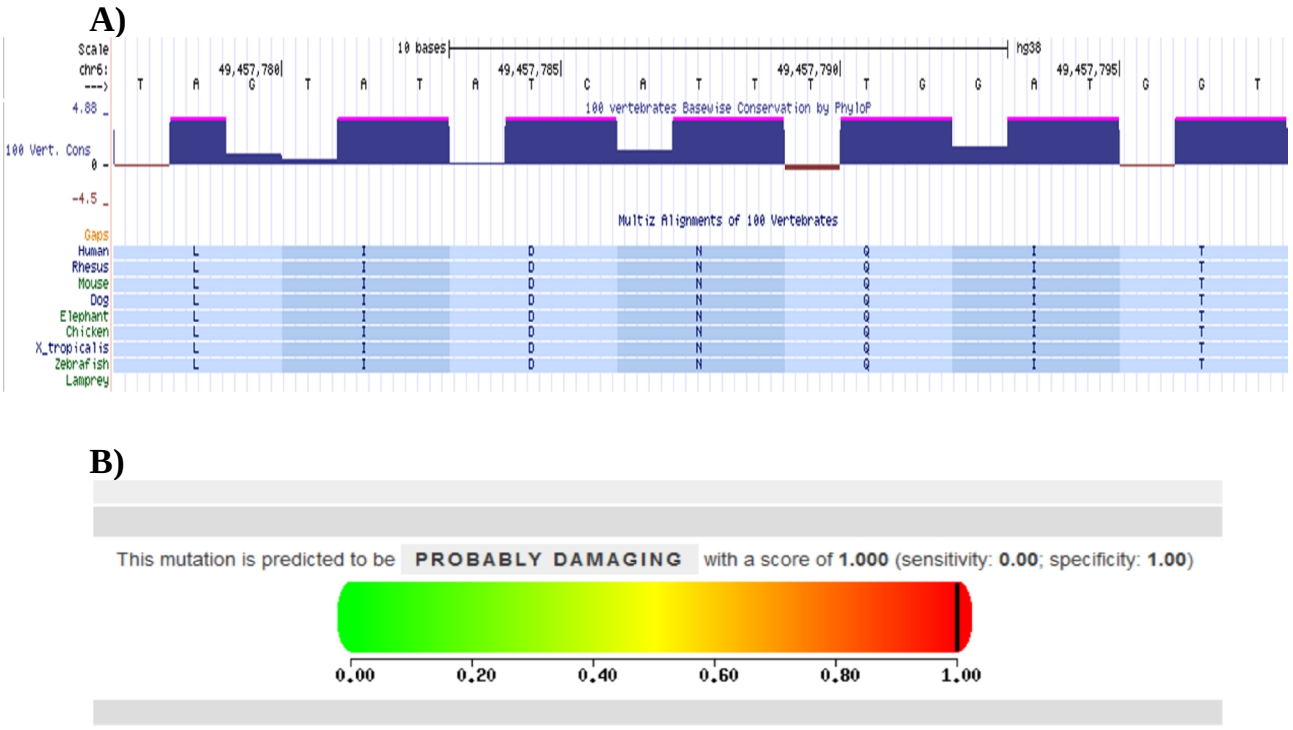


Figure 4.4: Result analyses by bioinformatic tools

Figure 4.4 Result analyses by bioinformatic tools. A) Multiple alignments of MCM protein for variable organisms from human to zebra fish showing the conservation of the mutant site N219Y. B) The black bar at the red region indicates the mutation is damaging

3- This variant was checked for segregation as shown in figure (4.1). The mutation, c.731T>A, segregate with the MMA in three families (MERC- T, I & AJ) in an autosomal recessive manner.

4- To confirm if this substitution was the causative mutation of the patients' muto phenotype, Acquaviva et al, 2001 performed transient expression studies of MCM activity in SCK2 muto cell line from patient fibroblasts. As seen in figure (4.5), this study revealed that the transfection of wild type expression vector (pCMVmut) results in increasing the propionate incorporation while the transfection with a vector containing 731 T>A substitution (pCMVmut731 T>A) showed a similar level of propionate utilization as cell line transfected with a vector containing null mutant MCM cDNA (pCMVmutNSCD238).. It is clear that the ability to restore MCM activity in muto patients was abolished by insertion of 731 T>A substitution in a wild-type MCM expression vector therefore this mutation is responsible for the phenotypes in those patients. (Acquaviva, Benoist et al. 2001)

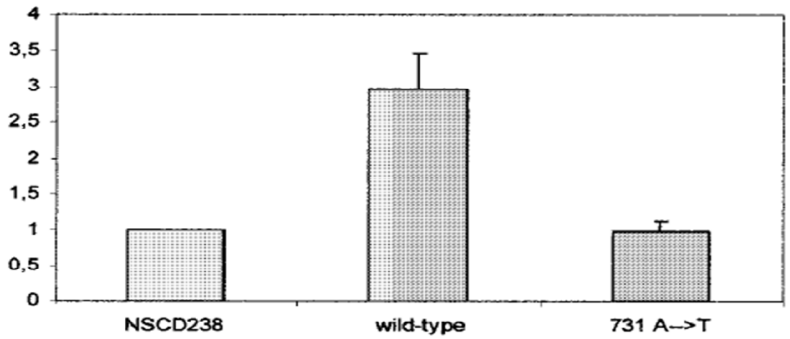


Figure 4.5: Results of transient expression of MCM

Fig.4.5: Results of transient expression of MCM activity in SCK2 mut^{o} cell line. MCM activity was measured based on [^{14}C]-propionate incorporation test, NSCD238 is a vector with MCM cDNA contains a null mutation. pCMVmut731 T>A; a vector containing 731 T>A substitution. And pCMVmut is wild-type expression vector. The results shown in SD and mean for duplicated three independent experiments. (Acquaviva, Benoist et al. 2001).

4.4.2 Splicing mutation validation:

1. This variant was checked for segregation in Family BB and AC and the pedigrees were shown in Figure (4.1.B) and (4.6) respectively. The mutation, IVS8+3a>g, segregates perfectly with the MMA in an autosomal recessive manner.

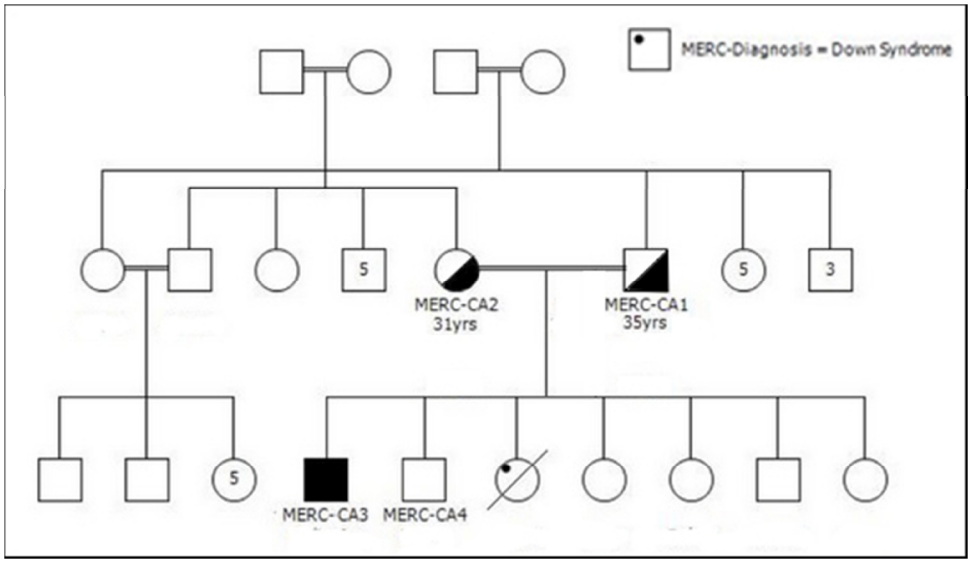


Figure 4.6: Pedigree of CA family

Fig. 4.6: Pedigree of CA family, showed a perfect segregation for the genotype (AA) with the phenotype in an autosomal recessive

■ : VV ◪ : NV □ : NN

2. To determine the influence of the second mutation on mRNA splicing, functional mRNA splicing analysis was done as illustrated previously. And the cDNA were PCR-amplified then the products of PCR were analyzes by electrophoresis on 2% agarose gel. As shown in Figure (4.7); one band of cDNA PCR product could be seen for CA3 on the agarose gel which was shorter than the normal control band. While his father CA1 had two separated bands one corresponding to a mutant allele and the other to wild type. As seen in Figure (4.8), direct sequencing of the mutated CA3 cDNA band confirmed that exon8 is totally skipped compared to the wild -type sample

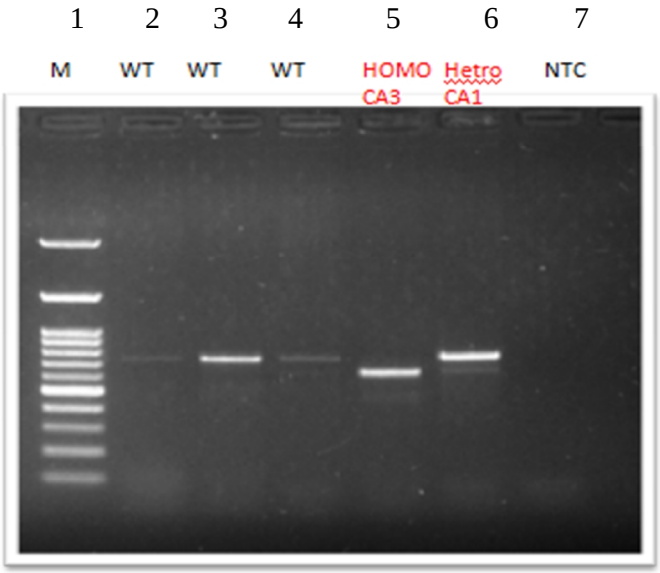


Figure 4.7: Agarose gel electrophoresis

Fig.(4.7): Agarose gel electrophoresis of cDNA for MUT gene of family (CA); (1) 100-bp ladder. (2, 3 and 4) are wild type, (5) cDNA PCR product for the patients or the homozygous sample 6) PCR product for the father 7) NTC "Negative Template Control).

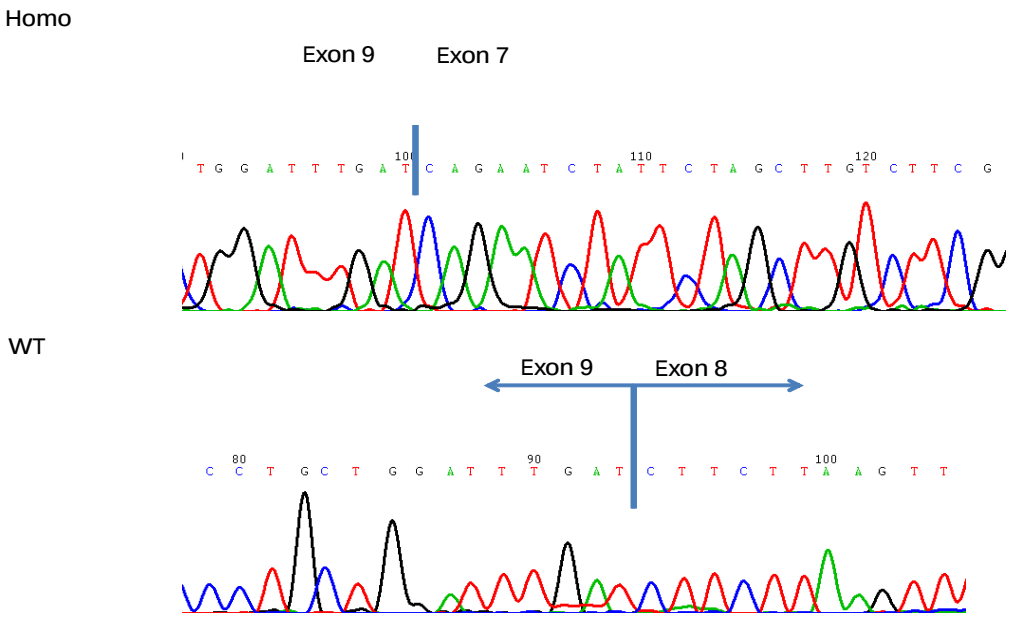


Figure 4.8: Aberrant splicing

Fig.4.8: Aberrant splicing (IVS8+3a>g) of MUT. CA3 mutant cDNA chromatogram showing exon8 skipping compared to a wild type sample.

4.5 Haplotype:

Haplotype analysis revealed that the three Palestinian patients homozygous for p.N219Y mutation were also homozygous for all studied markers around the MUT gene, therefore one shared haplotype was found; as shown in Figure (4.9). T3 and I3 patients who are from Al-Ubeidiya village (Bethlehem region) share an overlapping haplotype of 5.4 Mb of genomic DNA whereas AJ3 who is from Dura village (Hebron region) share the same haplotype but it is relatively shorter than that found in Al-Ubeidiya.

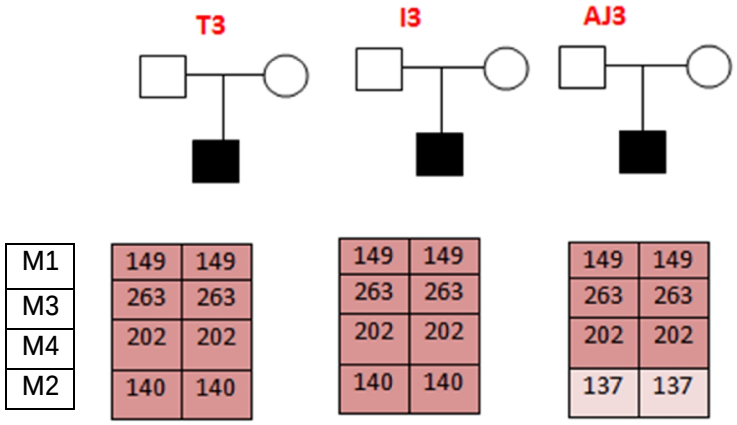
Interestingly, different haplotypes were found for p.N219Y substitution for French patients.

A)

Table 4.3: The positions of markers used in the haplotype analysis

Marker Names	Position	Distance relative to MUT
M1: D6S1632	46163131-46163481	3234942
M3: D6S1669	48037810-48038058	1360263
MUT gene	49398073-49431041	0
M4: D6S465	51164187- 51164518	1733146
M2: D6S1714	51556991-51557397	2125950

B)



C)

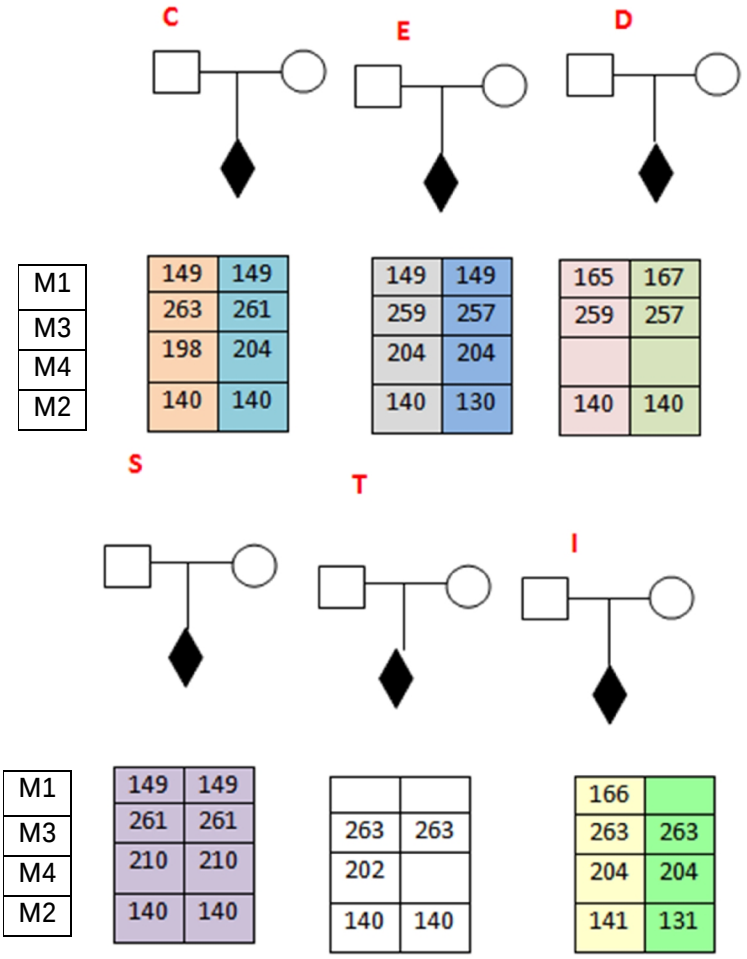


Figure 4.9: Haplotyping analysis results

Fig.4.9: Haplotyping analysis results; A) Table illustrating the marker codes and their positions arranged related to MUT gene position, B) the Palestinian (T3, I3 and AJ3) haplotypes results C) The French (C, E, D, S, T and I) haplotype results. Different colors mean different haplotypes while the same color indicates the same haplotype.

4.6 Screening results:

We did mutation analysis for N219Y for 888 individuals from Al-Ubeidiya village; 44 were heterozygous and the remaining was normal. Each volunteer has received a report containing his/ her result.

This means that the carrier frequency in that village is 4.95%.

Chapter Five:

5.1 Discussion and conclusion:

We started our research with Homozygosity mapping which was carried out in 2 individuals using 250K SNP microarray (affymetrix) in identifying MUT gene as a candidate genomic region associated with MMA phenotypes. This method is powerful for genes localization for autosomal recessive disorder within consanguineous families (Wang et al., 2009).

In this study we have identified two mutations using direct sequencing; splice and missense mutations. Both mutations have been previously reported in patients with enzymatically confirmed mut^o class MMA (vitamin B_{12} non-responsive MMA). These mutations were confirmed in affected individuals while absent in the unaffected of the studied Palestinian families with inherited Methylmalonic acidemia. Mutations segregated perfectly in an autosomal recessive pattern. In addition, 200 healthy Palestinian were tested for these two mutations, and no carriers were found.

The first mutation is the splicing site (IVS8+3a **à** g) mutation; which has been found in MERC-BB and MERC-CA families from Wad Rahal. The affected individuals are homozygous for this mutation which involves, on DNA level, change from A **à** G at the third nucleotide of the 5'-splice site of intron 8 (IVS8+3a **à** g). As mentioned earlier, this mutation has been reported previously in 2001 and also it was studied on mRNA level in order to investigate its influence on mutase expression. In that study, they mentioned (without data) that this mutation resulting in a complex rearrangement with skipping of exon 8 and retention of a 110-bp fragment derived from intron 7 (IVS7-152 to -43) (Berger et al., 2001).This is not consistent with our result which shows only a skipping of exon 8 without any involvement from intron 7 as shown in figure (4.8). It seems that the skipping of exon 8, which causes partial deletion in linker domain, encoded transcript with a relative function. This may interpret the late age of onset and the milder severity in affected patients. The region of Wad Rahal is being screened currently in order to investigate the carrier frequency for this mutation there.

The second mutation is a missense mutation (N219Y). It was observed in three families MERC-T, MERC-I and MERC-AJ with MMA. Transient expression study has proved that it is responsible for MMA phenotypes (Acquaviva et al., 2001). The affected patients were homozygous for this mutation which involves substitution A>T located in exon 3 at position 731 in the cDNA of MUT gene. On protein level, it results in the replacement of a conserved

asparagine residue by a bulky and hydrophobic tyrosine at position 219 (N219Y), this substitution does not have direct interaction with the substrate, however it is located at the fourth b-strand of the N-terminal (eight-stranded b/a barrel) which is a specific highly conserved secondary motif. The modeling analysis suggests that this mutation affects the enzyme conformation which may result in impairment of folding process and/or in poor protein stability (Acquaviva et al., 2001). Since MUT-N219Y mutation is common in the Caucasian population, it may suggest that it has an ancient origin (Sakamoto et al., 2007). Therefore, we performed haplotype analysis which revealed that this frequent mutation appeared to have been spread by founder effect in the Palestinian families. In contrast, French families had different haplotypes, and this suggests independent origins to this mutation. Also, this indicates that the N219Y mutation represents a more frequently occurring mutation (Peters et al., 2002); it might be a hot spot mutation region. This mutation has been frequently found in Al-Ubeidiya village; therefore screened the village to identify the carrier frequency and provide a consented approach towards testing and its future impact.

Although, MMA is a rare disorder, it is widely spread among the Palestinian population. Al-Ubeidiya is one of the Palestinian villages that suffer from this disorder where screening showed 4.95% frequency of N219Y carriers. The high frequency of this genetic disorder among the Palestinian population is due to a founder effect as the result of high consanguinity rates.

The haplotype analysis revealed that the N219Y mutation is inherited on a common haplotype throughout the Palestinian families, however, family (AJ) from Dura showed a shorter haplotype block which indicates an occurrence of crossover this may explain the longer lifetime of those patients.

Methylmalonic academia (MMA) is a serious metabolic disorder. However, there is a possibility to be controllable; the affected patients remain susceptible to life-threatening episodes of metabolic decompensation and to suffering from severe complications such as progressive renal failure, metabolic stroke and pancreatitis (Peters et al., 2002). Therefore, a region like Al-Ubeidiya is in much need for a genetic counseling through providing a definitive diagnostic tool.

This work is the first of its kind in Palestine to carry mutation analysis of the gene responsible for MMA. Through this study, we managed to establish a rapid molecular diagnostic tool through only a buccal swab for differential diagnosis of MMA to detect the

carriers and provide a prenatal diagnosis among the Palestinian families at risk of MMA. This is significant in many aspects; such as allowing a proper treatment, and counselling for not compatible marriages as a prophylactic step. In addition, the established markers that were used in the haplotype study provides a tool for PGD (Preimplantation genetic diagnosis) tests which is a new technology providing a genetic knowledge in advance before pregnancy takes place and it involves checking the genes and/or chromosomes of embryos using linked DNA markers to distinguish affected from healthy embryos. Moreover, it is recommended for the Palestinian Pediatric Hospitals to apply a Newborn Screening (NBS) to detect organic acidemias early in infancy; it just requires a blood spot to identify propionylcarnitine (C3) elevation.

Finally, we will provide our data to Ministry of Health in order to take the proper actions in the village and offer premarital MMA screening test. Hopefully our work will aid in decreasing the frequency rate of MMA carriers in the coming years within the Palestinian population.

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Appendices

- MMA Brochure:

كيفية انتقال المرض

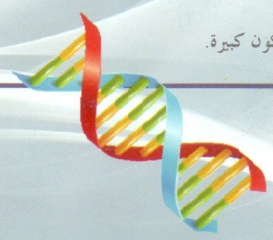
هذه الأمراض الوراثية تنتقل بما يعرف **بالوراثة المتنحية Autosomal Recessive** وهي تنتقل من الأبوين إلى أطفالهم حيث يكون كلا الأبوين حاملين للمرض، فلا يمكن أن ينتقل المرض من أحد الأبوين فقط، يجب أن يكونا حاملين لنفس الجين المعطوب لكي تحدث الإصابة.

احتمال تكرار الإصابة في كل مرة تحمل فيها الزوجة هي 25% واحتمال عدم تكرار الإصابة يكون 75%.

ما هي أهمية المعرفة عن مرض MMA

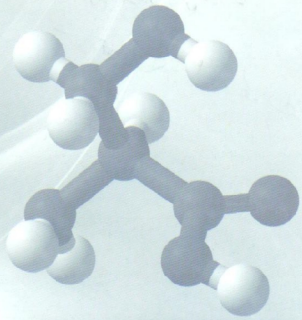
يعتبر هذا المرض نادر الانتشار ولكن في منطقتكم يوجد عدد من الأطفال الذين يعانون منه وهذا يعني أن هنالك الكثير من الأشخاص الذين قد يكونوا حاملين له.

وبالتالي، فإذا كنت/ي حامل/ة له، هنالك خطورة بإنجاب أطفال مرضى. وإن لم يكن أحد الوالدين حاملاً للمرض يكون الابن غير حامل له. أما في حالة أن يكون احد الوالدين حاملا لهذا الجين فان نسبة أن يكون الطفل حامل للمرض ستكون كبيرة.

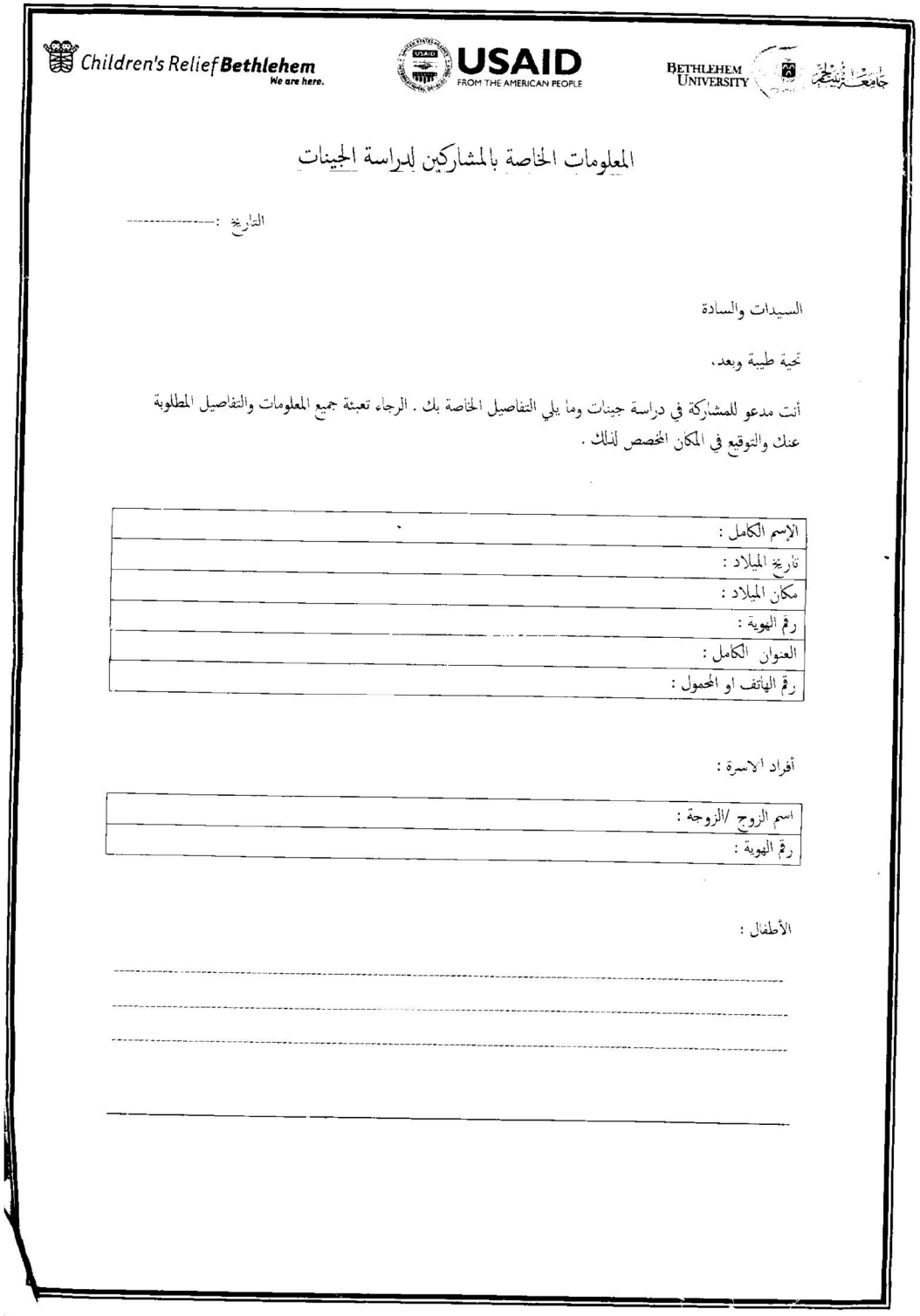


ما هو مرض حموضة الدم (MMA)

مرض حموضة الدم أو ما يعرف بالـ MMA هو عبارة عن خلل أيضي وراثي حيث يكون الجسم غير قادر على معالجة وتكسير بعض البروتينات والدهون وبالتالي تراكمها بشكل معين مما يؤدي إلى حدوث مضاعفات تظهر على شكل أعراض لهذا الخلل ألا وهي تأخر عقلي وجسدي للمريض مما يؤدي بالنهاية إلى الموت.



v Consent Form:



Children's Relief Bethlehem
We are here.

USAID
FROM THE AMERICAN PEOPLE

BETHLEHEM UNIVERSITY جامعة بيت لحم

المعلومات الخاصة بالمشاركين لدراسة الجينات

التاريخ :----------

السيدات والسادة

تحية طيبة وبعد،

أنت مدعو للمشاركة في دراسة جينات وما يلي التفاصيل الخاصة بك . الرجاء تعبئة جميع المعلومات والتفاصيل المطلوبة عنك والتوقيع في المكان المخصص لذلك .

الإسم الكامل :
تاريخ الميلاد :
مكان الميلاد :
رقم الهوية :
العنوان الكامل :
رقم الهاتف او المحمول :

أفراد الاسرة :

اسم الزوج /الزوجة :
رقم الهوية :

الأطفال :

Children's Relief Bethlehem We are here. USAID FROM THE AMERICAN PEOPLE Bethlehem University جامعة بيت لحم

نموذج الموافقة المسبقة لدراسة الجينات

إن هدف أي دراسة علمية طبية هو توسيع المعرفة المتوفرة وتطويرها في محاولة لمنع الإصابة بالأمراض أو التوصل إلى علاج مرض ما أو تخفيف الألم عن المرضى. وتحظى الدراسات الطبية التي تجرى على أبناء البشر بما فيها الدراسات الجينية التي تشمل على فحص المادة الوراثية (DNA) , على موافقة القانون بالشكل الذي يتم فيه حماية حقوق المشاركين بما في ذلك معلوماتهم الشخصية. والدراسة التي تمت دعوتك إلى المشاركة فيها حصلت على هذه الموافقة. نحن نشعر انه من الأهمية بما كان أن تفهم تفاصيل الدراسة وأغراضها وغايتها بحيث تكون موافقتك على المشاركة مبنية الفهم والدراية.

الرجاء أن تقرأ الشرح المبين أدناه بالكامل ولا تتردد في طلب الحصول على المزيد من المعلومات أو التوضيحات من عضو الطاقم الذي اتصل معك. وفي حال قبلت المشاركة في الدراسة.

يرجى الإجابة : نعم أو لا

1- لقد تلقيت فكرة واضحة عن المشروع البحثي ، والمتعلق بالتشخيص الوراثي وآثاره.----------

2- لقد تلقيت معلومات كاملة من الطبيب بشأن التفسيرات الممكنة والقيود المفروضة على اختبار الوراثة منها.--

3- أوافق على إرسال عينة الدم خاصتي الى مستشفى كاريتاس و مختبرات جامعة بيت لحم لاتمام الفحوصات- من المشروع و لتخزينها لاستخدامات بحثية فقط------

4- أقر بأن مشاركتي طوعية تماما. وأنا حر لسحب مشاركتي دون إبداء أي سبب. وفي هذه الحالة ، سيتم تجاهل البيانات الخاصة بي وعيناتي. ولا يشكل هذا الانسحاب أي خسارة في الاستحقاقات بالنسبة لي.--------

5- أنا اوكد الموافقة على سحب عينة الدم الوريدي -------- .

6- أوافق على أن نتائج الجينية سيتم تخزينها مع البيانات لأكثر من 10 سنوات في جامعة بيت لحم .-------

7- أتمنى أن أكون على إطلاع حول النتائج. لقد تم إبلاغي بأن النتائج تستند على مشروع بحثي ولا تلبي معايير التشخيص الجينية الروتينية.--------

التوقيع ----------

توقيع الشخص الذي تواجد عند التوقيع ----------

v Result report:

Moien N kanaan

HEREDITARY RESEARCH LAB

مختبر الأبحاث الوراثية

BETHLEHEM UNIVERSITY

جامعة بيت لحم

Community screening for MMA (Methylmalonic acidemia)

To: ID#

Date sample received: Result Date:

Result:

Disease	Gene (OMIM #)	Mutation	Result
Methylmalonic acidemia (MMA)	MUT (#609058)	N219Y	

Above are the results of your genetic test for the genetic cause of Methylmalonic acidemia (**MMA**) common in your community.

What is MMA?

Methylmalonic acidemia or aciduria is an inherited metabolic disorder in which the body is unable to process certain proteins and fats that accumulate and lead to more complications. Usually the symptoms appear in early infancy and vary from mild to life-threatening.

The affected infants can experience vomiting, dehydration, weak muscle tone (hypotonia), developmental delay, excessive tiredness (lethargy), an enlarged liver (hepatomegaly), and failure to gain weight and grow at the expected rate (failure to thrive). Long-term complications can include feeding problems, intellectual disability, chronic kidney disease, and inflammation of the pancreas (pancreatitis).This disorder can result in death in some cases if left undiagnosed or untreated.

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Moien N kanaan

How is MMA inherited?

The mode of inheritance is an autosomal recessive. There are two copies in each gene in our bodies. One inherited from the mother and one from the father. In a recessive disease, a child is affected only if both copies of the gene—one from each parent—is defective due to a change, or mutation. This can happed if each parent is a carrier, i.e. they have one normal copy of the gene and one defective copy. Carriers are not affected by the disorder but they can have an affected child if their spouse is also a carrier.

What was I tested for?

To date, several mutations in five known genes have been reported to cause **MMA.** In collaboration with Caritas Baby Hospital we found that in your the most common cause for this disease in your community is a specific mutation, called N219Y, in a gene called MUT. You were teated only for this mutation.
This means that the test does not rule out all causes of MMA, but other causes for MMA are rare.

What does my result mean?

Normal – You are not a carrier, and your children are not at increased risk to have MMA.

Carrier (hetereozygote) – You have one normal and one defective copy of the MUT gene. You could be at risk for having children with MMA, depending on your partner.

Recommendations:

1) Your partner or intended partner should be tested for MMA and genetic counseling or consultation with your physician is recommended before marriage and before pregnancy.
2) Refer your relatives for genetic testing of MMA.

Affected (homozygote) –Both copies of the gene are defective.A person with two defective copies is affected with MMA. As noted above, the severity of the disease is variable.

Recommendations:

1) Consult with your physician as soon as possible.
2) Refer your relatives for genetic testing of MMA.

Notes:

If there is a history of MMA in your immediate family (up to first cousins), genetic consultation is recommended.

If you have further questions, please do not hesitate to contact us.

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