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Deanship of Graduate Studies

Molecular diagnosis of Porphyria suspected patients from Hebron District, Palestine

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Molecular diagnosis of Porphyria suspected patients from Hebron District, Palestine

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Dedication

I dedicate this work to my father who didn't scrimp me from anything,

To my mother who flooded me with affection, for her prayers that are always helping me, for her endless support and love.

To my husband Majed, my sisters Anhar and Enmar and to my brother Noor-Aldeen, for their support, encouragement, patience and love.

To anyone who taught me a letter which participated in making me like what I am now.

To the shiny eyes of the patients who look for an answer

To anyone who has a dream, and fights for it.

Thank you all

Nawras Zeidan Hassan Fatouni

Declaration

I declare that the Master Thesis entitled "**Molecular diagnosis of Porphyria suspected patients from Hebron District, Palestine**" 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.

Signed:

Nawras Zeidan Hassan Fatouni

Date:22/12/2018

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Abstract

Background: The porphyrias are heterogenous disorders caused by abnormalities in the chemical steps of hemesynthesis, which is a vital molecule for all of the body's organs. Porphyria symptoms arise mostly from effects on either the nervous system or the skin. Effects on the skin occur in the form of cutaneous porphyrias. Proper diagnosis is often delayed because the symptoms are nonspecific. Skin manifestations can include burning, blistering and scarring of sun-exposed areas.

Objectives: To date there have been no reports on the molecular analysis of Porphyria in the Palestinian population. Therefore, the aim of this study was to confirm the clinical diagnosis of porphyria by biochemical and genetic tests in suspected patients from Hebron district and to determine the gene that cause the phenotype and clinical symptoms of cutaneous porphyria.

Method: 40 patients from Tafouh /Hebron city–who have been clinically diagnosed with cutaneous porphyria were recruited**,** Biochemical and genetic studies were performed using different molecular techniques like cloning, Polymerase chain reaction (PCR), Restriction fragment length polymorphism (RFLP), sequencing and next generation sequencing to investigate the causative mutations at DNA and mRNA levels.

Results: According to this study, the biochemical test that involves checking the total porphyrin and porphyrin precursor levels in urine give normal results for all the tested patients. Continually none of the Uroporphyrinogen decarboxylase (*UROD*NC_000001.11), uroporphyrinogen III synthase (*UROS*NC_000010.11), and Ferrochelatase (*FECH*NC_000018.10) genes give any

significant mutation using PCR-based techniques, Restriction fragment length polymorphism (RFLP), Sanger and Next generation sequencing.

In conclusion, deeper molecular tests is needed as doing exome/whole genome sequencing , epigentic modification analysis for these patients or invistigating a novel mutation that causes a new genetic disorder.

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Table of Abbreviations

Abbreviation	Full Word
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
mRNA	Messenger Ribonucleic Acid
µL	Microliter
M	Molar
PBS	Phosphate buffer saline
EDTA	Ethylene diamintetraacetic acid
TAE	Tris acetate EDTA
V	Volt
Bp	Base pair
°C	Celsius or centigrade degree
BLAST	Basic local alignment search tool
MW	Molecular weight
OMIM	Online Mendelian Inheritance in Man
Mu	Mutant
Wt	Wild type
SNP	Single Nucleotide polymorphism
IVS	Intervening Sequence
UROD	Uroporphyrinogen decarboxylase

UROS	Uroporphyrinogen III synthase
FECH	Ferrochelatase
PCT	Porphyria cutaneatarda
CEP	Congenital erythropoietic porphyria
EPP	Erythropoietic Protoporphyria
NGS	Next generation sequencing

Chapter 1

Introduction

1.1 Historical background

The porphyrias are heterogeneous disorders arising from predominantly inherited catalytic deficiencies of specific enzymes along the heme biosynthetic pathway (Frank et al., 1998; Wiederholt et al., 2006).The history of the porphyria started with the first allusion to the pigment so called Porphyrins by Scherer et al (1841). Hoppe-Seyler in (1871) explained the disease biochemically, he clarified that the iron free hematin described by Mulder previously (1844) was mixture of two substances, named hematoporphyrin which resulted from an error in the biosynthesis of hemoglobin. Gunther in (1911) defined this entity as haematoporphyria congenita and was the first who recognize it as an inborn error of metabolism(Desnick and Astrin, 2002; McCall-Anderson, 1898; Ramanujam and Anderson, 2015).The terms hamatoporphyrin which named by Hoppe-Seyler resulting from the Greek (Porphyries)meaning purple, after his nomenclature the term PORPHYRIN was subsequently used by others. (Ramanujam and Anderson, 2015).

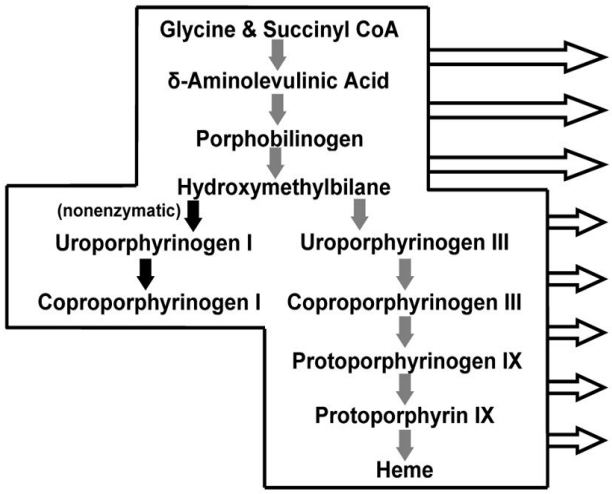
1.2 Biochemistry of Porphyria

1.2.1 Heme Biosynthesis (Porphyrin Biosynthesis)

The sequence of the biosynthetic pathway was excellently established by the middle of 1950s after many trials started by Sheminand Neubergerm who comprehended the primary description of how glysine was merged into heme (Goldberg et al., 2013).

Heme like chlorophyll are porphyrin, which considered as a primordial molecule and is one of the fundamental pigments of life, that play a critical role in various biological processes. Moreover, it acts as an essential cofactor for cytochromes, oxidases, peroxidases, catalases, hemoglobin and myoglobin in organisms (Tzou et al., 2014).

Its biosynthesis takes place mainly in the erythroblastic system (80%) and hepatocytes (15%) as well as in other tissues (5%), and it is formed by the condensation of 5-aminolevulinic acid (ALA) from glycine (Gly) and succinyl-CoA, catalyzed by δ-aminolevulinic acid synthase (ALAS). Two molecules of ALA are then condensed by ALA dehydratase (ALAD) yielding monopyrrole porphobilinogen (PBG). The enzyme hydroxymethylbilane synthase (HMBS, previously called PBG deaminase or PBGD) catalyzes the head-to-tail synthesis of four PBG molecules to form the linear tetrapyrrole HMB and discharges four molecules of ammonium then converted to uroporphyrinogen III in the presence of uroporphyrinogenIII synthase (*UROIIIS*) or spontaneously to uroporphyrinogen I. The uroporphyrinogen III undergoes decarboxylation by the cytoplasmic enzyme, uroporphyrinogen decarboxylase (*UROD*), to form coproporphyrinogen (Copro'gen) III and in the next step Coproporphyrinogen III oxidase (*CPOX*) then catalyses oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX. The next series of sequential decarboxylation reactions are carried out by a single cytosolic enzyme, called protoporphyrinogen oxidase (*PPOX*), to form protoporphyrin ;IX (PP). The final step of heme biosynthesis, catalyzed by the enzyme, ferrochelatase (*FECH*, also known as heme synthase), is the insertion of ferrous iron into PP to produce heme (Besur et al., 2014; Dailey and Meissner, 2013; Schubert et al., 2009; Szlendak et al., 2016). Step by step reactions are shown in Figure 1.1



Enzyme	Type of porphyria
δ-Aminolevulinic acid synthase	X-linked protoporphyria
α-Aminolevulinic acid dehydratase	ALAD porphyria
Porphobilinogen deaminase	Acute intermittent porphyria
Uroporphyrinogen III synthase	Congenital erythropoietic porphyria
Uroporphyrinogen decarboxylase	Porphyria cutanea tarda, hepatoerythropoietic porphyria
Coproporphyrinogen oxidase	Hereditary coproporphyria
Protoporphyrinogen oxidase	Variegate porphyria
Ferrochelatase	Erythropoietic protoporphyria

Figure1.1: The heme biosynthetic pathway showing intermediates, enzymes and types of porphyria associated with each enzyme.

1.2.2 Heme Biosynthesis Pathway Regulation

Regulation of the genes encoding the eight enzymes of the heme biosynthesis pathway can occur at the transcriptional, translational and post-translational levels. (Tzou et al., 2014).

The predominantly regulation occurs in the first step which is catalyzed by ALAS, the rate-limiting enzyme in the heme biosynthesis pathway, and regulated in several ways. Primarily, the enzyme is subject to inhibition by heme, although this may be of only minor significance in mammalian tissues. Furthermore, heme regulates at the transcriptional level in the liver cell where it seems to conjoin with an apo-repressorprotein(Jordan and Dailey, 1990).

A housekeeping promoter operated in all tissue types exists in ALAS1, PBGS, PBGD, and *UROS*. However, for ALAS2, PBGS, PBGD and *UROS*, erythroidspecific promoters drive gene expression. More notably, the alternative promoters found in PBGS, PBGD and *UROS* are locatedin intron 1, and an alternative splicing event is mandatory for the transcript generated from the housekeeping promoters and erythroid-specific promoters, consequently the presence of two genes, one encoding the liver mitochondrial enzyme and the other the erythroid enzyme, are regulated by altered tissue-specific mechanisms(Besur et al., 2014; Jordan and Dailey, 1990; Tzou et al., 2014).

1.3 Classification of Porphyria

Porphyria disorders are broadly classified as acute/inducible porphyrias or chronic cutaneous porphyriasas shown inTable1. (Karim et al., 2015; Thapar and Bonkovsky, 2008). Moreover, the classification is based on the main site of heme precursors production either in the liver (hepatic) or in bone marrow (erythropoietic). On the other hand, the classification can be according to the cardinal clinical features (either neuro-visceral or cutaneous) and/or by the enzyme defect (Besur et al., 2014; James and Hift, 2000; Magnus, 1968). Therefore, it is imperative to note that there is no one single simple classification of porphyrias, and the clinical features of various forms may be similar.

Disease	Primary enzymatic defect	Autosomal inheritance	Clinical features	
			Neurovisceral symptoms	*Photosensitivity dermatosis*
Acute/inducible porphyrias				
ALA-D deficiency porphyria	ALA dehydratase	Recessive	+	--
Acute intermittent porphyria	PBG deaminase	Dominant	+	--
Hereditary coproporphyria	Coproporphyrinogen oxidase	Dominant	+	+
Variegate porphyria	Protoporphyrinogen oxidase	Dominant	+	+
Chronic cutaneous porphyrias				
Congenital erythropoietic	Uroporphyrinogen III cosynthase	Recessive	--	++
Hepatoerythropoietic porphyria	Uroporphyrinogen decarboxylase	Recessive	+/-	+
Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Dominant (acquired variant exists)	--	+
Protoporphyria	Ferrochelatase	Dominant†	--*	+

Table 1.1 : Classification and Major Features of Human Porphyrias(Thapar and Bonkovsky, 2008).

†autosomal recessive inheritance has been described.

1.3.1 The hepatic porphyria

1.3.1.1 Clinical manifestations

Hepatic porphyrias comprise hereditary coproporphyria (HCP), variegate porphyria (VP), acute intermittent porphyria (AIP), aminolevulinic acid dehydratase deficiency porphyria (ADP), and porphyria cutaneatarda (PCT). Of these, ADP and AIP are categorized as acute porphyria but PCT as cutaneous, while VP and HCP presenting with both acute and cutaneous clinical manifestations (Arora et al., 2016; James and Hift, 2000).

In the case of the acute (neurologic) porphyrias, the origin of symptoms appears to be due to over production of a neurotoxic precursor (Bissell and Wang, 2015) , so the major manifestations of these disorders are acute neurologic attacks, which typically occur after puberty. Constipation, abdominal distension nausea, vomiting, tachycardia, hypertension, mental symptoms, extremity, neck, or chest pain, headache, muscle weakness, sensory loss, tremors, sweating, dysuria, and bladder distention are common (Balwani and Desnick, 2012; Szlendak et al., 2016).

Three of the acute hepatic porphyrias (acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), and variegate porphyria (VP)) are autosomal dominant disorders, affecting males and females equally. A fourth type, delta aminolevulinic aciduria (ALAD), is autosomal recessive and very rare (Bissell and Wang, 2015).

1.3.1.2 Pathogenesis of Acute Attacks

The pathogenetic mechanisms which cause the neurological dysfunction have stayed poorly understood. Obviously, stimulation of the acute attacks is prompted by severe fasting or dieting, alcohol, certain drugs (mainly barbiturates, hydantoins, rifampin, sulfonamides and endogenous steroid hormones, estrogen and progesterone), chemical compounds particularly paint, varnish, organic solvents, pesticides and other intercurrent illnesses or stress. So it is essential to recognize at-risk relatives and to offer asymptomatic heterozygote counseling to evade those factors, and other precipitants of the acute attacks (Balwani and Desnick, 2012; Besur et al., 2014;Yeung Laiwah et al., 1987).

1.3.2 Erythropoietic porphyrias

Porphyrins in red cells can cause photosensitive cell lysis, resulting in haemolytic anaemia. The two homozygous erythropoietic porphyrias, congenital erythropoietic porphyria (CEP) and hepatoerythropoietic porphyria (HEP), are accompanied by haemolytic anaemia of varying degrees. In contrast, erythropoietic protoporphyria (EPP), a heterozygous disease, infrequently associated with haemolytic anaemia. The effect of life-long anaemia in CEP or HEP may lead to compensatory increase of erythroid marrow, which may result in pathological fractures, vertebral compression or collapse, and shortness of stature. The haemolysis is also associated with varying degrees of splenomegaly and the production of pigment-laden gallstones (Sassa, 2006) .

1.3.2.1 Congenital Erythropoietic Porphyria (CEP)

Congenital erythropoietic porphyria (CEP; OMIM: 263700), also known as Gunther's disease, is a very rare with prevalence estimates to be less than one of million is affected with this disease (Ramanujam and Anderson, 2015; Thadani et al., 2000). . It is inherited as an autosomal

recessive trait in which the activity of uroporphyrinogen III cosynthase (URO-synthase), the fourth enzyme in heme biosynthesis is severely reduced (Mathews et al., 2001; Wiederholt et al., 2006).

The ~34-kb human URO-synthase gene was consigned to the narrow chromosomal region 10q25.3--- q26.3 and organized in 10 exons and 2 mRNA transcripts generated by alternative splicing, directed from unique housekeeping and erythroid-specific promoters, as well the expression arrays revealed that the housekeeping transcript was present in all tissues, while the erythroid transcript was only in erythropoietic tissues(Aizencang et al., 2000a; Aizencang et al., 2000b; Desnick and Astrin, 2002).Many mutations that cause CEP have been reported in the URO-synthase gene like missense and nonsense mutations, large and small deletions and insertions. In addition to that there are an altered splicing, frameshift, intronic branch poin mutations and erythroid-specific promoter mutations.(Desnick and Astrin, 2002; Fontanellas et al., 1996; Ged et al., 2004a; Xu et al., 1995; Zlotogora, 1997; Zlotogora, 2002).

The reduced URO-synthase activity leads to accumulation of uroporphyrin in other tissues that gives rise to skin photosensitivity, ulcers, erosions, erythrodontia, osteodystrophia, combining osteolysis and osteoporosis. Secondary infections of cutaneous lesions may induce to scarring, deformities, and disfigurement of the light-exposed parts of the body particularly on the forehead, upper cheeks, hands, ears, nose and eyelids. The severe photosensitivity can be noticed in infancy or childhood (Di Pierro et al., 2015; Sassa and Kappas, 2000; Thadani et al., 2000).

Few factors are responsible for the phenotypic inconsistency including the amount of the remaining URO-synthase activity, the haemolysis degree, consequent stimulation of erythropoiesis, and the exposure to ultraviolet light. Consequently, as in other porphyrias, a relationship between environmental factors and the deficient enzyme activity is essential in the clinical expression of the disease (Desnick and Astrin, 2002; Sassa and Kappas, 2000; Thadani et al., 2000).

Typical and severe CEP symptoms encountered within a Palestinian family in France due to novel mutation (S47P).Four siblings were shown to have the disease (Ged et al., 2004b).

1.3.3 Cutaneus Hepatic Porphyria

1.3.3.1 Porphyria CutaneaTarda (PCT)

Porphyria cutanea tarda (PCT; OMIM: 176100) is a hepato cutaneous porphyria triggered by a deficiency in uroporphyrinogen decarboxylase (*UROD*), this cytosolic protein is the fifth enzyme in the hemesynthesis pathway, and can be classified into severaltypes, familial PCT (fPCT) and sporadic PCT (sPCT) which being the most common. PCT is found in a sporadic form (sPCT) with no evident mutation and an inherited form, familial PCT (fPCT) with mutation in the gene *UROD*. The disease fPCT is inherited in an autosomal dominant way with reduced penetrance described by low *UROD* activity in all cells, the reduced or incomplete penetrance means that not all individual carrying a mutation in *UROD*-gene will show the clinical phenotype(Bulaj et al., 2000; Christiansen et al., 2016; Elder, 1998; Poblete-Gutiérrez et al., 2004).

The level of *UROD* activity in erythrocytes has conventionally been used to discriminate between fPCT and sPCT, because sPCT shows reduced *UROD* activity only in the liver while fPCT is characterized by low *UROD* activity in all cells(Aarsand et al., 2009)

The human *UROD* gene has been mapped to the short arm of chromosome 1 (1p34). The gene is ~3.4 kb and contains a single promoter and 10 exons. Its mRNA has 1.2 kb and encodes a 367 amino acid poly peptide with a molecular weight of approximately 41 kDa (Dubart et al., 1986).

Approximately 70 different mutations have been identified in *UROD* that are associated with F-PCT or the homozygous variant of PCT that named hepato erythropoietic porphyria [HEP]. PCT, perhaps the most common of all the porphyrias worldwide. Estimates of prevalence have fluctuated from one in 5000 to 25000 or one people in 10,000 (Aarsand et al., 2009; Ramanujam and Anderson, 2015).

PCT is characterized clinically by a photosensitive dermatosis associated with skin fragility which is considered a specific feature and blistering that usually presents with vesiculobullous eruptions on the hands and face, a minimal trauma is followed by superficial erosion soon covered by a crust, Hypertrichosis is often seen on the upper cheeks, ears and arms. Increased pigmentation of sun-exposed areas is common and signs of liver damage.

Porphyria cutanea tarda is associated with an increased incidence of the haemochromatosis gene mutation. Treatments for PCT include withdrawal of aggravating factors, phlebotomy and oral antimalarial medications.

The rare homozygous form of f-PCT is referred to as hepato erythropoietic porphyria (HEP), and is characterized by the onset in early childhood with more severe clinical features than PCT, resembling that of congenital erythropoietic porphyria(Gómez-Abecia et al., 2013).

1.3.3.1.1Hepatoerythropoietic Porphyria

Hepatoerythropoietic porphyria (HEP) is a severe form of cutaneous porphyria that manifest early in infancy, with similar frequency in females and males, UROD activity in HEP is exceedingly low, leading to the assumption that HEP maybe a homozygous form of PCT. The disorder is caused by homozygosity or compound heterozygosity for mutations of the uroporphyrinogen decarboxylase (URO-D) gene (Moran-Jimenez et al., 1996; Phillips et al., 2007).

HEP is characterized clinically by severe photosensitivity, skin fragility (bullae, erosions, and scarring) in sun-exposed areas, facial hypertrichosis, chronic anemia, polyarticular arthritis with high levels of circulating porphyrins, there may be a red/brown discoloration of teeth due to the deposition of porphyrins in the enamal layer of the developing tooth (erythrodontia)and pink-to-red-colored urine. HEP commonly presents in early childhood in contrast to PCT, which is mostly revealed in adults ,increased severity causing disfigurement(Cantatore-Francis et al., 2010; Meguro et al., 1994).

The disease arises worldwide and is inherited as an autosomal recessive trait either gene deletion, defective transcription, abnormal processing of the mRNA, unstable or untranslatable mRNA or that the protein coded for by the mutant gene is unstable in vivo that causing markedly lacking, but not absent, activity of the heme biosynthetic enzyme, uroporphyrinogen decarboxylase (UROD)(De Verneuil et al., 1986).

1.3.3.2. Erythropoietic protoporphyria (EPP)

Erythropoietic protoporphyria (EPP; OMIM: 177000) is an inherited disorder of heme biosynthesis that outcomes from a partial deficiency of ferrochelatase (encoded by the *FECH* gene), the last enzyme in the heme biosynthesis pathway. The human *FECH* gene is located on chromosome18q21.3 with 11 exons and 10 introns that catalyse the insertion of iron into protoporphyrin ring to generate the last product, haem (Balwani et al., 2014; Parera et al., 2009). EPP was first designated by Magnus in 1961, and was described by excessive production and consequent accumulation of metal-free protoporphyrin (PP) in erythrocytes, plasma, skin, and liver(Gouya et al., 2006; Lane et al., 2016a).

The main site of protoporphyrin overproduction is localized to the bone marrow, where erythrocytes are produced .Protoporphyrin, unlike other porphyrins, is lipophilic molecule and thus detached from the body only through hepatic excretion into bile or feces. In contrast to the other forms of cutaneous porphyria, the urinary porphyrins are usually normal (Thapar and Bonkovsky, 2008).

Clinically, EPP is described by photosensitivity that arises in early childhood and includes burning, swelling, itching, and painful erythema in sun-exposed areas. Chronic liver disease is an important complication in a minority of EPP patients, and in some cases liver transplantation has been performed(Balwani et al., 2014; Parera et al., 2009).

Repeated photosensitivity episodes cause alteration in skin appearance with everlasting changes, such as skin thickening with a waxy or leathery appearance, lichenification, grooving around the lips and areas of hyperkeratosis. These lesions are usually located on the dorsa of the hands and face, as these skin areas are usually exposed.

The condition is inherited as an autosomal dominant disorder, but recessive inheritance with two mutated *FECH* alleles has also been well-defined.

EPP is the third most common porphyria, with a general estimated incidence of two to five in 1,000,000; it is the most common porphyria in children. On the other hand, the autosomal dominant state has been indicated worldwide with prevalence between 1:75,000 and 1:200,000, it looks that males and females are likewise affected, furthermore the prevalence of autosomal recessive EPP has not been recognized but few studies in a small number of families propose that

up to 20% of patients may have autosomal recessive disease (Lane et al., 2016b; Lecha et al., 2009; Whatley et al., 2004).

1.4 Diagnosis of Porphyria

The diagnosis of CEP, PCT and HEP is established in probands by identification of raised up porphyrins in the urine (≈20 times of the usual upper limit) mainly uroporphyrin and heptacarboxylporphyrin besides to the increased erythrocyte zinc protoporphyrin level. On the other hand, the diagnosis of EPP is established by detection of markedly increased free erythrocyte protoporphyrin since the urinary porphyrins are usually normal. Even though each type of porphyria has a typical porphyrin excretion pattern, those excretion profiles of urinary and faecal porphyrins are sensitive but unspecific in differentiating porphyrias. Thus, the identification of pathogenic variants in UROS, UROD and *FECH* genes by molecular genetic testing approve the diagnosis(Balwani and Desnick, 2012; Danton and Lim, 2006; Meguro et al., 1994;Ramanujam and Anderson, 2015).

Table 1.2: Accumulation and excretion patterns of heme precursors and intermediates in body fluids

Porphyria	Enzyme Deficiency	Urine Precursors	Urine Porphyrins	Fecal Porphyrins	Erythrocyte Porphyrins	Plasma fluorescence
Acute Intermittent Porphyria (AIP)	HMB synthase (PBG deaminase)	PBG > ALA	Uro from PBG	Not increased	Not increased	618–622 nm
Variegate Porphyria (VP)	Protoporphyrinogen oxidase	PBG > ALA	Uro from PBG Copro III	Proto IX > Copro III	Not increased	626–628 nm
Hereditary Coproporphyria (HCP)	Coproporphyrinogen oxidase	PBG > ALA	Uro from PBG Copro III	Copro III	Not increased	618–622 nm
ALA Dehydratase Deficient Porphyria (ADP)	PBG synthase (ALA dehydratase)	ALA	Copro III	Not increased	Zn-proto	N/A
Porphyria Cutanea Tarda (PCT)	Uroporphyrinogen decarboxylase	Not increased	Uro, Hepta, Isocopro	Isocopro	Not increased	618–622 nm
Erythropoietic Protoporphyria (EPP)	Ferrochelatase	Not increased	Not increased	Proto IX	Proto IX	634–636 nm
Congenital Erythropoietic Porphyria (CEP)	Uroporphyrinogen III synthase	Not increased	Uro I, Copro I	Copro I	Uro I, Copro I, Proto IX, Zn-proto	618–622 nm

http://www.pinsdaddy.com/signs-and-symptoms-of-porphyria

1.5 Research significance

Porphyria is a group of at least eight metabolic disorders resulting from a malfunction in one of the eight steps in the body's synthesis of heme. In Palestine, several pateints were clinically diagnosed by inherited porphyria, all of them badly suffered from the clinical PCT symptoms.

This is the first genetic study on the Palestinian population focusing on the porphyria. The outcome of this research will contribute in identification of clinically asymptomatic heterozygous carriers within families. The study will enable correct and early diagnosis, which is required to develop a management plan that is appropriate to improve prenatal diagnosis for Porphyria families. It will provide them with a better genetic counseling correspondingly the outcome of this study and other future studies will help in drawing a genetics map for this disease in Palestine, which can be used in better advising and management of the Palestinian patients.

The objectives of this research are:

1. To confirm clinical diagnosis of porphyria by biochemical test and genetic study.
2. To determine the genes that cause the phenotypic characterization and symptoms.
3. To determine the molecular nature of the mutations that are correlated with the phenotypes in Palestinian patients and establish a diagnostic marker for mutation detection that is correlated with disease symptoms.

Chapter 2

Material and Methods

2.1 Working Plan

In this study we planned to reveal the molecular nature of the genetic mutations that is suspected to be the cause of cutaneous porphyria in suspected Palestinian patients who clinically diagnosed by porphyria based on their symptoms and family history.

Molecular genetic approaches that include single-gene or a multi-gene testing, and more comprehensive genomic testing like deep sequencing were used. In parallel, the identification of the unique pattern of overproduction of porphyrin and porphyrin precursors by biochemical test was performed using 24 hrs Urine collection / Porphyrin test.

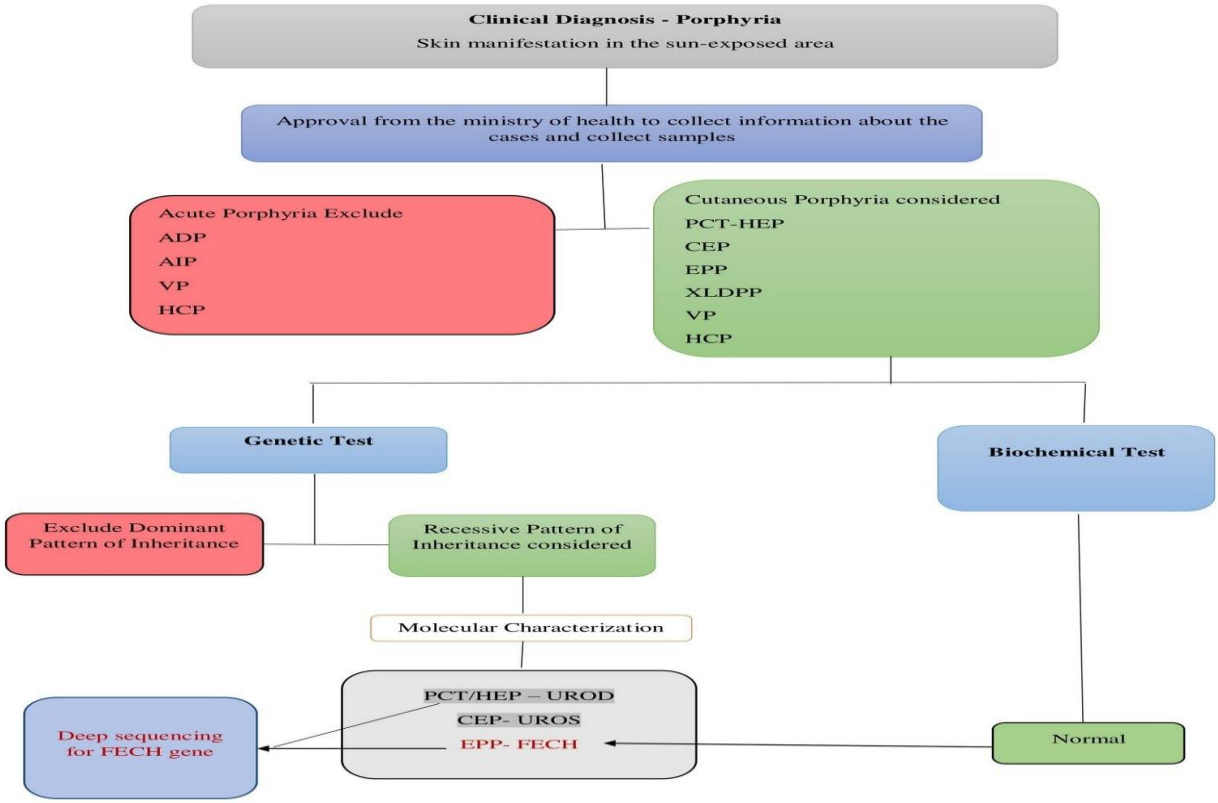


Figure 2.1: Flow chart demonstrates the working plan for our study

The porphyria types divided according to the symptoms and inheritance pattern, we followed the recessive pattern according to families' history of cutaneous symptoms, and the genetic studies for three genes were performed in parallel with biochemical test.

2.2 Patients and Controls

The study included all suspected Porphyria patients in Tafouh village- Hebron District. The population of the village around 10,597 persons in 2007[Palestinian Central Bureau of Statistics, 9002, General Population, Housing and Establishment Census, Ramallah, Palestine].

Forty related and unrelated Porphyria suspected patients, family members and three normal controls were included, Twenty two patients out of forty were enrolled in the study .The diagnosis of Porphyria was established according to the clinical presentation and followed by biochemical and genetic tests to confirm and specify the compatable disorder.

2.3Ethical issues

Patients and their families were informed about the aim of the study. The study was done in collaboration with the Palestinian Ministry of Health in Ramallah. The patients were clinically diagnosed as porphyria suspected patients based on the consensus of ministry of health dermatologist. All patients were invited to the primary health Clinic in order to participate in the study. Participation was on a voluntary base.

An agreement was assigned between Al-Quds University (Al-Quds Nutrition and Health Research Institute) and Medicare lab-Hebron branch to transfer the urine samples to Biolab in Jordan. One-liter light protected plastic containers were provided to the patients for urine collection, this step was approved correspondingly by the Ministry of health and Tafouh municipality.

2.4 Logistics of Sample Collection

2.4.1 Samples for Genetic analysis

Peripheral blood (5 ml) was taken from each patient. The drawn blood was placed in an ethylenediaminetetraacetic acid (EDTA) tube. The contents were mixed by inverting the tubes 5-8 times without shaking. Blood samples in EDTA tubes for DNA and RNA extraction were transferred immediately to Al-Quds Nutrition and Health Research Institute (ANHARI) Lab. A buffy coat separated and saved at 4-8 C^{o}at (ANAHRI) refrigerator until further analysis.

Our study included DNA and RNA investigation to cover all types of possible mutations including missense and nonsense mutations, large and small deletions and insertions. In addition to study any altered splicing that cause exon skipping, frame shift, intronic branch point mutations, etc.

2.4.2 Samples for Biochemical Studies

Accurate diagnosis of clinically overt porphyria requires identification of the unique pattern of overproduction of total porphyrin and the following precursors (Uroporphyrin Heptacarboxy porphyrin, Hexacarboxy porphyrin, Pentacarboxy porphyrin, CoproporphyrinI Coproporphyrin II).Therefore, fourteen patients participated in the biochemical test, (24-Hour) urine sample collection for Porphyrins fractions analysis was requested through Medicare lab- Hebron branch to transfer the samples to Biolab in Jordan. The special container was provided to the patients, moreover they were provided with the instruction how to collect the sample and to store it, which were described at the Medicare lab website guidelines [http://medicare.ps/en/test/porphyrins-fractions-urine].The collected samples were protected from light and kept refrigerated during collection.

2.4.3 Preparing DNA samples

1. Genomic DNA was extracted from blood Buffy Coat using a QIA amp DNA blood Mini kit according to the manufacturer's protocol. Control DNA was extracted from healthy people with no symptoms of porphyria and/or family history.
2. The concentration of extracted DNA was measured using Nano Drop ND.
3. The extracted DNA was stored at -20 C for mutation analysis.

2.5 Molecular DNA analysis

The DNA analysis was performed for three genes (*UROD*), uroporphyrinogen III synthase (*UROS*), and Ferrochelatase (*FECH*) according to the recessive pattern of inheritance in those genes to find the compatible mutation for the cutaneous symptoms.

2.5.1 UROD gene analysis on DNA and RNA levels

DNA analysis was done using the followed methods: cloning the whole gene (3.6Kb) including the exons and introns followed with Sanger sequencing and next generation sequencing. RNA analysis was done by production of cDNA from the extracted RNA followed by amplification of the spliced transcript.

2.5.1.1 Cloning UROD gene in a plasmid vector and Sanger sequencing (UROD gene amplification)

The molecular analysis of DNA and mutations screening have been made possible by the cloning of DNA since limited volume of blood sample was collected and several rounds of amplifications were needed to detect the suspected mutations.

2.5.1.2 PCR Master Mix set up and Condition of the PCR reaction

The *UROD* gene (3.6 Kb) for three patients from different families was amplified using Uro-DU forward and Uro-DL reverse newly designed primers (table 2.2),in a final concentration of 0.4µM. The LongAmp Hot Start Taq X2 Master Mix (M0533S, Lot:0061309) was used. PCR preparations were done as described in (table 2.1). The PCR reaction conditions were started

with initial denaturation for 30 second at 94ºC. Amplification was achieved in 36 cycles started from denaturation step for 20 seconds at 94ºC followed by 30 seconds at 55ºC for annealing and 3 min at 72ºC for extension, respectively. An additional extension step was performed for 10 minutes at 72 ºC. The reaction was then cooled down to 4ºC and either stored at -20 ºC or used directly for analysis. The reaction was carried out in special PCR tubes (0.2 ml Axygen Inc., USA) using Biometra thermocycler (TProfessional basic gradient).

Table 2.1: Setting up PCR reaction using LongAmp Hot Start Taq 2X Master Mix

Component	**Volume for one reaction (µL)**	**Final Concentration**
2X LongAmp Hot Start Taq	25	1X
Uro-DU Forward primer (10 µM)	2	0.4µM
Uro-DLReverse primer (10 µM)	2	0.4µM
Free nuclease water (up to 50 µL)	16	
DNA template	5	≈ 100 ng/µL

2.5.1.3 Detection and Purification of PCR product

The amplified gene of the expected size of (3.6 Kb) was separated by agarose gel electrophoresis. Agrose gel (1 %) (Seakem LE Agarose, Lonza cat#: 50004) was prepared in X1 TAE (Tris Acetate-EDTA buffer)(X10 TAE = 400nM Tris-HCL, pH 8.3; 200mM Na-Acetate; 20 mM EDTA). The agarose was boiled until it was well melted; ethidium bromide (0.5 mg/ml) was added when agarose suspension had cooled down, carefully mixed, poured into the agarose gel casting system (Bio-Rad, Consort EV245) and a comb was added to generate sample wells. PCR product (50ul) was mixed with 4 µl of the X6 DNA loading dye (Thermoscientific) and loaded into one gel well along with 4 µl of 1 kb molecular weight ladder marker, (Thermo scientific gene ruler 1 kb Plus DNA Ladder. The PCR products (10 ul) were visualized under UV light. A digital image of the gel was taken using a gel documentation system (Bio imaging system,Minilumi). The PCR product rest was kept frozen for Purification and preparing for cloning.

The Wizard® SV Gel and PCR Clean-Up System (Promega, Part#:9FB072) was used forPCR product purification. The steps were accomplished according to the manufacturer's instructions.

The purified samples were eluted in 50 µL of Nuclease-Free water and stored at -20 °C until further analysis.

2.5.1.4 Blunting, ligation reaction and cloning of UROD gene

The blunting step is essential for the PCR product since Taq polymerase product is an overhang product and thus further plasmid was prepared as blunt end according to the manufacturer's instructions (Thermo Scientific Clone JET PCR Cloning Kit, #K1231).The process of ligation was completed on ice by adding 1.4µL from pJET1.2/blunt Cloning Vector (50ng/ µL) and 1.4 µL from T4 DNA Ligase to the blunting reaction mixture. Schematic representation of the pJET1.2/blunt Cloning Vector was shown in figure 2.1

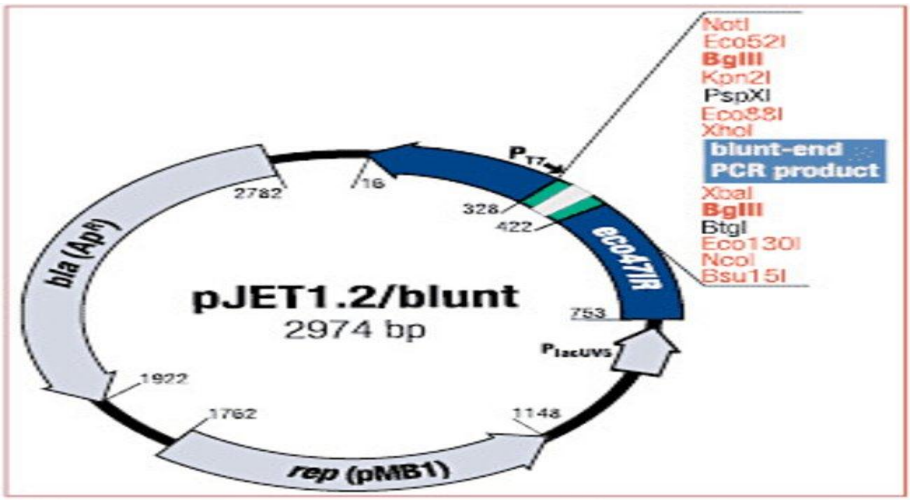


Figure 2.2: Map and features of pJET1.2/Blunt Cloning Vector

2.5.1.5 Competent cells preparation (DH5α)

Competent cells are -ready to use- bacterial cells that possess more easily altered cell walls by which foreign DNA can be passed through easily. Most types of cells cannot take up DNA efficiently unless they have been exposed to special chemical or electrical treatments to make them competent. The standard method for making the bacteria permeable to DNA involves treatment with calcium ions. Brief exposure of cells to an electric field also allows the bacteria to take up DNA and this process is called as electroporation. The competency can be obtained by creating pores in bacterial cells by suspending them in a solution containing high concentration

of calciumi.e$CaCl_2$. DNA can be forced into the host cell (DH5α*E. coli*) by heat shock treatment at 42^oC for the process of transformation. The protocol was followed as mentioned below:

Day1:

1.A portion from the top of the frozen glycerol of DH5α*E. coli* stock was scraped off by sterile disposal inoculating loop and inserted into 2ml LB broth (LB broth was prepared by adding 10 g from Trypton,5 g from Yeast Extract and 10 g NaCl in 1 Litre of distilled water, the obtained broth media was autoclaved at 121°C (249 °F) for around 20 minutes).

2. DH5α *E. coli* stock was retained back to -80C immediately.

3. The tube was incubated with shaking at 37 °C for 16-20 hours.

Day2:

4. 1ml culture broth was added to 49 ml of LB medium (1:50), to achieve bacterial log phase. Then it was incubated in a water bath at 37°C with shaking for 2.5 hours.

5. The turbidity was observed by eye and measured by spectrophotometer as it reach0.4OD at wavelength 550A.

6. The flask was placed in an ice for 10 minutes [After this point the cells should be never warmed].

7. The culture was transferred into pre-chilled 50ml falcon tube.

8. The centrifugation process was performed at 4000 rpm for 15 minutes at 4°C

9. The medium was removed, the cell pellet was washed with cold 50mM CaCl2 and finally re-suspended by gentle flicking in a final volume of 5ml of ice cold CaCl2 (50mM).

10. The tube was incubated on ice for 30 minutes then centrifuged at 2500 rpm for 10 minutes at 4°C

11. The medium was removed, the cell pellet was re-suspended in a final volume of 5ml ice cold CaCl2 50mM -by gentle flicking-including 7% DMSO. 0.5 ml were prepared in aliquots and stored in -80 °C for transformation.

2.5.1.6 Transformation

As shown in figure 2.2 the prepared competent cells (200 µl) were thawed directly on ice, then 2 µl of the ligation mixture was added. The reaction was incubated for 30 minutes on ice, incubated at 42°C for 1 min, transferred immediately on ice, and then 800 µl of LB was added to the tube and incubated for 1 hour at 37°C with 200 rpm shaking.

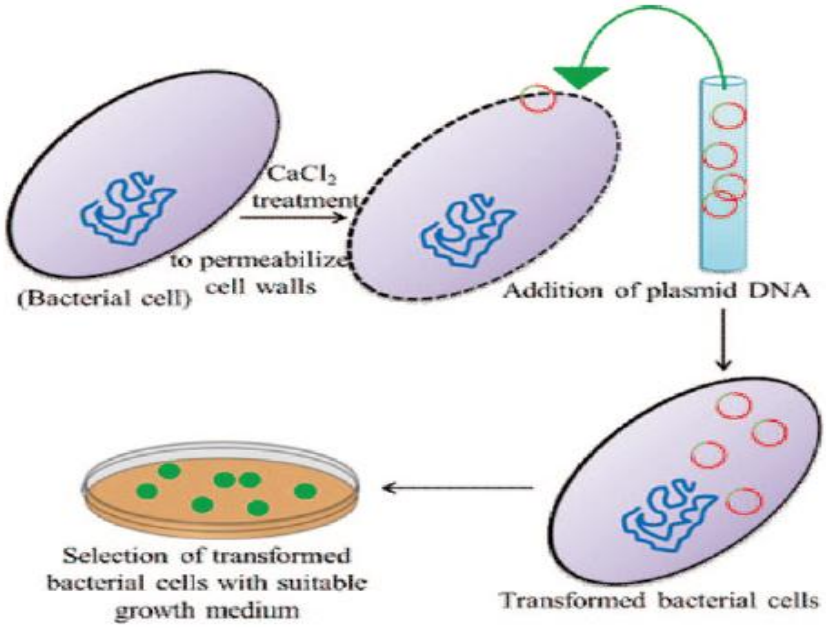


Figure 2.3: Preparation of competent cells by CaCl2 treatment and transformation

2.5.1.7 Positive selection of transformed DH5α cells

The vector contains lethal gene eco47IR enables positive selection of recombinant plasmid that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Re-circularized pJET1.2/blunt vector molecules lacking an insert express a lethal gene, which kills the host *E. coli* cell after transformation and keep only positive selection of recombinant plasmid.

To confirm the transformation process around 60µl of the transformed cells were plated on LB Agar contains Ampicillin for positive selection in Petri dishes (*E.coli* Fast Media, Fermentas Life Science Company). The plate was incubated at 37°C for 12-14 hours, at least 10 colonies after that was inoculated in 1-5 mL of LB medium supplemented with the appropriate selection antibiotic (Ampicillin). Then they were incubated overnight at 37°C while shaking at 200-250 rpm.

This step was prepared to enhance the growth rate. The turbidity was checked, then 500 µl from each suspension was taken, subsequently boiled at 95°C for 10 minutes, centrifugation process was done to get a DNA pellet which re-suspended in 70 µl free nuclease water.

PCR master mix and amplification reaction was done as mentioned previously in section 2.4.2.1but the Primers for *UROD* gene were replaced by the plasmid pJET1.2 forward and reverse sequencing primers, (10 µM)(Thermo Scientific Clone JET PCR Cloning Kit).

2.5.1.8 Plasmid extraction for DNA preparation from clones

This part was completed by using PureYield™ Plasmid Mini prep Zxcvbnm System, Promega, Part# 9FB093.The protocol was followed according to the leaflet by preparing lysate, washing and elution step then the eluted plasmid DNA was stored at –20°C. The required plasmid concentration was needed to perform sequencing step was ranged between (100-300 ng/µL).

2.5.1.9 UROD gene and Sanger sequencing

The screening for genetic changes were performed for full gene and for internal sequences that we designed as explained below in the table 2.2 and figure 2.3 to detect any homozygous mutation or compound heterozygotes that cause *PCT* or *HEP* a recessive form of *PCT*. In addition, primers were designed with consideration of overlaps to achieve complete sequence of the gene (see figure 2.3 below).

Table 2.2: Primer sets that used for UROD gene amplification and sequencing

Primer code	**Sequence 5'---3'**	**Reference**
pJET 1.2 F	5'-CGACTCACTATAGGGAGAGCGGC-3'	Thermo Scientific CloneJET PCR Cloning Kit ,#K1231
Uro-DU	5'-TATGGACCTGGCTGGATAAGACTGTTGGT-3'	Mendez,M.,Sorkin1998
UroDR3	5'-CGGGAGTGTAGTCTGTTTCCT-3'	Newly designed
Uro-DMF	5'-GGTAGATAGCGGTCCTGGAC-3'	Newly designed
Uro-DM2F	5'-TCCTTCTATCAGTCCAGTCAAGG-3'	Newly designed
Uro-DMR	5'-AACCACCTCATAGCCAGCTT-3'	Newly designed
Uro-DL	5'GGGACAATCTTTCACAAACAAAACTACAC-3'	Mendez,M.,Sorkin1998
pJET 1.2 R	5'-AAGAACATCGATTTTCCATGGCAG-3'	Thermo Scientific CloneJET PCR Cloning Kit ,#K1231

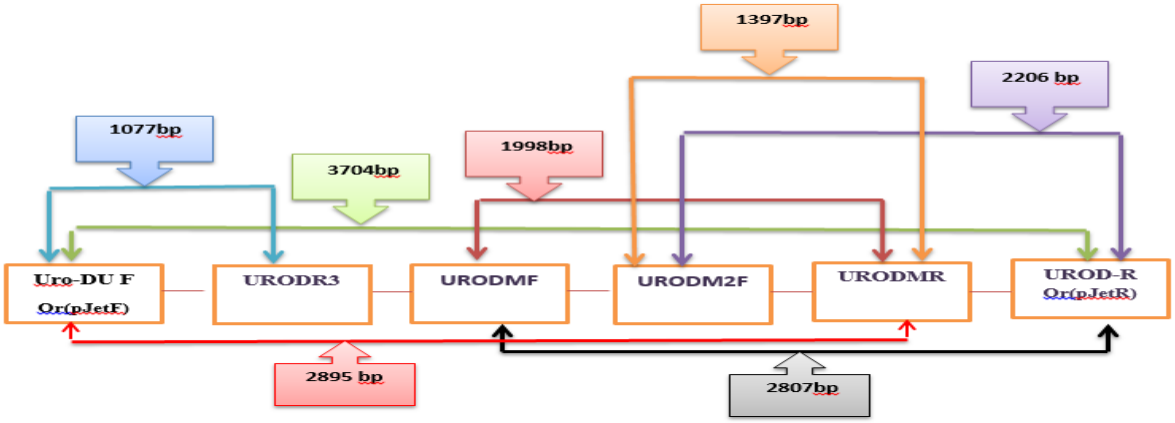


Figure 2.4 : Distribution of primer sets cross UROD gene and the PCR product size.

2.5.1.10 UROD gene and next generation sequencing

For next generation sequencing (NGS), several PCR reactions target regions in the whole gene were determined. These regions were amplified by using Long Amp Hot Start TaqX2 Master Mix (M0533S/Lot:0111503).

The master mix reaction was done according to the kit leaflet as well as the PCR reaction for large sizes of PCR products was started with initial denaturation step for 30 second at 94ºC. Amplification was achieved in 33 cycles started from denaturation step for 20 seconds at 94ºC followed by 20 seconds at 55ºC for annealing and 5 minutes at 65 ºC for extension respectively. An additional extension step was performed for a further 10 min at 65 ºC, to assure a complete extension of the amplified product. The reaction was then cooled down to 4ºC and either stored at -20 ºC or used directly for sequencing.

For each patient all PCR products were combined then it was purified by using AGENCOURT® AMPURE® XP beads (X1) , DNA library was prepared using Nextera XT kit (Illumina) according to manufacture protocol. Deep sequencing was done using Mid throughput Nextseq kit usigNextSeq500 Illumina machine.

2.5.2 UROS gene analysis

2.5.2.1 RNA Extraction and cDNA production of UROS gene

Previously a novel mutation T>C was detected in exon 3 of the *UROS* gene, the substitution of serine by proline at the amino acid residue 47 (S47P , rs397515527), hot spot regions within exon 10,4, intron and exon 2,all were examined in this study, on the other hand multiple alternatively spliced mRNAs were detected earlier, thus mRNA was examined also in this study.(Ged et al., 2004a)

Genomic RNA was extracted immediately when the samples were received from buffy coat using Nucleospin RNA (DNA, RNA, Protein purified) commercially available kits according to the manufactures instructions. Red cell lysis solution was used (EpicentreKit:Master pure DNA purification kit for blood version II , Cat.NoMB711740) to get rid from RBCs.The extracted RNA (50ng/μL) was converted directly to cDNA using a commercial kit (First Strand cDNA Synthesis using ProtoScript II Reverse Transcriptase, M0368).

2.5.2.2Amplification of cDNA – UROS gene and Sanger sequencing

The amplification process was performed using PCR Q5® High-Fidelity DNA Polymerase kit (New England Biolab, M0491).Specific DNA regions were amplified using specific primers that were prepared for exons and hotspot areas within *UROS*gene, F1 primer was excluded since it was cross reacted with a genomic DNA sequence and F2 primer was used instead. The PCR conditions and gel electrophoresis were followed as mentioned in the kit leaflet. The sequences of forward and reverse primers, the annealing temperatures, and the size of PCR product are described in the (table 2.3) below.

Table 2.3: Newly designed Primers code, sequences and sizes for each target for *UROS* gene

Primer code	Sequence 5'---3'	PCR product Size (bp)
IntEx2-3 FW	TCTTTCCGGAACCATAAACG	1190
IntEx2-3 REV	CACTGAAGCTGTGGGCACT	
Ex4 FW	AAATTCAGTGCCCACAGCTT	701
Ex4 REV	AGAAGTGCAGCTGCTTCTGG	
Ex10 FW	CAGAAGCTGGGCTAGAATCG	442
Ex10 REV	GTCCTCAGGTGCTTCCACT	
F1-mRNA FW	ATGGCGGTGCGCATGCGCGA	1347(F1R)
F2-mRNA FW	ATTGCTCCTGCAGCCTTTT	1204(F2R)
R-mRNA REV	AAACCACACTGACTACCTGTGC	
EX3a FW	TCAGAGGAACTGACGCAGAA	314
EX3a REV	CCTGGAGCCAGGTGAAGTTA	

2.5.3FECH gene analysis

After receiving a biochemical results for (24-Hour) urine sample collection for Porphyrins fractions analysis, the results were compatible with biochemical characteristics of Erythropoietic Protoporphyria (EPP) which oriented us toward screening *FECH* gene.

Screening patients for absence of the IVS3-48C allele to select those with the T/T genotype for mutational analysis of the *FECH* gene should identify most patients with autosomal recessive disease.

Patients and control subjects were genotyped for the FECHIVS3-48C/T polymorphism using a set of newly designed primer (GTGTTGTGTGTCCTGAATCTT) and (GAATGGTGCCAGCTTACTAAA) to obtain a 282 bp PCR product.

The amplification process was performed using Thermo scientific X2 PCR master mix kit (Lot: 00364507) according to the kit instructions.

The PCR reaction started with initial denaturation step for 2 minutes at 95ºC. Amplification was achieved in 35 cycles started from denaturation step for 25 seconds at 95ºC followed by 35 seconds at 53ºC for annealing and 40 seconds at 72ºC for extension respectively. An additional extension step was performed for a further 5 min at 72 ºC, to assure a complete extension of the amplified product. The reaction was then cooled down to 4ºC and either stored at -20 ºC for analysis by sequencing and RFLP technique. PCR products was loaded and captured as previously described.

2.5.3.1 Restriction fragment length polymorphism for FECHIVS3-48C/T polymorphism detection

The PCR product was digested with *TseI* restriction enzyme (1 ul) Cat #:R0591S). The digested product was loaded on 2% agarose gel and captured as previously mentioned in section 2.4.2.2

2.5.3.2 FECH gene and Next generation sequencing

After Restriction fragment length polymorphism (RFLP) was performed for FECHIVS3-48C/T polymorphism a comprehensive genetic analysis was done for *FECH* gene by deep sequencing in order to detect any probable mutation.

The steps were followed as mentioned in section 2.4.4.But a slight changing was done for smaller size of PCR product by modifying the extension time 1 minute instead of 5 minutes for the long sequences as explained in the table below.

Table 2.4: Newly designed Primers code, sequences, sizes for each target and the extension time for FECH gene

Primers code	Primers' sequences	Target size/bp	Extension time/ Minutes
EXON1- PCR1	GGAGCGGGCTTCTAGCTC CAGACCCACTTACCGCTCTG	408	1
EXON2-PCR2	GCCTGCAGAGAAATGCTAGG GCTATTGAACGGAAGCCAAG	405	1
Exon34-PCR3 Intron3	TGTGACGGCAGTGAAAAGAG AATAGCAGCAGCCTCAATCC	2522	5
Exon56-PCR4 Intron 5	CCGTCAGTGCCATAGGAAAT CAAACCCAGAAGGGATGAGA	3900	5
Exon789- PCR5 Intron 7,8	ATGCTGAGAGGCTGGACTGT GAGGACACCGTACATGCAAA	5200	5
Exon10,11-PCR6 Intron 10	GGAAGGGGAAGGGACATAAG CTGTCCCTGGAGACCAGAAG	1650	1
Exon 1 –PCR2	AGTCCAGCAGGTTTTGCAGT ACCTTCCCTCGCGTAAT	710	1

Pool of the same sample of PCR 1-6 was collected, purification and library preparation was done as mentioned above. DNA sequencing was done as described above on Nextseq 500 machine from Illumina.

2.6 Bioinformatics analysis

PCR amplicons were examined by direct automated bidirectional sequencing using the same set of primers used for PCR amplification, the results were analyzed using bioinformatics tools: Bioedit Program was used for sequence quality determination: http://www.mbio.ncsu.edu/Bioedit/bioedit.html

NCBI website was used for DNA sequence blast analysis: http://www.ncbi.nlm.nih.gov , and for the multiple alignments and homology predication multiple sequence alignment by Florence Corpet program was also used http://multalin.toulouse.inra.fr/multalin. For NGS analysis free online galaxy program was used: https://usegalaxy.org/ and DNA sequences assembly using Geneious bioinformatics software.

Chapter 3

Results

3.1 Patients and Controls

The history of the targeted families have been started since 65 years ago, symptomatic patients were noticed after few months of birth when the child start crawling and becomes exposed to the sunlight.

The Patients age ranges from several months to 65 years, all patients were related to three families with a high percentage of consanguineous marriage. Some of them showing a sever photosensitivity accompanied by scarring and mutilation. Males and females are likewise affected. Photos for some patients are shown in appendix D explaining the symptoms.

Primarly after the family Pedigrees followed the mode of inhiritance considered as recessive inhiritance pattern.The parents appeared normal without any symptoms but one or more siblings were affected. Different patients from different families have been chosen for genetic screening according to the age group and severity of symptoms (figure 3.1).

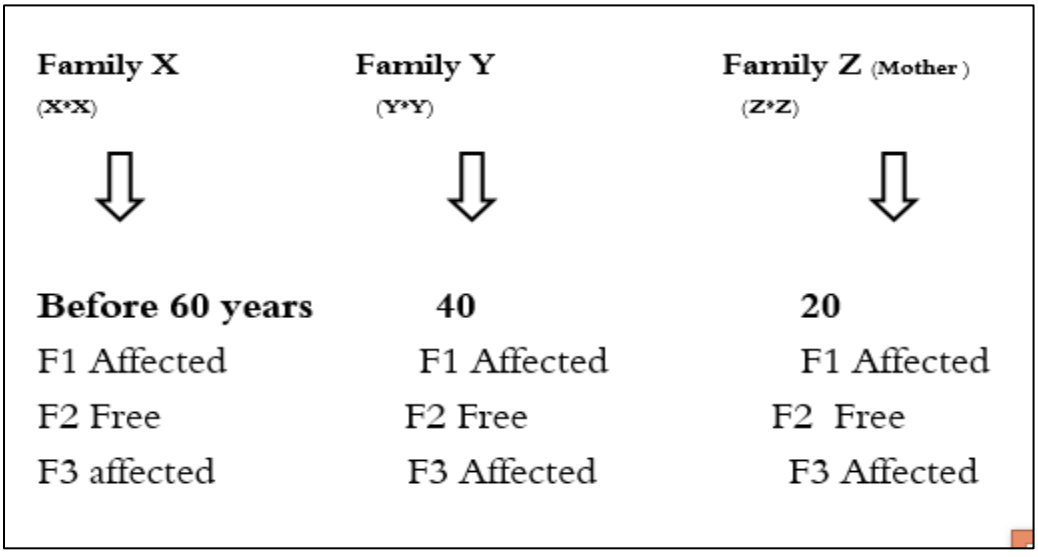


Figure 3.1: Family history and the progression of disease

3.2 Biochemical Test result

Table 3.1: The porphyrin and porphyrin precursor levels in urine/24 hrs. Urine collection for fourteen patients from three different families

#	Patient ID	Age(Y)	Gender	Uroporphyrin (ug/24 hr)	Heptacarboxyporphyrin (ug/24 hr)	Hexacarboxyporphyrin (ug/24 hr)	Pentacarboxyporphyrin (ug/24 hr)	Coproporphyrin I (ug/24 hr)	Coproporphyrin II (ug/24 hr)	Total Porphyrins
1	**TMZ**	**9**	**F**	**3.7**	**2.1**	**5.0**	**0.16**	**3.5**	**7.3**	**22.0**
2	**TAZ**	**14**	**M**	**7.9**	**7.4**	**8.0**	**1.50**	**12.5**	**0.7**	**37.8**
3	**TMYZ**	**46**	**F**	**6.2**	**0.5**	**4.0**	**1.0**	**2.0**	**0.9**	**14.1**
4	**TNJK**	**26**	**F**	**4.5**	**1.7**	**7.0**	**2.0**	**0.4**	**2.2**	**17.2**
5	**TYK**	**7**	**M**	**2.7**	**4.7**	**4.0**	**0.10**	**6.0**	**1.2**	**19.1**
6	**TIZ**	**15**	**M**	**6.4**	**1.3**	**1.0**	**0.097**	**0.1**	**0.4**	**9.4**
7	**TST**	**10**	**M**	**5.2**	**5.1**	**7.0**	**0.52**	**0.4**	**2.1**	**20.1**
8	**TMT**	**10**	**M**	**2.2**	**3.1**	**4.0**	**0.16**	**0.2**	**2.3**	**11.6**
9	**TNET**	**25**	**F**	**4.0**	**2.8**	**4.0**	**0.15**	**0.3**	**0.2**	**12.0**
10	**TRK**	**16**	**F**	**26.6**	**1.8**	**5.0**	**0.05**	**0.8**	**0.3**	**35.1**
11	**TIK**	**21**	**M**	**4.6**	**2.1**	**3.0**	**0.36**	**3.6**	**7.9**	**21.6**
12	**TOK**	**22**	**M**	**2.7**	**0.7**	**8.0**	**0.24**	**16.7**	**0.8**	**29.5**
13	**TKHZ**	**5**	**M**	**6.3**	**5.4**	**5.0**	**0.2**	**8.2**	**16.2**	**41.8**
14	**TNT**	**16**	**F**	**5.3**	**2.7**	**5.0**	**0.53**	**0.2**	**0.05**	**15.1**

Biochemical testing involves checking the total porphyrin and porphyrin precursor levels in urine. This method is used to diagnose all types of porphyria as each hase a distinctive pattern of raised levels. As we noticed from the table above all the values in the range without any elevation in any precursor, accordingly this result leaded us to screen the *FECH* gene that is responsible about Erythropoietic Protoporphyria (EPP) symptoms.

3.3 DNA molecular analysis

3.3.1UROD molecular DNA Analysis:

3.3.1.1Cloning UROD gene in a plasmid vector

The Clone JET PCR Cloning Kit features the novel positive selection cloning vector pJET1.2/blunt. This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate.

Table 3.2: cloning selection results for three patients

Patients' Code	Clones Result							
LT	Clone1 -ve	Clone2 -ve	Clone3 -ve	Clone4 +ve	Clone5 +ve	Clone6 -ve	Clone7 -ve	Clone8 +ve
KZ	Clone1 +ve			Clone2 +ve		Clone3 –ve		
LKH	Clone1 –ve			Clone2 +ve		Clone3 –ve		

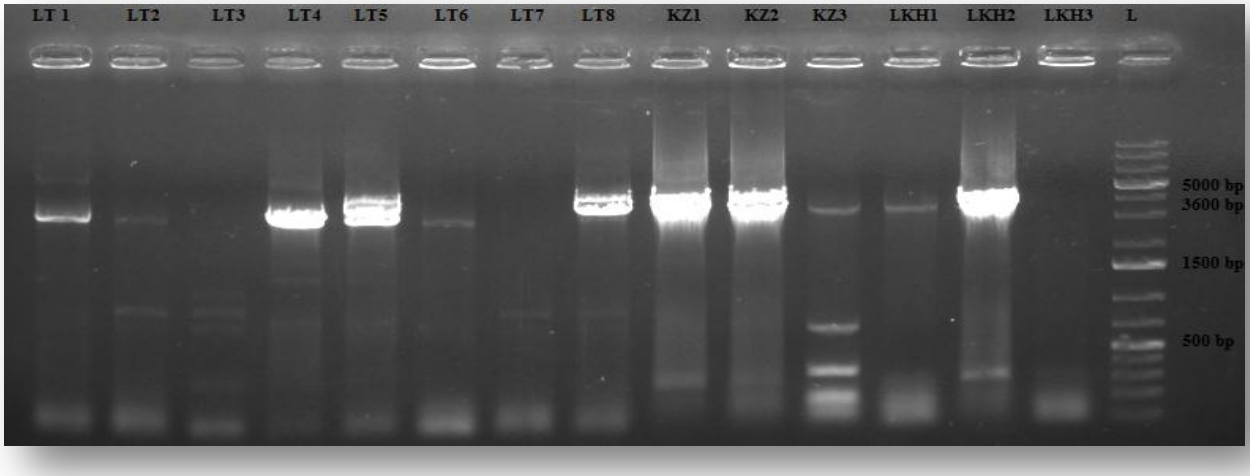


Figure 3.2: Detection of positive clones for three patients, the table above demonstrates the results for each clone

After extraction of plasmid was done DNA concentrations were in range (57.3 – 100.6) ng/ul, aPCR products were loaded in the gel below. But for sequencing separate parts of the gene were amplified and prepared for sequencing according to the map in Figure 2.3

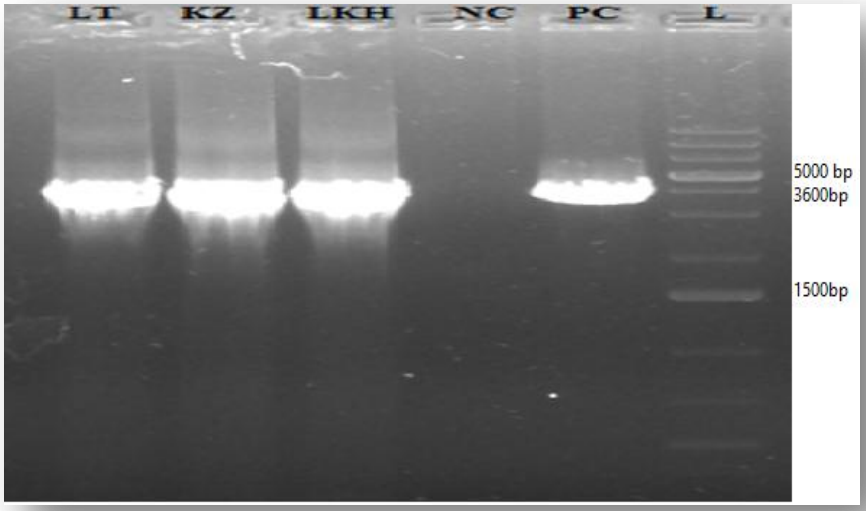


Figure 3.3: Full UROD gene (3.6 Kb) gene image. From different patients' Samples,1-3 as shown, Lane 4 negative control. lane 5 positive control.

3.3.1.2 UROD gene sequencing and screening

Amplification reactions were performed to the UROD gene using different sets of primers as mentioned previously.

The sequencing results were manipulated and the gene asssemply was performed for 3 patients KZ, LTand LKH, after assemply completed the total size of the collected fragment was equal to the size of the gene 3.6 kb , all single nucleotide polymorphysms existing in the gene were documented in specific table for each patient subsecuantly.We started our research by investigation the variation in UROD gene because PCT is the most common type of cutaneous porphyria around the world.

The alignment was done by using a RefSeqGene on chromosome1, Homo sapiens uroporphyrinogen decarboxylase (UROD), NG_007122.2.

Table 3.3: SNPs in UROD Gene for KZ patient

Position #	SNPs	Location	Position regarding +1	Clinical significant	rS #	Notes
4846	T>A	5'UTR	263 -	N/A	2236576	Reported
5503	T>C	Intron 1	394			Not reported
5533	Hetero C\G	Intron 1	424		184463721 for (C>T)(N/A)	Not reported
5604	C>T Comp (C>A)	Intron1	495	N/A	12749939	Reported
6083	A>G	Intron 3	974			Not reported
6943	G>T	Intron6	1835	N/A	**11211066**	Reported
6946	A>G	Intron6	1838			Not reported
7045	G>T	Intron6	1937	N/A	6698485	Reported
7219	T>C	Intron6	2110			Not reported
7749	Com G>A A>G	Intron8	26439			Not reported
7904	C>T Com(G>A)	Intron 9	2794	N/A	6429553	Reported
8000	T>C com (A>G)	Intron 9	2891	N\A	13948	Reported

Table 3.4: SNPs in UROD Gene for LKH patient

Original positin#	SNPs	Loction	Position regarding +1	Clinical significant	rS #	Notes
4816	A>G	5'UTR	293-			Not reported
4875	T>C	5'UTR	234-			Not reported
5474	G>A Com(C>T)	Intron 1	365			Not reported
7045	G>T Com(C>A)	Intron6	1936	N/A	6698485	Reported
7254	A>G Com(T>C)	Intron6	2145			Not reported
7759	A>G Com(T>C)	Intron7	2650			Not reported
7904	C>T	Intron9	2795	N/A	6429553	Reported
7927	T>C	Intron9	2818			Not reported
8177	delC	Intron9	3068			Not reported

Table 3.5: SNPs in UROD Gene for LT patient

Position #	SNPs	Position	Position regarding +1	Clinical significant	rS #	Notes
4846	T>A	5'UTR	263 -	N/A	2236576	complement(4846) reported
5503	T>C	Intron1	394			Not reported
5605	G>A	Intron1	496			Not reported
6083	A>G	Intron 3	974			
6943	G>T	Intron6	1834	N/A	11211066	Reported
6946	A>G	Intron6	1837			Not Reported
7045	G>T	Intron6	1936	N/A	6698485	
7219	T>C	Intron6	2110			Not Reported
7749	A>G	Intron8	264			Not reported
7904	C>T	Intron9	2795	N/A	6429553	Reported
8000	T>C	Intron9	2891	N/A	13948	Reported
7927	T>C	Intron9	2818			Not reported
8177	delC	Intron9	3068			Not reported

The variations that were detected in the examined samples for three patients from different families have no significant effect even though some of them were reported, continuously we went through advanced method (Next Generation sequencing) to detect any mutation that could participate in the symptoms.

3.3.1.3 UROD next generation sequencing and screening

The consenses sequences that were obtained from the Next generation sequencig for UROD gene shows some SNPs without any clinical significance as illumenated in the table below, the consenses sequences was compared to the reference sequence (Homo sapiens uroporphyrinogen decarboxylase, RefSeqGene on chromosome 1 NCBI Reference Sequence: NG_007122.2).

Table 3.6: SNPs were detected in UROD by NGS

SNP #	Reference SNP (rs #)	RefSNP Alleles	Clinical Significance	Position	Note
5604	12749939	C/T	N/A	Intron 1	Khand my
6943	11211066	G/T	N/A	Intron6	Khand my
7045	6698484	G/T	N/A	Intron 6	Khand my
7904	6429553	C/T	N/A	Intron 9	Khand my
8000	13948	C/T	N/A	Intron 9	Khand my
7442	Not Reported	T/K	-	Intron7	Normal in Kh

3.3.2UROS molecular DNA analysis

Amplification reactions were performed to the cDNA-UROS and DNA gene in both directions using different sets of primers as mentioned previously .The sequencing results were manipulated and the gene asssemply was performed .

We choosed exon 4 ,10 , Intron2, Exon2 and Exon3 regions for amplification and sequencing since those targets contain tho most common mutation that cause congenital erythropoietic porphyria (CEP) adding to that exon three contains the (S47P) a novel mutation that was detected in four siblings of a Palestinian family in France.

All the sequences were compared with a RefSeqGene on chromosome10, Homo sapiens uroporphyrinogen III synthase (*UROS*), NG_011557.1. All the alignment results for all patients from different three families were normal without any significant SNP

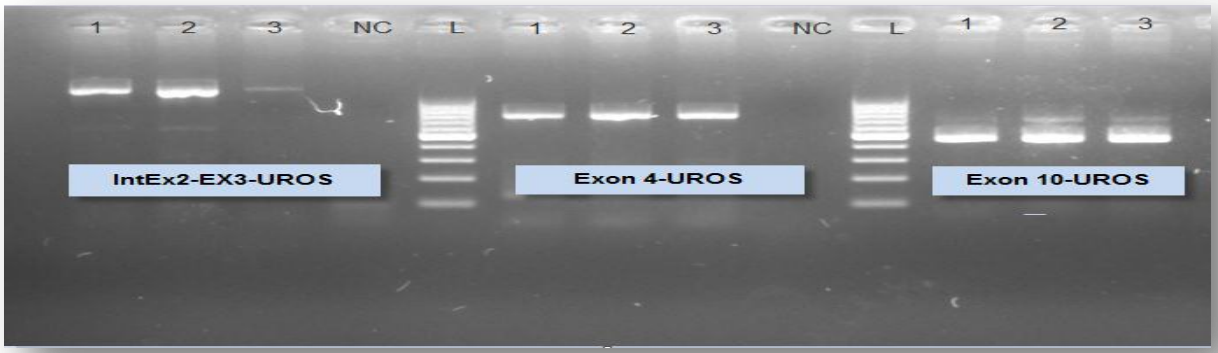


Figure 3.4: Three hot spot areas of *UROS* gene were amplified for sequencing, Samples for three unrelated patients1-3 as shown, Lane 4 negative control

Regarding to the cDNA amplification for four unrelated patients and one control, The figure below shows that the cDNA for affected patient and normal control unchanged furthermore the product size equal the Coding region mRNA (1354 bp) so there is no exon skipping.

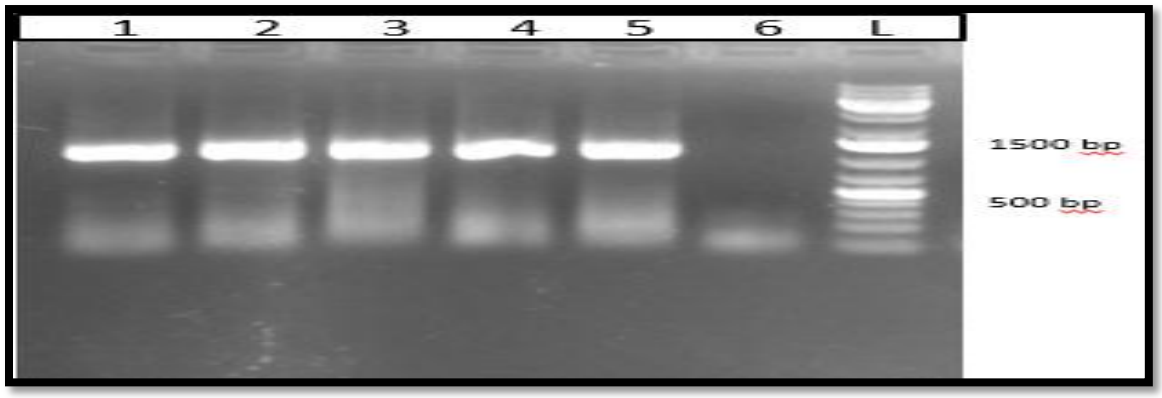


Figure 3.5: cDNA was amplified for UROS gene for four unrelated patients (Lane1-4)

Lane5 for a control, lane6 for a negative control (Product size for each target illustrated in table 2.3)

3.3.3 FECH molecular DNA analysis

We explored the most common mutation that cause the autosomal recessive pattern of inheritance in EPP(SNP; IVS3-48C/T) allele that appears to explain the occurrence of photosensitivity in most EPP families, so we amplified the region in intron three that contains this SNP,as illumenated in the figure below with 282 bp for the target size, but we didn't find the SNP.

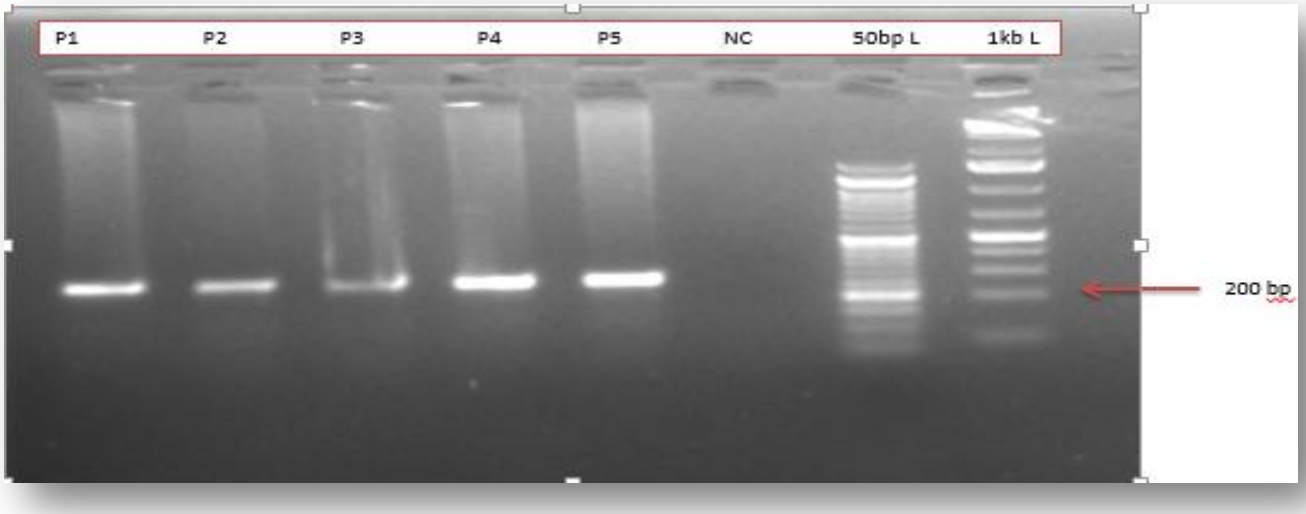


Figure 3.6: Intron3 (IVS3-48C/T allele) was amplified from different sample patients 1-5, using specific primers. Lane 6 negative control, PCR product 282 bp

After that the product was restricted by restriction enzyme TSeI, and we got the pands with a total sequence size 282bp so allele C present, while allele T the mutant one not present which give the following pattern of restriction (29 and 253 bp).

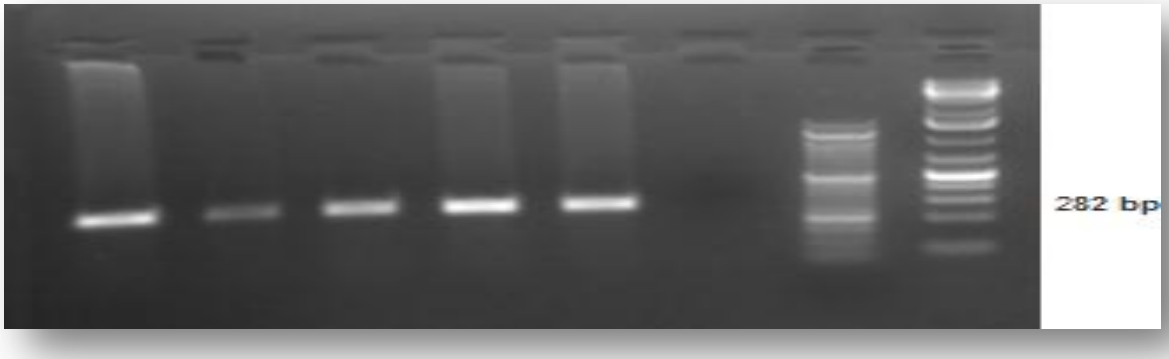


Figure 3.7: Restriction fragment length polymorphism (RFLP) analysis after digestion the PCR product by TSeI, enzyme. Product was loaded on 3% agarose gel. 1 to 5 represent samples sequence and a total sequence size 282bp was obtained , 100 bp ladder was used .

Finally we amplified the FECH gene in separet target as shown in table 2.4, the gel image below shows some of the PCR product that were ready for deep sequencing.

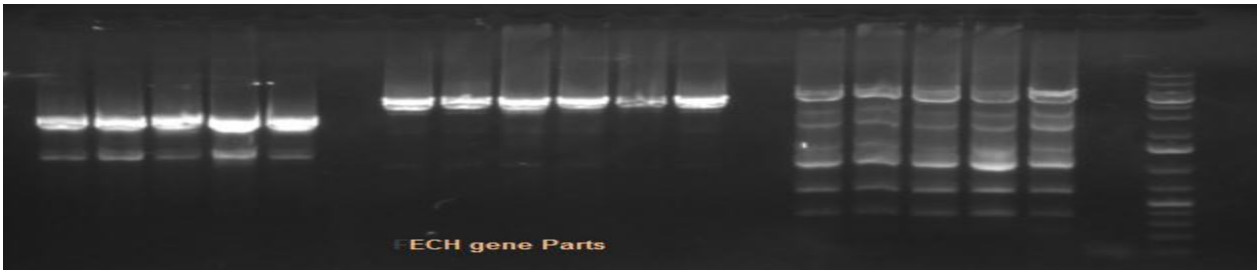


Figure 3.8: PCR product for FECH gene, ready for Next generation sequencing.(Product size for each PCR reaction illustrated in table 2.4).

The consenses sequences that were optained from the Next generation sequencing for two unrelated patients shows that FECH gene in the amplified regions was normal without any significant variation, the consenses sequences was compared to the reference sequence (Homo sapiens ferrochelatase (FECH), RefSeqGene on chromosome 18,NCBI Reference Sequence: NG_008175.1).

Chapter 4

Discussion and conclusions

4.1 Discussion

In this study we investigated the molecular basis of cutaneous porphyria with recessive mode of inheritance or reduced (incomplete) penetrance that include familial porphyria cutaneous tarda (fPCT), Hepatoerythropoietic porphyria (HEP), Congenital erythropoietic porphyria (CEP) and erythropoietic protoporphyria (EPP).

The samples were tested from different patients, families, ages and the severity of the symptoms were diverse.

During this investigation and genetic studies on *UROD* and *UROS* genes, a biochemical test (24Hours urine collection) was performed in specialist lab in Jordan for fourteen patients, the results illuminated in section 3.2 showed that all porphyrin precursors and total porphyrin were within the normal range, on the other hands the biochemical results in the normal range without increasing in porphyrin or porphyrin precursors oriented us to go through *FECH* gene screening according to the criteria in (table 1.2).

The screening of three unrelated patients for UROD gene was performed by cloning the gene followed by PCR and sequencing of full gene using DNA.

The molecular heterogeneity of type II PCT is demonstrated by different mutations reported in the *UROD* gene, remarkably, in the majority of the reported cases of fPCT, mutations are unique in each individual family.

In this study we found different variations some of them were reported the others not, one of them was in the 5'UTR region which was reported in the Genbank under rs:2236576 without any pathogenic effect.

The substitution of serine by proline at the amino acid residue 47 (S47P) was present at the homozygous state in four patients of a Palestinian family in the present study, we investigated this mutation in our patients particularly the CEP that caused by a genetic changing in *UROS* gene which was compatible with the mode of inheritance of our patients. However, no mutations were detected in all tested sequences. Based on literature review, we summarized the most common mutations that can cause CEP and three region in *UROS* gene were examined without any detectable mutation.

Subsequently the mRNA was examined by converting it to cDNA to confirm the presence of all exons without any skipping especially in exon4 and exon10.

This finding nominated *FECH* gene for screening because EPP is the only type of cutaneous porphyria that gives a normal porphyrin level in a biochemical test and that related to the nature of its precursor (Protoporphyrin) which is unlike other porphyrins, is lipophilic molecule and thus detached from the body only through hepatic excretion into bile or feces, accordingly in contrast to the other forms of cutaneous porphyria the urinary porphyrins are usually normal.

The *FECH* gene screening was started to identify the most common mutation that was reported widely and cause the autosomal recessive pattern of inheritance in EPP (SNP; IVS3-48C/T) allele that appears to explain the occurrence of photosensitivity in most EPP families, but the sequencing results showed a normal sequence in the tested patients.

To screen the *FECH and UROD* genes deeply we used Next generetion sequencing technology and the multiple alignment of a consences sequenses of two patients with the reference sequence of the genes gave a normal result and no significant mutation was detected.

This final result complicated the interpretation of the results and leaded us to perform further investigation to make matching between the biochemical and genetic results adding to that there are variations with regard to diagnostic stratigies and clinical intrpretation even between these specialized labooratories and diagnostic centers.

4.2.Conclusions and Recommendations

Porphyria is not a single disease but a group of at least eight disorders. The diagnosis of porphyria can be difficult. As a group, these diseases are rare, and the signs and symptoms of the disease may mimic other more common diseases. Adding to that, this is the first time where such a study is performed in Palestine to diagnose those patients, so there are no baseline investigations, and moreover there is a lack of porphyria investigation in the Arab population was also observed.

So the screening of a mutation in at least three genes the smallest one around 3600 bp is a huge job and requires a lot of resources and tests with different molecular techniques.

Respectively we have to focus on many facts about this disease regarding the regulation of the genes encoding the eight enzymes of heme biosynthesis pathway that can occur at transcriptional, translational and post translational levels adding to that the relationship between different genes like Human hemochromatosis protein (*HFE*) gene.

In addition, further studies need to confirm our mutations finding in vitro, such as in vitro expression testing of normal and mutant genes in tissue culture cells including GFP or luciferase expression reporters.

Mutations in the *HFE* gene that cause hereditary hemochromatosis increase the risk of developing the most common form of porphyria, Porphyria Cutanea Tarda. So it is recommended to investigate the mutations in *HFE* gene to confirm or exclude this relationship.

Also when the biochemical test for erythrocytes becomes available it is recommended to test the patients' blood porphyrin especially for EPP confirmation or exclusion in a definite manner.

Also in porphyria, there is a lot of intervening sequences mutation that affect the gene or the enzyme activity, so it will be helpful to investigate the areas that not covered in our study and to do prokaryotic expression studies.

Finally it's highly recommended to follow up the patients and provide them with a proper treatment that can reduce the photosensitivity symptoms.

In conclusion, deeper molecular tests are needed such as exome/whole genome sequencing and epigentic modification analysis for all genes that involved in heme synthesis pathway. However,

new genetic disorder with symptoms similar to that of cutaneous porphyria should be considered and investigated to complete our work and achieve the main objectives.

4.3 Limitations

We faced many limitations during the sample collection, because the patients were disappointed related to the negative feedback from the previous researchers.

On the other hand the financial issues delayed the biochemical test so many genetic studies were performed before receiving the biochemical results.

References:

Aarsand, A.K., Boman, H., Sandberg, S., 2009. Familial and sporadic porphyria cutanea tarda: characterization and diagnostic strategies. Clinical chemistry55, 795-803.

Aizencang, G., Solis, C., Bishop, D.F., Warner, C., Desnick, R.J., 2000a. Human uroporphyrinogen-III synthase: genomic organization, alternative promoters, and erythroid-specific expression. Genomics70, 223-231.

Aizencang, G.I., Bishop, D.F., Forrest, D., Astrin, K.H., Desnick, R.J., 2000b. Uroporphyrinogen III synthase. An alternative promoter controls erythroid-specific expression in the murine gene. The Journal of biological chemistry275, 2295-2304.

Arora, S., Young, S., Kodali, S., Singal, A.K., 2016. Hepatic porphyria: A narrative review. Indian journal of gastroenterology : official journal of the Indian Society of Gastroenterology35, 405-418.

Balwani, M., Bloomer, J., Desnick, R., of the NIH-Sponsored, P.C., Network, R.D.C.R., 2014. Erythropoietic protoporphyria, autosomal recessive.

Balwani, M., Desnick, R.J., 2012. The porphyrias: advances in diagnosis and treatment. Hematology. American Society of Hematology. Education Program2012, 19-27.

Besur, S., Hou, W., Schmeltzer, P., Bonkovsky, H.L., 2014. Clinically important features of porphyrin and heme metabolism and the porphyrias. Metabolites4, 977-1006.

Bissell, D.M., Wang, B., 2015. Acute Hepatic Porphyria. Journal of clinical and translational hepatology3, 17-26.

Bulaj, Z.J., Phillips, J.D., Ajioka, R.S., Franklin, M.R., Griffen, L.M., Guinee, D.J., Edwards, C.Q., Kushner, J.P., 2000. Hemochromatosis genes and other factors contributing to the pathogenesis of porphyria cutanea tarda. Blood95, 1565-1571.

Cantatore-Francis, J.L., Cohen-Pfeffer, J., Balwani, M., Kahn, P., Lazarus, H.M., Desnick, R.J., Schaffer, J.V., 2010. Hepatoerythropoietic porphyria misdiagnosed as child abuse: cutaneous, arthritic, and hematologic manifestations in siblings with a novel UROD mutation. Archives of dermatology146, 529-533.

Christiansen, A.L., Aagaard, L., Krag, A., Rasmussen, L.M., Bygum, A., 2016. Cutaneous Porphyrias: Causes, Symptoms, Treatments and the Danish Incidence 1989–2013. Acta dermato-venereologica96, 868-872.

Dailey, H.A., Meissner, P.N., 2013. Erythroid heme biosynthesis and its disorders. Cold Spring Harbor perspectives in medicine3, a011676.

Danton, M., Lim, C.K., 2006. Porphyrin profiles in blood, urine and faeces by HPLC/electrospray ionization tandem mass spectrometry. Biomedical chromatography : BMC20, 612-621.

De Verneuil, H., Grandchamp, B., Romeo, P.H., Raich, N., Beaumont, C., Goossens, M., Nicolas, H., Nordmann, Y., 1986. Molecular analysis of uroporphyrinogen decarboxylase deficiency in a family with two cases of hepatoerythropoietic porphyria. Journal of Clinical Investigation77, 431.

Desnick, R.J., Astrin, K.H., 2002. Congenital erythropoietic porphyria: advances in pathogenesis and treatment. British journal of haematology117, 779-795.

Di Pierro, E., Russo, R., Karakas, Z., Brancaleoni, V., Gambale, A., Kurt, I., Winter, S.S., Granata, F., Czuchlewski, D.R., Langella, C., Iolascon, A., Cappellini, M.D., 2015. Congenital erythropoietic porphyria linked to GATA1-R216W mutation: challenges for diagnosis. European journal of haematology94, 491-497.

Dubart, A., Mattei, M., Raich, N., Beaupain, D., Romeo, P., Mattei, J., Goossens, M., 1986. Assignment of human uroporphyrinogen decarboxylase (URO-D) to the p34 band of chromosome 1. Human genetics73, 277-279.

Elder, G.H., 1998. Porphyria cutanea tarda. In: Seminars in liver disease, pp. 67-75.

Fontanellas, A., Bensidhoum, M., Enriquez de Salamanca, R., Moruno Tirado, A., de Verneuil, H., Ged, C., 1996. A systematic analysis of the mutations of the uroporphyrinogen III synthase gene in congenital erythropoietic porphyria. European journal of human genetics : EJHG4, 274-282.

Frank, J., Wang, X., Lam, H.M., Aita, V.M., Jugert, F.K., Goerz, G., Merk, H.F., Poh-Fitzpatrick, M.B., Christiano, A.M., 1998. C73R is a hotspot mutation in the uroporphyrinogen III synthase gene in congenital erythropoietic porphyria. Annals of human genetics62, 225-230.

Ged, C., Megarbane, H., Chouery, E., Lalanne, M., Megarbane, A., de Verneuil, H., 2004a. Congenital erythropoietic porphyria: report of a novel mutation with absence of clinical manifestations in a homozygous mutant sibling. The Journal of investigative dermatology123, 589-591.

Ged, C., Mégarbané, H., Chouery, E., Lalanne, M., Megarbane, A., de Verneuil, H., 2004b. Congenital erythropoietic porphyria: report of a novel mutation with absence of clinical manifestations in a homozygous mutant sibling. Journal of Investigative Dermatology123, 589-591.

Goldberg, A., McColl, K.E., Moore, M., Rimington, C., 2013. Disorders of porphyrin metabolism. Springer Science and Business Media.

Gómez-Abecia, S., Morán-Jiménez, M.-J., Ruiz-Casares, E., Henriques-Gil, N., García-Pastor, I., Garrido-Astray, M.-C., de Salamanca, R.E., Méndez, M., 2013. Familial porphyria cutanea tarda in Spain: Characterization of eight novel mutations in the UROD gene and haplotype analysis of the common p. G281E mutation. Gene522, 89-95.

Gouya, L., Martin-Schmitt, C., Robreau, A.-M., Austerlitz, F., Da Silva, V., Brun, P., Simonin, S., Lyoumi, S., Grandchamp, B., Beaumont, C., 2006. Contribution of a common single-nucleotide polymorphism to the genetic predisposition for erythropoietic protoporphyria. The American Journal of Human Genetics78, 2-14.

James, M.F., Hift, R.J., 2000. Porphyrias. British journal of anaesthesia85, 143-153.

Jordan, P.M., Dailey, H.A., 1990. Biochemistry of porphyrins. Molecular Aspects of Medicine11, 21-37.

Karim, Z., Lyoumi, S., Nicolas, G., Deybach, J.-C., Gouya, L., Puy, H., 2015. Porphyrias: a 2015 update. Clinics and research in hepatology and gastroenterology39, 412-425.

Lane, A.M., McKay, J.T., Bonkovsky, H.L., 2016a. Advances in the management of erythropoietic protoporphyria–role of afamelanotide. The application of clinical genetics9, 179.

Lane, A.M., McKay, J.T., Bonkovsky, H.L., 2016b. Advances in the management of erythropoietic protoporphyria - role of afamelanotide. Appl Clin Genet9, 179-189.

Lecha, M., Puy, H., Deybach, J.C., 2009. Erythropoietic protoporphyria. Orphanet journal of rare diseases4, 19.

Magnus, I. 1968. The Porphyrias: Photobiological Aspects of Porphyria (SAGE Publications).

Mathews, M.A., Schubert, H.L., Whitby, F.G., Alexander, K.J., Schadick, K., Bergonia, H.A., Phillips, J.D., Hill, C.P., 2001. Crystal structure of human uroporphyrinogen III synthase. The EMBO journal20, 5832-5839.

McCall-Anderson, T., 1898. Hydroa aestivale in two brothers, complicated with the presence of haematoporphyrin in the urine. Brit J Dermatol10, 1-4.

Meguro, K., Fujita, H., Ishida, N., Akagi, R., Kurihara, T., Galbraith, R.A., Kappas, A., Zabriskie, J.B., Sassa, S., Toback, A.C., 1994. Molecular defects of uroporphyrinogen decarboxylase in a patient with mild hepatoerythropoietic porphyria. Journal of investigative dermatology102, 681-685.

Moran-Jimenez, M., Ged, C., Romana, M., De Salamanca, R.E., Taieb, A., Topi, G., D'Alessandro, L., De Verneuil, H., 1996. Uroporphyrinogen decarboxylase: complete human gene sequence and molecular study of three families with hepatoerythropoietic porphyria. American journal of human genetics58, 712.

Parera, V.E., Koole, R.H., Minderman, G., Edixhoven, A., Rossetti, M.V., Batlle, A., de Rooij, F.W., 2009. Novel null-allele mutations and genotype-phenotype correlation in Argentinean patients with erythropoietic protoporphyria. Molecular Medicine15, 425.

Phillips, J.D., Whitby, F.G., Stadtmueller, B.M., Edwards, C.Q., Hill, C.P., Kushner, J.P., 2007. Two novel uroporphyrinogen decarboxylase (URO-D) mutations causing hepatoerythropoietic porphyria (HEP). Translational Research149, 85-91.

Poblete-Gutiérrez, P., Mendez, M., Wiederholt, T., Merk, H.F., Fontanellas, A., Wolff, C., Frank, J., 2004. The molecular basis of porphyria cutanea tarda in Chile: identification and functional characterization of mutations in the uroporphyrinogen decarboxylase gene. Experimental dermatology13, 372-379.

Ramanujam, V.M., Anderson, K.E., 2015. Porphyria Diagnostics-Part 1: A Brief Overview of the Porphyrias. Current protocols in human genetics86, 17 20 11-26.

Sassa, S., 2006. Modern diagnosis and management of the porphyrias. British journal of haematology135, 281-292.

Sassa, S., Kappas, A., 2000. Molecular aspects of the inherited porphyrias. Journal of internal medicine247, 169-178.

Schubert, H.L., Erskine, P.T., Cooper, J.B., 2009. 5-Aminolaevulinic acid dehydratase, porphobilinogen deaminase and uroporphyrinogen III synthase. Tetrapyrroles, 43-73.

Szlendak, U., Bykowska, K., Lipniacka, A., 2016. Clinical, Biochemical and Molecular Characteristics of the Main Types of Porphyria. Advances in clinical and experimental medicine : official organ Wroclaw Medical University25, 361-368.

Thadani, H., Deacon, A., Peters, T., 2000. Diagnosis and management of porphyria. Bmj320, 1647-1651.

Thapar, M., Bonkovsky, H.L., 2008. The diagnosis and management of erythropoietic protoporphyria. Gastroenterology and hepatology4, 561-566.

Tzou, W.S., Chu, Y., Lin, T.Y., Hu, C.H., Pai, T.W., Liu, H.F., Lin, H.J., Cases, I., Rojas, A., Sanchez, M., You, Z.Y., Hsu, M.W., 2014. Molecular evolution of multiple-level control of heme biosynthesis pathway in animal kingdom. PloS one9, e86718.

Whatley, S.D., Mason, N.G., Khan, M., Zamiri, M., Badminton, M.N., Missaoui, W.N., Dailey, T.A., Dailey, H.A., Douglas, W.S., Wainwright, N.J., Elder, G.H., 2004. Autosomal recessive erythropoietic protoporphyria in the United Kingdom: prevalence and relationship to liver disease. J Med Genet41, e105.

Wiederholt, T., Poblete-Gutierrez, P., Gardlo, K., Goerz, G., Bolsen, K., Merk, H.F., Frank, J., 2006. Identification of mutations in the uroporphyrinogen III cosynthase gene in German patients with congenital erythropoietic porphyria. Physiological research55 Suppl 2, S85-92.

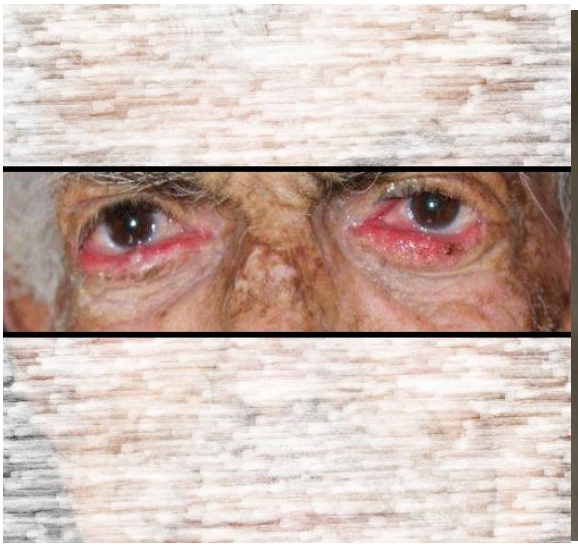
Xu, W., Warner, C.A., Desnick, R.J., 1995. Congenital erythropoietic porphyria: identification and expression of 10 mutations in the uroporphyrinogen III synthase gene. The Journal of clinical investigation95, 905-912.

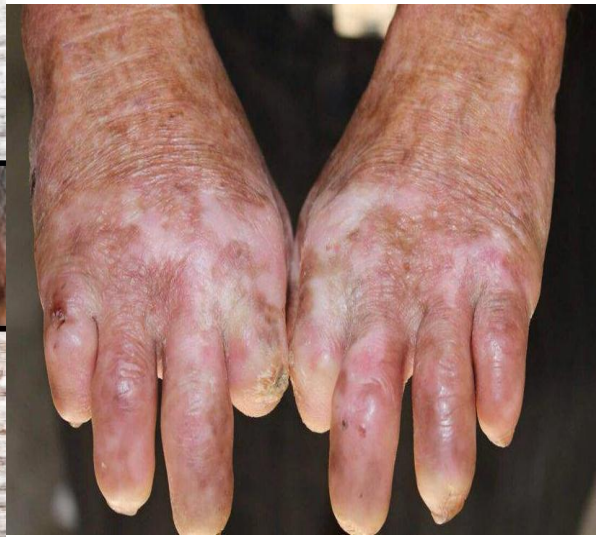
Yeung Laiwah, A.C., Moore, M.R., Goldberg, A., 1987. Pathogenesis of acute porphyria. The Quarterly journal of medicine63, 377-392.

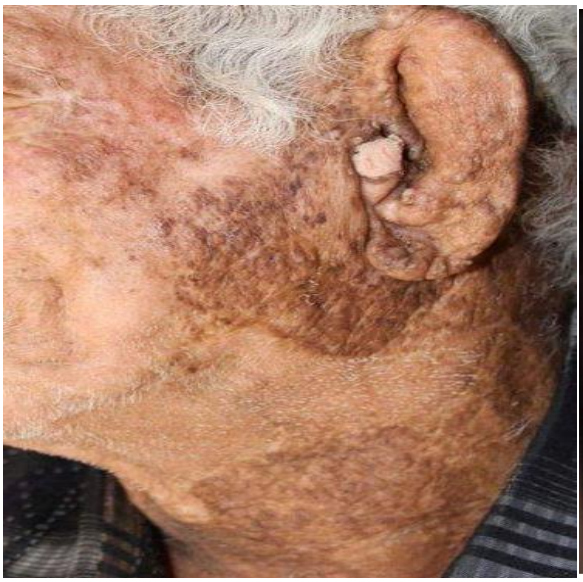
Zlotogora, J., 1997. Autosomal recessive diseases among Palestinian Arabs. Journal of medical genetics34, 765-766.

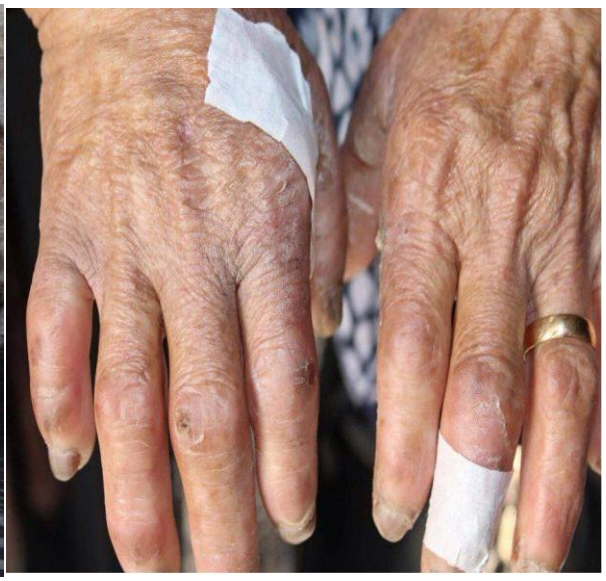
Zlotogora, J., 2002. Molecular basis of autosomal recessive diseases among the Palestinian Arabs. American journal of medical genetics109, 176-182.

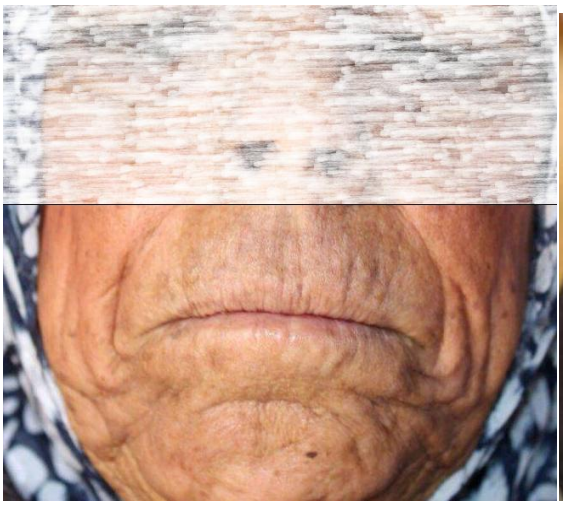
Appendix A : Patients' Photos shows a mild to severe symptoms

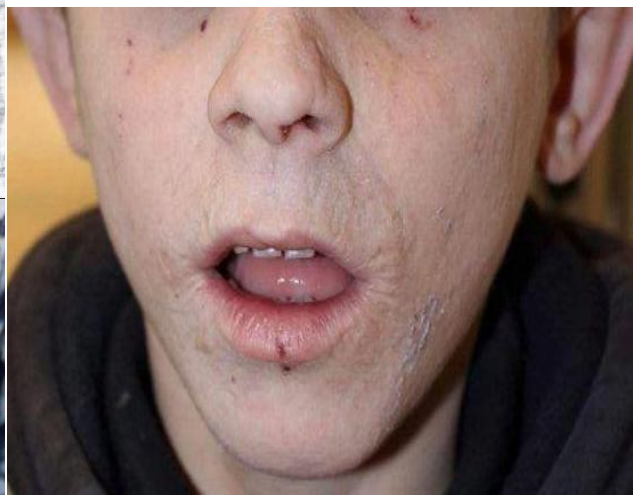


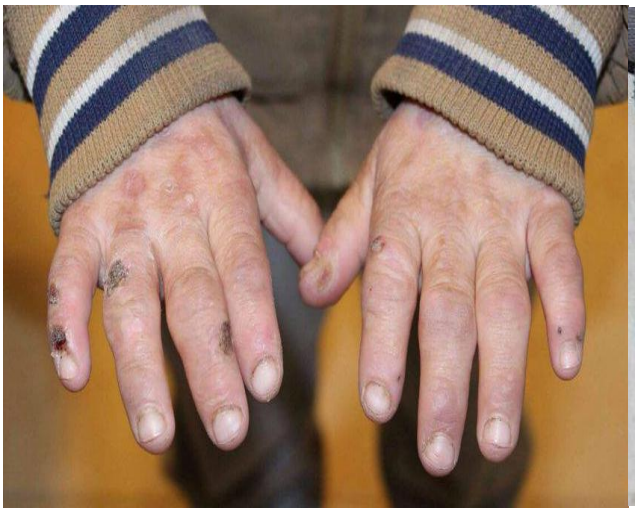


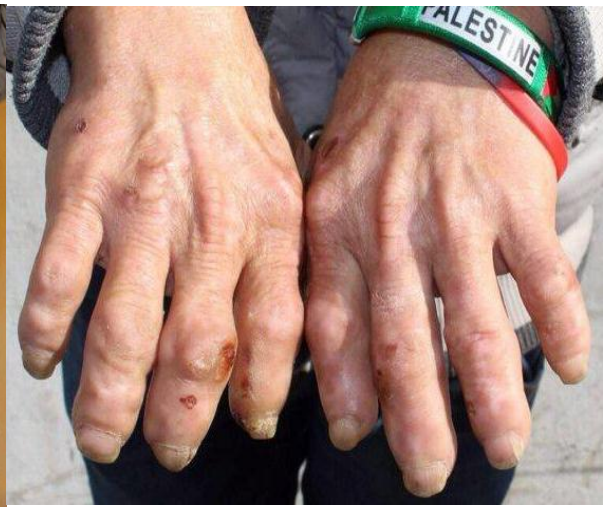











PALESTINE

التشخيص الجزيئي للمرضى المشتبه باصابتهم بمرض البورفيريافي منطقة الخليل، فلسطين

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الملخص

البورفيريات هي اضطرابات غير متجانسة تنجم عن تشوهات في الخطوات الكيميائية التي تؤدي إلى إنتاج الهيم، حيث يعتبر من اهم الجزيئات الحيوية الضرورية لجميع أجهزة الجسم ووظائفه الحيوية. تنشأ أعراض البورفيريا في الغالب من آثار قد تتركز على الجهاز العصبي أو الجلد. هناك انواع متعددة للمرض تظهر بصور عدة ولكن الانواع التي تصاحبها اعراض جلدية هي (fPCT, HEP, CEP and EPP). غالبا ما يتأخر التشخيص الصحيح لأن الأعراض غير محددة. يمكن أن تشمل المظاهر الجلدية حرق، وانتفاخات وتندب خاصة في المناطق المعرضة لأشعة الشمس.

حتى الآن لم يكن هناك أي تقرير او دراسة عن التحليل الجزيئي الجيني للبورفيريا في المرضى الفلسطينيين. وكان الهدف من هذه الدراسة هو تأكيد التشخيص السريري للبورفيريا من خلال اختبارات الكيمياء الحيوية والدراسات الوراثية للمرضى المشتبه بهم وتحديد الجين الذي يسبب التوصيف المظهري والأعراض في منطقة الخليل.

وسيشارك التشخيص المؤكد وراثيا في الحد من الآثار الفظيعة للمرض، وسيسهم بشكل كبير في صحة المرضى الفلسطينيين وحالات الطفرة غير المتجانسة سريريا غير المتناظرة داخل الأسر، وسيمكن التشخيص الصحيح والمبكر من وضع خطة لإدارة المشكلة والتقليل من عدد المواليد الجدد المصابين ، وتوجيه المصابين لنمط حياة مناسب للتخفيف من الاعراض وشدتها، وتقديم المشورة الجينية الأفضل للأسر المعرضة لإنجاب اطفال مصابين.

وقد شارك 40 مريضا تقريبا من مدينة تفوح- الخليل في هذه الدراسة، وأجريت الدراسات الوراثية والبيوكيميائية باستخدام تقنيات جزيئية مختلفة للتشخيص والكشف عن الطفرات المسببة للمرض.

واظهرت النتائج وجود عدد من الطفرات دون اثر تشخيصي على المرضى، مما يقودنا الى المزيد من البحث من خلال طرق اكثر تقدما عن طفرات او مؤثرات جينية قد تسبب الاعراض الموجودة عند المرضى او التوجه للكشف عن طفرات تتعلق بمرض اخر سواء كان معروفا او جديدا وذلك استكمالا لما قمنا به من اجل تحقيق كافة الاهداف المرجوة .