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**MORE EVIDENCE FOR H<sub>2</sub>O<sub>2</sub>-MEDIATED  
OXIDATIVE STRESS IN VITILIGO -  
INCREASED EPIDERMAL DNA DAMAGE /  
REPAIR**

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## Summary

Nowdays there is a plethora of evidence for H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress in the epidermis as well as in the system in patients with vitiligo (for review see (Schallreuter, Bahadoran et al. 2008). Xanthine dehydrogenase / xanthine oxidase (XDH / XO) catalyses the oxidative hydroxylation of hypoxanthine to xanthine followed by xanthine to uric acid, the last two steps in purine degradation pathway. Under oxidative conditions, XDH is converted to XO. The reactions catalysed by this enzyme generate H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>, yielding in the presence of ROS accumulation, allantoin from uric acid. Therefore XO has been considered a major biologic source of oxygen-derived free radicals in many organs. The presence of XO in the human epidermis has not been shown so far. In this study several techniques were utilised to nail the presence and activity of XO in epidermal melanocytes and keratinocytes.

The enzyme is regulated by H<sub>2</sub>O<sub>2</sub> in a concentration dependent manner, where concentrations of 10<sup>-6</sup>M upregulate activity. Importantly, the results showed that the activity of XO is little affected by H<sub>2</sub>O<sub>2</sub> in the mM range. H<sub>2</sub>O<sub>2</sub>-mediated oxidation of tryptophan and methionine residues in the sequence of XO yields only subtle alterations in the enzyme active site. These findings are in agreement with enzyme kinetics in the presence of 10<sup>-3</sup>M H<sub>2</sub>O<sub>2</sub>. Since uric acid is the end product of XO activity and this can be oxidised to allantoin by H<sub>2</sub>O<sub>2</sub>, we wanted to know whether allantoin is formed in the epidermis of patients with vitiligo. In order to address this issue, we utilised HPLC/mass spectrometry analysis. Analysis of epidermal cell extracts from suction blister tissue identified the presence of allantoin in patients with acute vitiligo, while this product was absent in healthy controls. In conclusion, our results provide evidence for functioning epidermal XO in the human epidermis which

can be a major source for the production of H<sub>2</sub>O<sub>2</sub> contributing to oxidative stress in vitiligo.

In addition, this thesis also demonstrates for the first time the presence of XO in melanosomes, and we showed that both 7BH<sub>4</sub> and 7-biopterin inhibit XO activity in a concentration dependent manner. Moreover, XO has the potential to bind to 6/7BH<sub>4</sub> and 6/7-biopterin from the pterin/tyrosinase inhibitor complex. This discovery adds another receptor independent mechanism for regulation of tyrosinase within the melanocyte similar to  $\alpha/\beta$ -MSH as shown earlier (Moore, Wood et al. 1999; Spencer, Chavan et al. 2005).

Since the entire epidermis of patients with vitiligo is under H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress, oxidative DNA damage would be highly expected. This thesis shows for the first time that epidermal 8-oxoG levels as well as plasma level of this oxidised DNA base are significantly increased in patients compared to healthy controls. We have shown that epidermal cells from patients with vitiligo respond to oxidative DNA damage via the overexpression of p21 and Gadd45 $\alpha$  leading to a functioning increased short-patch base-excision repair (BER), while increased apoptosis can be ruled out due to lower caspase 3 and cytochrome c response compared to healthy controls. Our results show that patients develop effective DNA repair machinery via hOgg1, APE1 and DNA polymerase $\beta$ . Taking into consideration that these patients do not have an increased prevalence for solar-induced skin cancers, our data suggest that BER is a major player in the hierarchy to combat H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress preventing ROS-induced tumourigenesis in the epidermis of these patients.

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**Figure 45.** Inhibition / activation of tyrosinase, the key enzyme in melanogenesis

**Scheme 1.** Up-regulated wild type p53 as the main conductor of ROS-mediated DNA damage / repair in vitiligo

**Table 1.** Sources for H<sub>2</sub>O<sub>2</sub> production in the human epidermis in the mM range

**Table 2.** Effect of H<sub>2</sub>O<sub>2</sub>-mediated oxidation on proteins (enzymes, albumin), peptides and hormones (aromatic steroids) transcription (protein expression) in the human epidermis

**Table 3.** The quantities of each solution required for preparation of polyacrylamine gels for SDS-PAGE.

## Abbreviations

4a-OH-BH <sub>4</sub>	4a-carbinolamine
6BH <sub>4</sub>	(6R)-L-erythro 5, 6, 7, 8 tetrahydrobiopterin
7BH <sub>4</sub>	(7R, S)-L-erythro 5, 6, 7, 8 tetrahydrobiopterin
8-OHdG	8-hydroxy-2'-deoxyguanosine
8-oxoG	8-oxoGuanine
α-MSH	α-melanocyte stimulating hormone
β <sub>2</sub> -AR	β <sub>2</sub> -adrenoreceptor
AchE	acetylcholineesterase
ACTH	adrenocorticotropin hormone
APE1	apurinic / apyrimidinic endonuclease 1
BchE	butyrylcholinestrase
BH <sub>2</sub>	dihydropteridin
BSA	bovine serum albumin
DAPI	4, 6-diamino-2-phenylindole
DHI	dihydroxyindole
DHICA	dihydroxyindole-2 carboxylic acid
DHPR	dihydropteridine reductase
DNA polβ	DNA polymerase beta
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamino tetra acetic acid
EGF	epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay

EMU	epidermal melanin unit
FAD	flavin adenine dinucleotide
FITC	fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFRP	GTP cyclohydrolase I feedback regulatory protein
GR	glutathione reductase
GTP	guanosine triphosphate
GTPCH-I	GTP cyclohydrolase I
hOgg1	human oxo-glycosylase 1
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
iNOS	inducible nitric oxide synthase
L-DOPA	L-dihydroxyphenylalanine
LAT	large neutral amino acid transporter
MAO	monoamine oxidase
MSRA	methionine sulfoxide reductase A
MSRB	methionine sulfoxide reductase B
Mo	molybdenum
MAO-A/B	Monoamine oxidase A/B
Moco	molybdopterin cofactor
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NADP <sup>+</sup>	nicotinamide adenine dinucleotide phosphate
NDS	normal donkey serum
O <sub>2</sub> <sup>•-</sup>	superoxide anion
OH <sup>•</sup>	hydroxyl radical
PAH	phenylalanine hydroxylase

PBS	phosphate buffered saline
PCD	pterin 4a-carbinolamine dehydratase
PDGF	platelet derived growth factor
P/S	penicillin/streptomycin
POMC	pro-opiomelanocortin peptide
qBH <sub>2</sub>	quinonoid dihydropterin
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
SPF	sun protection factor
TBS	tris buffered saline
TGF $\beta$	transforming growth factor
TH I	tyrosine hydroxylase I
TLC	thin layer chromatography
TNF $\alpha$	tumour necrosis factor $\alpha$
TR	thioredoxin reductase
TRITC	tetramethyl rhodamine isothiocyanate
TRP-1&2	tyrosine related protein 1&2
TYR	tyrosinase
UVA	ultraviolet A
UVB	ultraviolet B
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

## List of Publications, Presentations and Posters

### Publications:

1. **Wood JM, Decker H, Hartmann H, Chavan B, Rokos H, Spencer JD, Hasse S, Thornton MJ, Shalhaf M, Paus R, Schallreuter KU**  
Senile hair graying: H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair  
**FASEB J.** 2009 Feb 23. [Epub ahead of print].
2. **KU Schallreuter, S Hasse, B Chavan, M Shalhaf, JD Spencer, JM Wood**  
Cholesterol regulates melanogenesis in human epidermal melanocytes and melanoma cells  
**Exp Dermatol. In press.**
3. **MM Abou Elloof\*, M Shalhaf\*, NCJ Gibbons, B Chavan, and KU Schallreuter**  
DNA binding capacity of upregulated epidermal wild type p53 in vitiligo is enhanced by combined H<sub>2</sub>O<sub>2</sub>- mediated oxidation and nitration  
**FASEB J.** submitted.
4. **Mohammad Shalhaf, Nicholas C.J. Gibbons, John M.Wood, Derek J. Maitland, Hartmut Rokos, Souna M Elwary, Lee K. Marles and Karin U. Schallreuter**  
The presence of epidermal allantoin further supports oxidative stress in vitiligo  
**Exp Dermatol.** 2008 Sep;17(9):761-70. Epub 2008 Mar 4.



## **Conference talks:**

- **Oxidative stress and 8-hydroxydeoxy guanosine in the human epidermis**  
Frontiers of Cutaneous Biology – Neuroendocrine Stress Response in the Human Skin in Greifswald, Germany, August 2007.

## **Abstracts:**

- **H<sub>2</sub>O<sub>2</sub>-mediated oxidation affects epidermal xanthine oxidase**  
68<sup>th</sup> Annual Meeting of the Society of Investigative Dermatology in Los Angeles, California, May 2007.

# 1.0 INTRODUCTION

## 1.1 The human skin

### 1.1.1 Structure

The human integumentary system including skin and its appendages is the largest and heaviest organ of the body with thickness ranging from 1.5 to 4 mm. The size of the skin is around 2 m<sup>2</sup> in area constituting almost 6% of the total body weight (5 to 6 kg). It is made up of different layers of tissues guarding underlying muscles and organs. The importance of this organ in retaining normal homeostasis can be pointed out through its multiple functions. The skin plays the first line of defense against external pathogens, mechanical injuries and dehydration. It is also involved in insulation and temperature regulation of the body, reception of environmental messages through a variety of nerve endings, provision of a waterproof barrier, excretion via sweat glands, and protection via absorption of ultraviolet radiation (UVR) by the pigmentary system. Moreover, the skin contains an immuno-regulatory network and this organ plays an important role in psycho-emotion (Holbrook and Wolff 1993; Gartner 2001; Elias 2005; Tobin 2006).

Histologically, human skin consists of two tissue types. The external layer is a stratified non-vascularised epidermis with an underlying basement membrane and the internal connective tissue layer, the dermis. Both epidermis and dermis collaborate with each other to maintain the properties of both tissues. Below these layers lies the hypodermis or subcutis (subcutaneous adipose layer), which is usually not classified as a layer of skin (Holbrook and Wolff 1993; Gartner 2001; Tobin 2006).

### **1.1.2 The epidermis**

The epidermis, the outer layer of the skin which is continuously being cornified and rejuvenated in 28-30 days cycle, is made up of an ectodermally derived (Weiss and Zelickson 1975) stratified squamous keratinised epithelium with an underlying basal lamina, approximately 0.4 mm (eyelids) to 1.5 mm (palms and soles) thick (Holbrook and Wolff 1993). Keratinocytes in different stages and positions form the majority of cells, constituting the epidermis. Melanocytes, Langerhans cells and Merkel cells are other cells present at different levels. The basement membrane separates the epidermis from the dermis (Holbrook and Wolff 1993; Gartner 2001). Due to cell differentiation of keratinocytes five morphologically distinct strata are identified (Holbrook and Wolff 1993; Gartner 2001).

#### **1.1.2.1 Stratum basale**

The stratum basale (or synonym stratum germinativum) is directly over the basement membrane of the epidermis immediately above the dermis. This layer consists of a single layer of undifferentiated mitotically active, tall, cuboidal columnar epithelial cells with a large nucleus. Their cytoplasm can contain pigmented melanosomes transferred from neighbouring melanocytes. The keratin filaments are formed into fine bundles in the vicinity of the nucleus. This layer is responsible for proliferation by generating new cells. With the beginning of differentiation, the stratum spinosum is formed (Holbrook and Wolff 1993; Gartner 2001).

#### **1.1.2.2 Stratum spinosum**

The stratum spinosum consists of polyhedral KCs and it is located above the stratum basale. The cytoplasm of the cells in this layer of the epidermis contains the same cellular organelles as the cells in the basale layer as well as a rounded nucleus. Moreover, large bundles of intermediate filaments (cytokeratin) compared to cells in the stratum basale exist in their cytoplasm (Holbrook and Wolff 1993; Gartner 2001).

#### **1.1.2.3 Stratum granulosum**

This stratum consists of 1-2 rows of flattened keratinocytes which are still metabolically active and they still have a nucleus. Their cytoplasm is characterised by large and irregularly shaped keratinohylin granules in which bundles of keratin filaments pass through. Programmed destruction of these cells yields differentiated cornified cells accompanied by the loss of the nucleus as well as other cellular organelles except keratin filaments (Holbrook and Wolff 1993; Gartner 2001).

#### **1.1.2.4 Stratum lucidum**

This layer is present only in thick skin (palms and soles), consisting of a thin layer of flattened cells sitting above the stratum granulosum. The cells in this layer lack cytoplasmic organelles and nuclei, but they contain densely packed keratin filaments (Gartner 2001).

#### **1.1.2.5 Stratum corneum**

This layer is the most superficial layer of the skin and is composed of several layers of flattened and keratinised (cornified) cells without nuclei and organelles but filled with keratin filaments. This layer of the epidermis provides mechanical protection to the skin and plays a major role as barrier (Holbrook and Wolff 1993; Gartner 2001; Elias 2005).

### **1.1.3 The keratinocyte**

Keratinocytes are derived from the superficial ectoderm. They form the largest population of cells present in the epidermis. These cells all contain keratin filaments in their cytoplasm giving structure and forming desmosomes (desmosomal junctions) with neighbouring cells (Koch and Roop 2004). Keratinocytes are continuously keratinised, turning over and finally sloughing off from the surface of the epidermis (Gartner 2001). Therefore they need to be renewed. As a result, they are highly mitotically active in the basal layer of the epidermis. The epidermal turnover takes approximately 28-30 days. The keratin pairs in the cells indicate the position of the cells in epidermis and also their status of differentiation. There are different keratins expressed in different layers of the epidermis. Keratin 5 and 14 are expressed in the stratum basale whereas keratin 1 and 10 exist in suprabasal layers (Koch and Roop 2004). Keratinocytes, both differentiated and undifferentiated, release numerous factors which could stimulate the melanocyte in a paracrine fashion by binding to their respective receptors on the melanocyte membrane leading in turn to changes in melanocyte proliferation, differentiation, dendritogenesis and melanin synthesis (Prunieras 1969; Yaar and Gilchrest 1991; Yaar, Grossman et al. 1991; Yada, Higuchi et al. 1991; Imokawa, Yada et al. 1992; Schallreuter, Wood et al. 1992; Imokawa, Yada et al. 1996; Abdel-Naser 1999; Imokawa 2004; Hirobe 2005; Schallreuter, Kothari et al. 2007).

#### **1.1.4 The melanocyte**

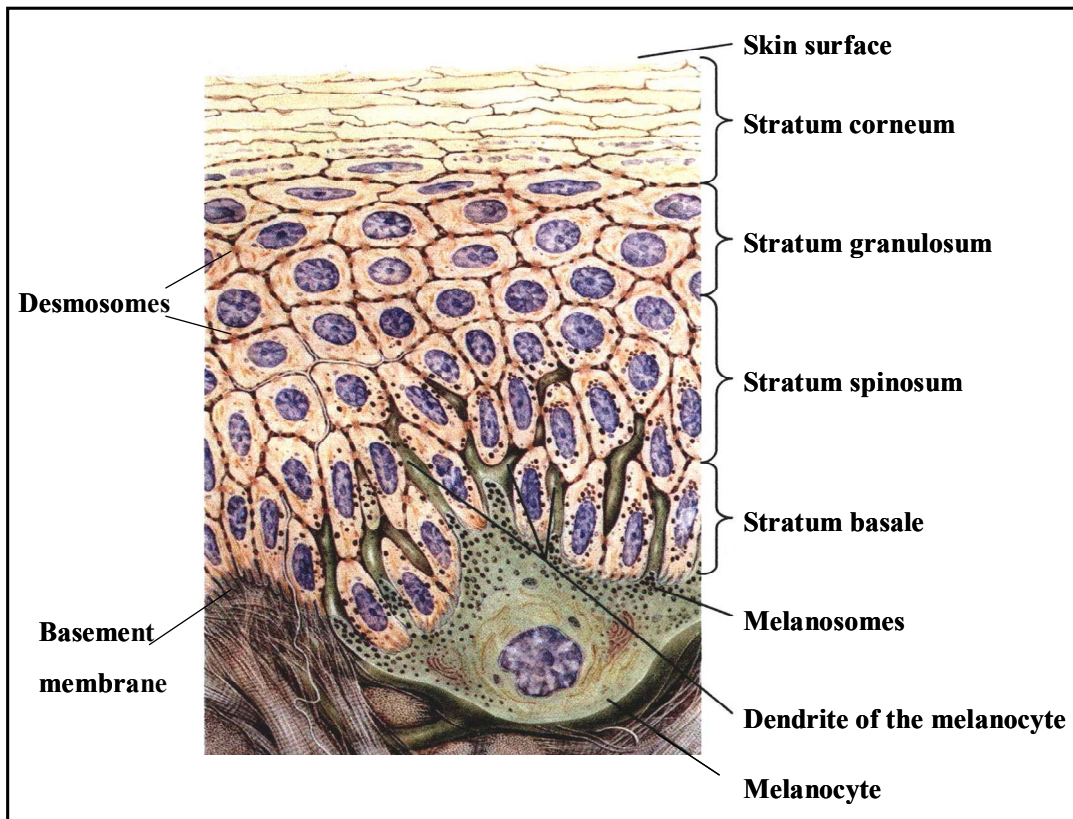
Melanocytes are the pigment synthesising cells, giving the skin its colour. These dendritic cells are derived from melanoblasts originating from the neural crest (Cramer 1991). Melanocytes reside in the stratum basale among the other cells with the average density of 1000 to 2000 melanocytes/cm<sup>2</sup>. Although this density varies in different regions of the human skin, the total number of melanocytes in different racial groups is somewhat constant (Thody 1993). Melanocytes are round to columnar cells with a pale-staining cytoplasm and an ovoid nucleus. Their elongated dendrites extend among neighbouring epidermal cells, facilitating the transfer of melanosomes (the specific organelle where melanin is synthesised) to the keratinocytes. In this way, melanin is distributed into the suprabasal layers of the epidermis (Holbrook and Wolff 1993; Thody 1993; Gartner 2001). In human epidermis, approximately 36 basal and suprabasal keratinocytes are functioning in close working relationship with one melanocyte. This organisation is called “epidermal melanin unit (EMU)” (Fitzpatrick and Breathnach 1963; Fitzpatrick, Miyamoto et al. 1967). Both cell types are in close symbiotic relation during melanogenesis (Fitzpatrick and Breathnach 1963; Hadley and Quevedo 1966).

### **1.1.5 Langerhans cells and Merkel cells**

Langerhans cells are also dendritic cells which are primarily located in suprabasal layers. They originate from precursors in the bone marrow and account for 2 to 8 percent of the epidermal cell population. These cells are a part of a mononuclear phagocyte system and involved in a variety of T-cell responses. Langerhans cells function in the immune response, they are responsible for the recognition, uptake, processing and presentation of foreign antigens to T-lymphocytes present in the lymph nodes in their vicinity. Furthermore, it has been proposed that the Langerhans cells, melanocytes and keratinocytes form an “epidermal tripod” which is characterised by functional interaction (Holbrook and Wolff 1993; Gartner 2001).

Merkel cells are other dendritic cells which are scattered among the keratinocytes of the stratum basale. These cells are differentiated from epithelial cells in the early human fetus. Unmyelinated sensory nerves traverse the basal lamina to approach the Merkel cells, forming Merkel cell-neurite complexes which function as mechanoreceptors. Apart from that, the Merkel cell function is still unclear (Holbrook and Wolff 1993; Gartner 2001).





**Figure 1**

**The structure of human epidermis**

The picture illustrates different cell types and layers in the epidermis (modified from (Christophers and Braver 1987)).

### **1.1.6 The dermis**

The dermis (synonym Corium) makes up the bulk of the skin and it derives from the mesoderm. The dermis is tightly connected to the epidermis by a basement membrane. The thickness of the dermis varies from 0.6mm in the eyelids to 3mm on the palms and soles (Gartner 2001). The dermis is composed of a combination of fibrous, filamentous and dense irregular collagenous connective tissues, which altogether support the epidermis and connect the skin to the underlying hypodermis. These tissues also give the skin its elasticity, flexibility and strength. The major types of fibrous connective tissues in the dermis are collagen and elastin. The dermis also contains the neural and vascular networks, lymphatic systems and its appendages including sebaceous and sweat glands, hair follicles and nails. The most abundant cells in the dermis are fibroblasts, besides macrophages and mast cells. In addition, blood-derived cells including plasma cells and leukocytes can enter the dermis in response to various stimuli. On the basis of different types of fibrous connective tissue organisation, cell density and nerve and vascular patterns dermis is divided into two layers. The upper layer adjacent to the epidermis is the papillary layer and is composed of small bundles of small-diameter collagen fibrils. The deeper layer is the reticular layer is much denser compared to the papillary layer and is made up of large bundles of large-diameter collagen fibrils which accounts for the most thickness of the dermis and also skin on the whole. The papillary dermis has a higher metabolic activity compared to the reticular dermis due to its high density of mesenchymally derived fibroblasts which are responsible for synthesis and degradation of collagen and also fibrous and non-fibrous connective tissue matrix proteins. The border between the two regions is distinguished with a straight plane of vessels and the subpapillary plexus (Holbrook and Wolff 1993; Gartner 2001).

### **1.1.7 The hypodermis (subcutis)**

Conventionally, the hypodermis is the subcutaneous adipose layer underlying directly the dermis. It is composed of fibrous dermal connective tissue containing varying amounts of adipose tissue (Holbrook and Wolff 1993; Gartner 2001).

## **1.2 Skin colour**

### **1.2.1 History**

The first recorded document about a human pigmentation disorder, most likely vitiligo, dates back to around 2200 BC. From that time, the origins of skin and hair colour were an enigma and it was just with the discovery of melanocytes in the 19<sup>th</sup> century that this unsolved mystery was uncovered. This significant discovery allowed investigators to find out more about the melanocytes' origin and development, the structure of melanosomes, their translocation into neighbouring keratinocytes as well as the epidermal melanin unit. These discoveries then justified the differences between human skin phototypes (Westerhof 2006).

### 1.2.2 Inherited versus induced skin colour

The differences in human skin colour are due to variation in epidermal melanin synthesis which can be attributed to many factors such as climates, genetic architecture and also ultraviolet radiation (UVR). Thus the majority of scientists agree that the differences in human skin colour reflect biological adaptations to some aspect of the environment (Jablonski and Chaplin 2000; Barsh 2003). In principle, the human skin colour is genetically determined and that is generally called “constitutive skin colour” (Bennett and Lamoreux 2003). However, it is important to note that inherited skin colour can be also influenced by certain environmental factors such as long-term exposure to UV light. Increase in melanin formation due to external stimuli is termed facultative skin pigmentation (Kaidbey and Kligman 1978; Yamaguchi, Takahashi et al. 2006; Miyamura, Coelho et al. 2007; Yamaguchi, Brenner et al. 2007). Due to higher UV radiation in tropical areas dark skin has emerged to provide more protection against the harmful effects of UV radiation, such as sunburn and skin cancer (Fitzpatrick 1965). In regions with low UV exposure mostly fair skin is present to allow UV-induced biosynthesis of vitamin D<sub>3</sub> to occur (Harris, Soteriades et al. 2000; Jablonski and Chaplin 2000). Pigmentation in response to UV light is commonly known as tanning (Fitzpatrick, Becker et al. 1950; Eller and Gilchrist 2000). Two different mechanisms of tanning are known: immediate tanning and delayed tanning. Immediate tanning can occur within minutes after exposure to UVA (320-400nm) and is believed to be due to oxidation and/or polymerization of existing melanin. Delayed tanning takes several days or longer to become apparent mirrors the pigmentary response to UVB (280-320nm) by the increase in *de novo* production of melanin via the melanogenic pathway (Tadokoro, Yamaguchi et al. 2005; Young 2006; Wolber, Schlenz et al. 2008).

### **1.2.3 Classification**

Human skin colour formed the basis for 6 categories. Fitzpatrick used two parameters for this classification (Fitzpatrick 1988) based on the ability of skin to tan (the result of exposure to ultraviolet radiation) and the inherited skin colour (white, brown or black skin). According to this classification, skin phototype I is very sensitive to ultraviolet light, burns easily and never tans. People who have skin phototype I have the greatest chance of experiencing photoaging and developing skin cancer. Skin phototype II is also very sensitive to ultraviolet light and burns easily but tans minimally. People with skin phototype II are also in the high risk category for photoaging and skin cancer. Skin phototype III is rather sensitive to ultraviolet light, burns moderately and gradually tans to light brown. People with Skin Phototype III also are in the high risk category for photoaging and skin cancer but are not as susceptible as those with types I and II. Skin Phototype IV burns minimally and always tans well to moderately brown. People with skin phototype IV have slightly less of a chance of getting photoaging and skin cancer but are still commonly diagnosed with these diseases. Skin Phototype V rarely burns but tans easily to dark. People with skin phototype V have much less of a chance to develop photoaging and skin cancer although it is still a possibility. And finally skin phototype VI never burns and always tans darkly. Individuals with this skin phototype are still susceptible to photoaging and skin cancer (Fitzpatrick 1988). In this context it is noteworthy that skin colour is not the only basis for effective UV-response. It is possible that even dark skin can experience sunburn. It is also possible that dark skin can be sensitive to sun (KUS personal communications). These observations suggest that pigment is not the only defence mechanism against UV radiation.

#### **1.2.4 Different types of pigment**

Three different pigments contribute to skin colour; i.e. carotenoids, haemoglobins and melanins. Nevertheless, melanin which is present in melanocytes and keratinocytes accounts for most of the variation in the visual appearance of human skin (Fitzpatrick, Becker et al. 1950; Szabo 1954; Stamatias and Kollias 2004; Miyamura, Coelho et al. 2007).

#### **1.2.4.1 Melanin**

Melanin pigments, which are polymorphous biopolymers, are synthesised from the metabolism of the amino acid L-phenylalanine in melanogenesis (Schallreuter and Wood 1999). There are a number of different types of melanin including eumelanin, pheomelanin, neuromelanin, and mixed melanin pigment which differ in proportions and bonding patterns (Prota, Crescenzi et al. 1970; Prota and Thomson 1976; Prota and d'Ischia 1993; Prota 2000). Eumelanin accounts for brown/black hair whereas pheomelanin is in association with red/blond hair. However, only small differences in melanin production can remarkably affect hair colouration (Prota and Thomson 1976; Prota 1992; Prota 2000; Slominski, Tobin et al. 2004; Tobin 2006). Both eumelanin and pheomelanin exist in the human epidermis and they can be produced within the same melanocyte (Thody, Higgins et al. 1991; Jimbow, Lee et al. 1993; Le Pape, Wakamatsu et al. 2008). Neuromelanin is a dark pigment which is produced in the substantia nigra or dark matter of the human brain but is also present in the locus coeruleus of the human brain (Prota and d'Ischia 1993). The common structural property among all types of melanins is arrangement of repeating units linked by carbon-carbon bonds. Nevertheless, there are differences in their chemical, structural and physical properties (Prota 1992; Prota 1995; Ito 2003; Slominski, Tobin et al. 2004).



#### **1.2.4.1.1 Eumelanin**

Eumelanin is primarily formed from the metabolism of L-tyrosine which is produced from L-phenylalanine by intracellular phenylalanine hydroxylase (EC 1.14.16.1, PAH) (Schallreuter and Wood 1999). This pigment behaves like a potent anti-oxidant defence for melanocytes as it has semiquinone units in its structure responsible for its redox status with both reducing and oxidising capabilities towards oxygen radicals, thus protecting the cell from UV-induced oxidative damage by scavenging UV-generated ROS (Sarna and Sealy 1984; Korytowski, Kalyanaraman et al. 1986; Wood and Schallreuter 1991; Tobin and Thody 1994; Prota 1995; Sarna 1998; Gutteridge and Halliwell 2000). Moreover, eumelanin is the most photo-protective melanin despite its sun protection factor (SPF) is only 1-2 (Kaidbey and Kligman 1978; Hill 1992; Wood, Jimbow et al. 1999; Kadarko, Kavanagh et al. 2003). Additionally, melanin has the ability to chelate cations; it can bind to iron and copper ions preventing the Haber-Weiss reaction to occur, in which metal ions react with  $H_2O_2$  to generate  $OH^\bullet$  radicals (Goldstein and Czapski 1986).

#### **1.2.4.1.2 Pheomelanin**

Pheomelanin is yellow to reddish-brown pigment and is abundant particularly in large quantities in blond and red hair (Prota, Crescenzi et al. 1970; Chedekel, Smith et al. 1978). It is formed by a deviation in the eumelanin pathway. The formation of pheomelanin is due to the high concentration of L-cysteine and glutathione which are actively transported through the melanosomal membrane. The conjugation of this amino acid with dopaquinone leads to the formation of cysteinyl-dopa which serves as the starting point of pheomelanogenesis (Slominski, Tobin et al. 2004). Pheomelanin is photo-labile with photolysis products including mostly singlet oxygen followed by superoxide anion ( $O_2^{\bullet-}$ ), hydroxyl radicals ( $OH^{\bullet}$ ) and hydrogen peroxide ( $H_2O_2$ ) (Wood, Jimbow et al. 1999). Unlike eumelanin, this pigment is actually phototoxic rather than having a photoprotective role. Therefore, it may contribute to UV-induced skin damage (Thody, Higgins et al. 1991).

#### **1.2.4.1.3 Neuromelanin**

Neuromelanin is present in the substantia nigra and locus coeruleus of the human brain. This pigment is synthesised by polymerisation of dopamine with L-cysteine (Bazelon, Fenichel et al. 1967; Prota and d'Ischia 1993; d'Ischia and Prota 1997).

## **1.3 Melanogenesis**

Melanogenesis (pigmentation) occurs in melanocytes within specialised intracellular organelles called melanosomes. This event involves a sequence of oxidoreduction reactions and intramolecular transformations which finally leads to the synthesis and deposition of melanin.

### **1.3.1 The melanosome**

Melanosomes are lysosome-related organelles in the melanocytes in which the biosynthesis of melanin pigments occur. These spherical, membrane-coated vesicles are originated from the endoplasmic reticulum. They were first described by Seiji in 1961 as organelles where the pigment is packaged (Seiji, Fitzpatrick et al. 1961; Seiji, Shimaio et al. 1961; Orlow 1995; Barral and Seabra 2004). The size of melanosomes varies among human races and is genetically determined. Black skin contains larger melanosomes compared to other lightly pigmented skin. Melanosomes mature in a four staged process corresponding to the degree of melanisation and morphology. In stage I of eumelanogenesis melanosomes (eumelanosomes) are spherical vesicles containing a poorly organised internal structure. In stage II melanosomes become ellipsoidal with a well developed internal structure containing organised lamellae and microvesicles but without melanin formation. In pheomelanosomes melanin is synthesised already in this stage. In stage III melanisation begins on the lamellae and melanosomes have a well organised internal structure with the accumulation of melanin. Melanosomes in stage IV are completely filled with melanin (Holbrook and Wolff 1993; Thody 1993; Slominski, Tobin et al. 2004).

### **1.3.2 Melanosomal transfer to neighbouring keratinocytes**

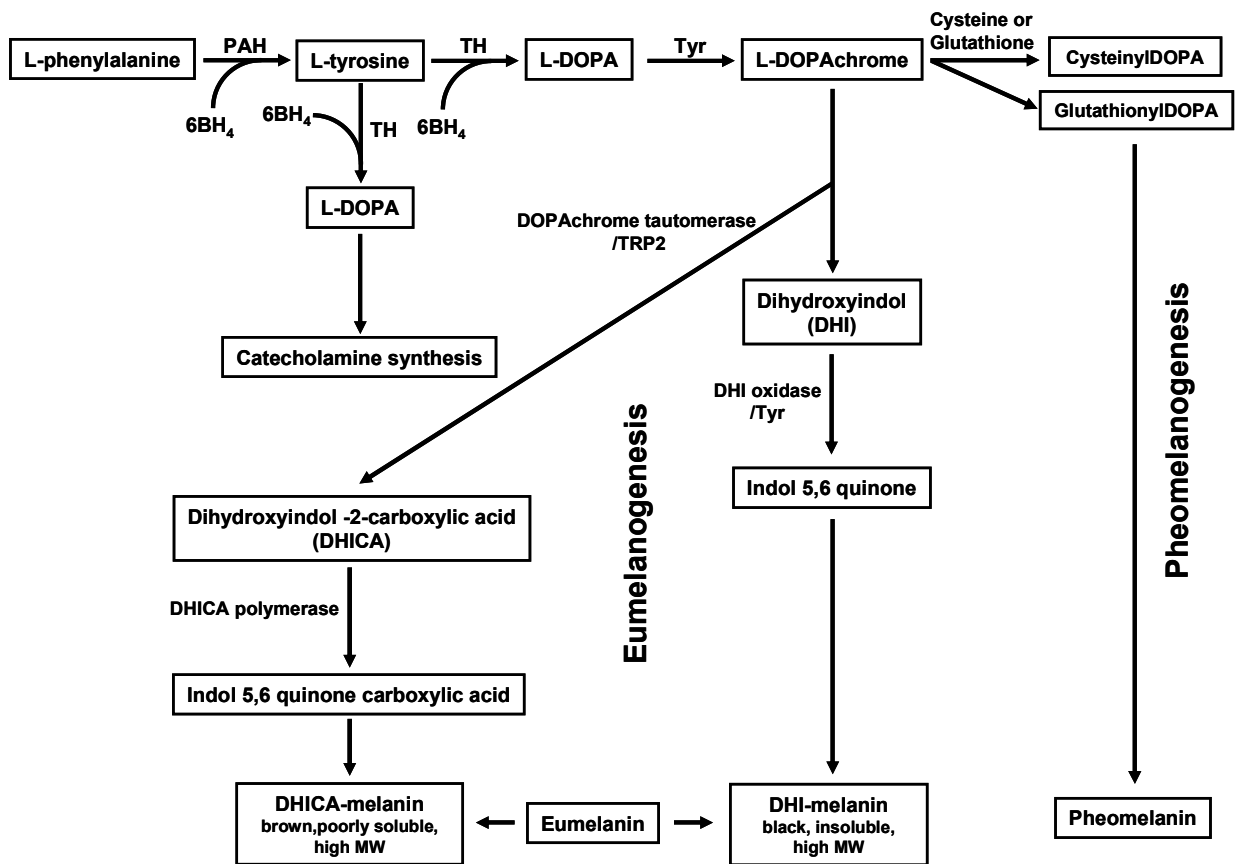
Visible pigmentation requires the transfer of melanin granules from melanocytes to neighbouring keratinocytes; the process in which melanin pigments are dispersed throughout the basal and suprabasal layers. Melanosomal translocation was first described by Prunieras in 1969 (Prunieras 1969). Although there have been many attempts to study this process over the past 40 years, the exact mechanism(s) involved in this complex phenomenon is yet to be understood. Once fully melanised and matured, the melanosomes are moved from the perikaryon of the melanocytes to the tip of their dendrites which are in contact with associated keratinocytes in each epidermal melanin unit. Mammalian melanocytes contain 10nm intermediate filaments in their cytosol described as “microtrabeculae” (Schliwa, Euteneuer et al. 1979) which are longitudinally located in the dendrites (cytoskeleton) and they specifically transfer the melanosomes from the perikaryon onto the dendrites. Previous reports have suggested that melanosomes move bi-directionally along the mentioned microtubules (Barral and Seabra 2004). Subsequently, the melanosomes travel from the dendrites into the keratinocytes. To date, three hypotheses have been suggested. Once melanosomal translocation to keratinocytes is initiated, keratinocytes may phagocytose the melanosome-filled dendritic tips of melanocytes (Cruickshank and Harcourt 1964; Mottaz and Zelickson 1967; Klaus 1969; Okazaki, Uzuka et al. 1976; Yamamoto and Bhawan 1994). The other hypothesis is that melanosomes may be directly released into the keratinocytes by melanocytes via a temporary membrane fusion and opening a cytoplasmic channel (Garcia, Flynn et al. 1983). Another hypothesis is that melanocytes first discharge the melanosomes into the extracellular space and then keratinocytes phagocytose them (Swift 1964; Yamamoto and Bhawan 1994). Once the translocation of matured melanosomes into keratinocytes is

completed, they interact with lysosomes of keratinocytes. Then while the keratinocytes are moving towards the skin surface, melanosomes are degraded within secondary lysosomes by lysosome hydrolase (Holbrook and Wolff 1993).

### 1.3.3 Melanin biosynthesis

The biosynthesis of melanin involves a series of complex events. The velocity and specificity of this process are under the regulation of many enzymes including phenylalanine hydroxylase (EC 1.14.16.1, PAH), tyrosine hydroxylase (EC 1.14.16.2, TH) and tyrosinase (EC 1.14.18.1, Tyr) (Fitzpatrick, Becker et al. 1950; Lerner and Fitzpatrick 1950; Fitzpatrick and Lerner 1954; Fitzpatrick, Seiji et al. 1961; Fitzpatrick 1965; Protá and Thomson 1976). The availability of the amino acid L-tyrosine initiates melanin biosynthesis. This amino acid can either be transported from the extracellular space into the melanocytes through the plasma membrane via facilitated diffusion rather than active transport (Schallreuter and Wood 1999) and further from the cytosol into the melanosomes (Potterf, Muller et al. 1996; Potterf and Hearing 1998), or can be formed in the melanocytes by hydroxylation of L-phenylalanine via intracellular phenylalanine hydroxylase in the presence of the cofactor (6R)-L-5, 6, 7, 8 tetrahydrobiopterin (6BH<sub>4</sub>) (Kaufman 1957; Kaufman 1958; Kaufman 1959; Schallreuter and Wood 1999). Because L-tyrosine is highly demanded for melanogenesis to proceed and this demand is not fully provided by facilitated diffusion since its serum levels are too low (Schallreuter, Chavan et al. 2005), thus the amino acid L-phenylalanine is actively taken up by melanocytes from the extracellular space via a large neutral amino acid transporter type 1 (LAT-1) or type 2 (LAT-2) to be converted to L-tyrosine via intracellular PAH (Schallreuter and Wood 1999). Therefore L-tyrosine pool in melanocytes is mainly supplied by the hydroxylation of L-phenylalanine to L-tyrosine via intracellular PAH (Schallreuter and Wood 1999). Taken together, melanogenesis can be initiated either directly from the hydroxylation of intracellular L-tyrosine to L-DOPA, or from the hydroxylation of L-phenylalanine to L-tyrosine. L-DOPA is a precursor for the synthesis of both

melanins and catecholamines via separate pathways. It has been shown that both human melanocytes and keratinocytes hold the full capacity for catecholamine biosynthesis (Schallreuter, Wood et al. 1992; Gillbro, Marles et al. 2004). The type of melanin synthesised is determined by the enzymatic archive available for the melanocytes. For instance, in central nervous system, L-DOPA is either decarboxylated by aromatic amino acid decarboxylase (AAD) to produce catecholamines or oxidised to form neuromelanins (for review see (Slominski, Tobin et al. 2004). The hydroxylation of L-tyrosine to L-DOPA via tyrosine hydroxylase I is an obligatory and rate-limiting step in melanin biosynthesis (Marles, Peters et al. 2003). L-DOPA is then oxidised to dopaquinone, the pathway which is common in eumelanogenesis and pheomelanogenesis (Prota 1992; Prota 2000). The formation of eu- or pheomelanin is directly determined by the presence/absence of cysteine which is actively transported through the melanosomal membrane via the cysteine/glutamate exchangers (Sato, Ito et al. 1987; Benathan, Virador et al. 1999; Potterf, Virador et al. 1999). When the concentration of cysteine or glutathione is high, it is conjugated to dopaquinone yielding to the formation of pheomelanin (Prota 1995; Ito 2003). In eumelanogenesis, dopaquinone is further transformed to leukodopachrome, followed by a series of oxidoreduction reactions as well as polymerisation which finally leads to the formation of eumelanin (Pawelek 1991; Prota 1992; Ito 2003).



**Figure 2**

### Melanogenesis pathways

The modified Raper-Mason scheme for the biosynthesis of eumelanin and pheomelanin from L-phenylalanine in the presence of 6BH<sub>4</sub>, PAH, TH and tyrosinase. **PAH:** Phenylalanine hydroxylase (Schallreuter and Wood 1999), **TH:** tyrosine hydroxylase, **TYR:** Tyrosinase, **TRP1&2:** Tyrosine Related Protein 1&2, **6BH<sub>4</sub>:** (6R)-L-erythro 5, 6, 7, 8 tetrahydrobiopterin.



### **1.3.3.1 Phenylalanine hydroxylase**

Phenylalanine hydroxylase (PAH) catalyses the hydroxylation of the amino acid L-phenylalanine to L-tyrosine. The enzyme's activity depends on the presence of tetrahydrobiopterin as cofactor and molecular oxygen (dioxygen) as additional substrate (Kaufman 1958; Kaufman and Levenberg 1959; Teigen and Martinez 2003). The active site of this enzyme contains an iron atom, where it binds directly to dioxygen to be activated. Therefore, the transformation of the iron atom from the ferric form to the ferrous form is essential for the activation of the enzyme (Teigen, Froystein et al. 1999; Teigen and Martinez 2003). Also, activation of the enzyme needs pre-incubation with the substrate which enables phosphorylation of amino acid Serine-16 as well as tetramerisation of the protein to occur (Daubner, Hillas et al. 1997; Miranda, Teigen et al. 2002; Miranda, Thorolfsson et al. 2004). Moreover, the natural cofactor tetrahydrobiopterin (6BH<sub>4</sub>) is involved in pre-reduction of the active site of the enzyme (Teigen and Martinez 2003). This cofactor regulates the enzyme activity as well as being essential in catalysis (Schallreuter, Wood et al. 1994; Schallreuter, Slominski et al. 1998) and recently it has been indicated that 6BH<sub>4</sub> has a chaperone-like effect on PAH synthesis by protecting it from degradation via shielding the active site from ROS (Pey, Perez et al. 2004) and also from enzyme auto-activation (Thöny, Ding et al. 2004). Hence PAH is under absolute control by the 6BH<sub>4</sub> concentration in the cytosol of melanocytes.

### 1.3.3.2 Tyrosine hydroxylase

Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu, Levitt et al. 1964). It is responsible for catalysing the hydroxylation of the amino acid L-tyrosine to L-DOPA which is a precursor to dopamine in the process of the adrenaline (epinephrine) synthesis. The same as PAH, this enzyme is iron-containing and tetrahydrobiopterin-dependent which uses molecular oxygen to hydroxylate its substrate (Ellenbogen, Taylor et al. 1965; Fitzpatrick 1999; Dunkley, Bobrovskaya et al. 2004). In order for the enzyme to become catalytically active, in the presence of  $O_2$ ,  $6BH_4$  reduces the iron atom from the ferric form ( $Fe^{3+}$ ) to ferrous form ( $Fe^{2+}$ ) in its active site. Furthermore, only recently it has been indicated that the phosphorylation of TH at Serine-40 increases the enzyme's activity *in vitro*, *in situ* and *in vivo* (Dunkley, Bobrovskaya et al. 2004). It has been reported that the human epidermis express only a functioning TH isoform I, while the other isoforms are absent (Marles, Peters et al. 2003). The enzyme is sitting site by site with tyrosinase. TH I activity has been identified in melanosomes. Since tyrosinase requires L-DOPA for activation, it has been proposed that TH I activity is tightly coupled to tyrosinase in melanosomes to initiate melanogenesis (Marles, Peters et al. 2003).

### 1.3.3.3 Tyrosinase

Mammalian tyrosinase is a melanosomal membrane bound and copper-containing enzyme which holds a central position in melanin biosynthesis (Lerner, Fitzpatrick et al. 1948; Fitzpatrick, Becker et al. 1950; Wang and Hebert 2006). This enzyme was discovered for the first time by Bourquelot and Bertrand in 1896 (Raper and Wormald 1923), but it was Raper who discovered that melanin is formed from L-tyrosine via tyrosinase (Raper and Wormald 1923; Happold and Raper 1925; Raper and Wormald 1925; Raper 1926; Raper 1927). This enzyme which is found in epidermal melanocytes as well as the pigment epithelia of the retina, iris, and ciliary body of the eye, catalyses three distinct reactions in the melanogenic pathway; hydroxylation of L-tyrosine to L-DOPA followed by the rapid oxidation (dehydrogenation) of the latter to L-DOPAquinone (Lerner, Fitzpatrick et al. 1948; Pomerantz 1966), and also oxidation (dehydrogenation) of 5, 6 dihydroxyindole (DHI) and 5, 6 dihydroxyindole-2-carboxylic acid (DHICA) into the eumelanin precursors indole-5, 6-quinone and indole-5, 6-quinone carboxylic acid respectively (Korner and Pawelek 1982; Wang and Hebert 2006). Furthermore, tyrosinase can also react with UV light generated oxygen radicals such as superoxide anion (Wood and Schallreuter 1991; Prota 1992). Tyrosinase mRNA shows no difference in different skin phototypes (Fitzpatrick classification), thus its protein expression in the epidermis among all human skin phototypes does not vary (Iwata, Corn et al. 1990; Iozumi, Hoganson et al. 1993). Hence, the formation of melanin depends either on the substrate supply or on metabolic inhibitors and activators of this enzyme (Wood and Schallreuter 1991; Iozumi, Hoganson et al. 1993). The active site of tyrosinase contains two copper atoms which introduce dioxygen into the substrates (tyrosine or L-DOPA) (Lerner, Fitzpatrick et al. 1950; Wang and Hebert 2006; Ullrich and Hofrichter 2007). Inactive

form of the enzyme requires the reduction of these two copper atoms (from  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$ ) in order to become fully activated (Lerner, Fitzpatrick et al. 1950) and in this reduction step, L-DOPA is the most efficient electron donor necessary to start the hydroxylation process of L-tyrosine although other compounds such as ascorbic acid, dopamine and superoxide anion radicals are potentially able to activate the enzyme (Wood and Schallreuter 1991). As stated above TH isoenzyme I is expressed in the melanosomal membrane of the human melanocytes side by side with tyrosinase (Marles, Peters et al. 2003). This implies a coupled reaction, where L-DOPA produced by this isoform of TH facilitates tyrosinase activation which consequently initiates melanogenesis (Marles, Peters et al. 2003). However, it was shown when  $\text{O}_2^{\bullet-}$  replaced  $\text{O}_2$  as substrate, the activation of tyrosinase to oxidise L-tyrosine to dopachrome was 40-fold increased but this increase depends on the removal of  $\text{H}_2\text{O}_2$  from the reaction mixture by catalase (Wood and Schallreuter 1991). This observation highlights the role of UV-generated  $\text{O}_2^{\bullet-}$  in melanogenesis (Wood and Schallreuter 1991).

#### **1.3.3.3.1 Tyrosinase activity is pH dependent**

Tyrosinase activity is tightly related to pH. Almost at neutral pH the enzyme displays maximum enzymatic activity with little activity below pH 5 (Hearing and Ekel 1976; Townsend, Guillery et al. 1984; Saeki and Oikawa 1985; Ancans, Hoogduijn et al. 2001; Ancans, Tobin et al. 2001; Watabe, Valencia et al. 2004; Wang and Hebert 2006). Therefore, due to the existence of acidic pH (as low as 5) in pre-melanosomes (Bhatnagar, Anjaiah et al. 1993; Puri, Gardner et al. 2000), neutralization of the normally acidic environment seems vital for tyrosinase activity (Ancans and Thody 2000). For this purpose, melanosomes own a proton pump that regulates intramelanosomal pH (Ancans, Hoogduijn et al. 2001). By pumping  $H^+$  out from the melanosomes, the pH switches from 5 to 6.8 initiating melanogenesis which, in turn is optimal for tyrosinase activity where lower pH is optimal for TH function (Ancans, Hoogduijn et al. 2001; Ancans, Tobin et al. 2001; Watabe, Valencia et al. 2004).

#### **1.3.3.3.2 Tyrosinase activity is influenced by 6BH<sub>4</sub>**

The cofactor 6BH<sub>4</sub> has been reported to down regulate tyrosinase activity by an uncompetitive mechanism only when L-tyrosine is substrate whereas when L-DOPA is substrate, 6BH<sub>4</sub> does not inhibit tyrosinase activity. This indicates that there are two separate binding sites for L-tyrosine and L-DOPA on this enzyme (Wood, Schallreuter-Wood et al. 1995; Olivares, Garcia-Borron et al. 2002). The tyrosinase-6BH<sub>4</sub> inhibitory complex can be reactivated by  $\alpha$ -MSH binding directly to 6BH<sub>4</sub> (Moore, Wood et al. 1999; Schallreuter, Moore et al. 1999). Moreover, it was shown that UVB light oxidises 6BH<sub>4</sub> to 6-biopterin, reactivating tyrosinase. This is considered as a 'photo-switch' allowing melanogenesis to proceed (Schallreuter, Wood et al. 1998).

## 1.4 Pteridines in pigmentation

Pteridines were discovered more than 100 years ago, when Sir Frederick Gowland Hopkins isolated two distinct yellow and white pigments from butterfly wings, but Wieland and Schöpf were the first who introduced those pigments as “pteridines” in 1925 for the first time and named them xanthopterin and leucopterin according to their colours. These chemical compounds perform many roles in colouration in the biological world. Pterins also function as cofactor in enzymatic catalysis. Tetrahydrobiopterin, the major pterin in vertebrates, is involved in the hydroxylation of aromatic compounds and synthesis of nitric oxide. Molybdopterin, which contains pterin and molybdenum is involved in biological hydroxylations (Nichol, Smith et al. 1985).

### 1.4.1 6BH<sub>4</sub> in the human epidermis

The importance of this cofactor in the human epidermis has been demonstrated by Schallreuter and colleagues in 1994. Both epidermal melanocytes and keratinocytes hold the full capacity to synthesise and recycle 6BH<sub>4</sub> (Schallreuter, Wood et al. 1994). 6BH<sub>4</sub> plays three major roles in melanin biosynthesis in order to regulate the supply of the melanin precursors and substrates. It functions as an essential cofactor for PAH and TH isoform I and it is a potent uncompetitive inhibitor for tyrosinase. But this inhibition is reversible by UVB photo-oxidation of the pterin ring in the presence of O<sub>2</sub> from 6BH<sub>4</sub> to quinonoid dihydropterin (qBH<sub>2</sub>) followed by the formation of 7, 8 dihydropterin (BH<sub>2</sub>), and finally to 6-biopterin in addition to the production of O<sub>2</sub><sup>•-</sup> (Kaufman 1957; Kaufman 1959; Kaufman and Levenberg 1959; Moore, Wood et al. 1999). It must be noted that neither qBH<sub>2</sub> nor BH<sub>2</sub> binds or inhibits tyrosinase (Wood,

Schallreuter-Wood et al. 1995). Also, 6BH<sub>4</sub> plays an important role as a cofactor in the synthesis of neurotransmitters serotonin and the catecholamines (Kaufman 1957; Kaufman 1959; Kaufman and Levenberg 1959). This cofactor is the immediate electron donor for hydroxylation of the amino acids phenylalanine, tyrosine and tryptophan in the presence of molecular oxygen. 6BH<sub>4</sub> is synthesised *de novo* by three enzymes: GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase (Nichol, Smith et al. 1985; Kaufman 1997; Thöny, Auerbach et al. 2000).

#### **1.4.2 7BH<sub>4</sub> in the human epidermis**

(7R)-L-erythro-5, 6, 7, 8 tetrahydrobiopterin (7BH<sub>4</sub>), the abiogetic isomer of 6BH<sub>4</sub> is produced nonenzymatically in very low levels during the recycling process of this cofactor (Curtius, Alder et al. 1990). These two isomers can directly control tyrosinase activity via a specific binding domain on the enzyme. 7BH<sub>4</sub>, however, is twice as effective as 6BH<sub>4</sub> in inhibiting tyrosinase activity (Wood, Schallreuter-Wood et al. 1995). Moreover, under certain pathologic conditions in the epidermis such as vitiligo, due to defective *de novo* synthesis, recycling and regulation of 6BH<sub>4</sub>, 7BH<sub>4</sub> can accumulate to mM concentrations inhibiting PAH activities by a competitive mechanism thus preventing the turnover of L-phenylalanine to L-tyrosine resulting in a build up of L-phenylalanine in the epidermis (Davis, Ribeiro et al. 1992; Schallreuter, Wood et al. 1994; Pey, Martinez et al. 2006). The presence of 7BH<sub>4</sub> in the melanosome in physiological concentrations has been shown. However,  $\alpha$ -MSH cannot reactivate the tyrosinase-7BH<sub>4</sub> inhibitor complex. But  $\beta$ -melanocyte stimulating hormone ( $\beta$ -MSH) can bind 7BH<sub>4</sub> reactivating tyrosinase from the inhibitor complex (Spencer, Chavan et al. 2005). These findings highlight the role of

7BH<sub>4</sub> in the regulation of melanogenesis suggesting for the first time physiological function for this protein.

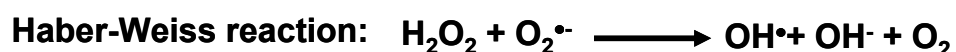


## 1.5 Oxidative stress

Helmut Sies coined the term oxidative stress for the first time in 1985, which defines the disturbance in prooxidant-antioxidant balance in favour of the former, indicating the damage to cells and thereby to the organs and tissues of those cells (Cadenas and Sies 1985; Sies and Cadenas 1985). This condition is caused by reactive oxygen species (ROS), very small and highly reactive molecules due to their unpaired electrons. These include superoxide anion ( $O_2^{\bullet-}$ ), singlet oxygen, hydroxyl radical ( $OH^{\bullet}$ ) and hydrogen peroxide ( $H_2O_2$ ). ROS are generated in cells by several mechanisms (Halliwell 1989; Halliwell and Gutteridge 1989; Smith, Marks et al. 2004).  $H_2O_2$  is a very reactive molecule, however,  $O_2^{\bullet-}$  and  $H_2O_2$  can also generate hydroxyl radical ( $OH^{\bullet}$ ) which is a much more reactive ROS especially in the presence of transition metals such as iron or copper and this happens through the Fenton reaction and the Haber-Weiss reaction (Haber and Weiss 1932; Goldstein and Czapski 1986). ROS are generally formed as natural byproducts of the normal metabolism of oxygen. Therefore low levels of ROS are continuously present in cells under physiological conditions (Thannickal and Fanburg 2000). However, the cellular redox status is precisely regulated and living cells are capable to maintain ROS within non-toxic levels via their antioxidant defence machinery which consist of multiple enzymatic and non-enzymatic systems. The toxic effects of ROS become evident when the rate of their generation exceeds the defence capacities of cells (Cadenas and Sies 1985; Halliwell 1989; Poli, Leonarduzzi et al. 2004; Glantzounis, Tsimoyiannis et al. 2005).

### 1.5.1 Oxidative stress in the human epidermis

Human skin is in continuous contact with endogenous and exogenous ROS produced by physical, chemical and biological reactions. Therefore it requires neutralising mechanisms to regulate its redox status and subsequently to maintain its redox homeostasis. These mechanisms include enzymes such as superoxide dismutase, glutathione reductase, glutathione peroxidase, thioredoxin reductase, thioredoxin peroxidase and catalase and non-enzymatic systems including small trapping molecules such as thiols (glutathione and thioredoxin) vitamins C and E, the amino acids L-methionine and L-tryptophan, 6- and 7-tetrahydrobiopterins and lipoic acid (Schallreuter 2005). Moreover, melanin itself contains free radical traps. Here it is noteworthy to mention that there is a close correlation between skin colour and individual free radical defence capacity as darker skin phototypes (Fitzpatrick classification) express significantly more effective mechanisms against UV induced damage (Schallreuter 2005). The most potent ROS is the hydroxyl radical ( $\text{OH}^\bullet$ ) as it reacts with nucleotide bases causing in turn irreversible damage to DNA. ROS also react with fatty acids and amino acids leading finally to disturbance in the cell membrane and protein function, enzyme deactivation and disruption of transcription (Smith, Marks et al. 2004). This radical is produced from  $\text{H}_2\text{O}_2$  by the Fenton and Haber-Weiss reaction (Haber and Weiss 1932; Goldstein and Czapski 1986):



As shown above the production of  $\text{OH}^\bullet$  in the epidermis depends on the presence of  $\text{H}_2\text{O}_2$  and this highlights the role of  $\text{H}_2\text{O}_2$  in the skin. Therefore it is noteworthy to mention that although  $\text{H}_2\text{O}_2$  is not a free radical, it is a strong reactive oxidising agent that can generate  $\text{OH}^\bullet$  in the presence of transition metals i.e.  $\text{Fe}^{2+}$  (Goldstein and Czapski 1986).

## 1.6 Vitiligo

### 1.6.1 What is Vitiligo?

Vitiligo is a disfiguring, progressive and acquired idiopathic non-infectious skin and hair disorder characterised by depigmented lesions as white patches with variable size, shape and location (Lerner 1959; Lerner and Nordlund 1978; Le Poole and Boissy 1997; Westerhof, Njoo et al. 1997; Schallreuter 2005; Schallreuter, Bahadoran et al. 2008). The incidence of vitiligo is 0.5 to 2% worldwide and the precise aetiology is complex and not fully understood. The exact mechanisms involved in the occurrence of the disease are still a matter of controversy, however, a number of hypotheses have been put forward, mainly the autoimmune, neural, genetics and oxidative stress. All of these theories are related primarily to the loss of functional melanocytes leading to a lack of melanin production (Westerhof and d'Ischia 2007; Schallreuter, Bahadoran et al. 2008). This depigmentation disorder can occur anywhere on the body, regardless of sex or race and can involve the entire epidermis. Also the course of the disease is different in individuals (Bystryń 1997; Castanet and Ortonne 1997; Tobin, Swanson et al. 2000; Passeron and Ortonne 2005; Schallreuter 2005). Vitiligo is defined by the disturbance in the function of some or all of melanocytes in the epidermis leading to the appearance of white cutaneous spots (Bystryń 1997; Passeron and Ortonne 2005; Schallreuter 2005). Wood's light (UVA 351nm) is utilised in order to diagnose vitiligo due a distinct fluorescence in depigmented lesions which is owed to the presence of oxidised pteridines (Schallreuter, Wood et al. 1994).

### **1.6.2 The autoimmune hypothesis**

The autoimmune hypothesis suggests there are specific auto-antibodies involved in the progress of the disease as it has been indicated that these auto-antibodies exist in patients with vitiligo against specific antigens on the melanocyte cell surface leading to their destruction (Naughton, Eisinger et al. 1983; Kemp, Waterman et al. 2001; Kemp, Waterman et al. 2002).

### **1.6.3 The neural hypothesis**

This hypothesis states that the released catecholamines from nerve endings disturb the pigment. It is believed that these neural end products are involved in 30% of patients with vitiligo (Bose 1994).

### **1.6.4 The autocytoxicity hypothesis**

Autocytoxicity or the Self Destruction is another hypothesis for the incidence of vitiligo. This hypothesis believes that some of the phenolic and catecholic compounds as well as free radicals which are formed from the complex oxidation reactions in the process of melanin biosynthesis can be toxic for melanocytes initiating apoptosis (Boissy and Manga 2004; Westerhof and d'Ischia 2007).

### 1.6.5 Vitiligo, a model for oxidative stress

Over the last decade vitiligo has been used as a model disease for oxidative stress (Schallreuter, Wood et al. 1991; Schallreuter, Wood et al. 1994; Maresca, Roccella et al. 1997; Schallreuter 2005; Dell'anna and Picardo 2006; Schallreuter, Bahadoran et al. 2008). In particular it has been shown that  $H_2O_2$  plays a major role in the pathogenesis of this disease. Numerous sources of  $H_2O_2$  generation have been identified and it has been shown *in vivo* that epidermal  $H_2O_2$  concentration can be in the mM range (Schallreuter 2005; Schallreuter, Bahadoran et al. 2008). It has been well documented that the entire epidermis of patients with vitiligo is under oxidative stress (Schallreuter, Moore et al. 1999). Not only melanocytes, but keratinocytes and Langerhans cells are disturbed in this disease as well. Moreover, catalase (EC 1.11.1.6) levels have been shown to be significantly reduced in both lesional and non-lesional epidermis of patients (Schallreuter, Wood et al. 1991; Schallreuter, Moore et al. 1999). Maresca et al. also reported a similar observation under *in vitro* condition using MCs originated from non-lesional epidermis of patients with vitiligo (Maresca, Roccella et al. 1997). Under normal physiological conditions, catalase disproportionates  $H_2O_2$  to  $H_2O$  and  $O_2$  (Aronoff 1965). In addition, the activity of glutathione peroxidase (EC 1.11.1.9), an important enzyme for the efficient removal of  $H_2O_2$  in the presence of low catalase is also disturbed along with thioredoxin reductase (EC 1.8.1.9) activity, resulting in the accumulation of higher concentrations of  $H_2O_2$  (Schallreuter, Pittelkow et al. 1986; Schallreuter, Hordinsky et al. 1987; Beazley, Gaze et al. 1999). Another target for  $H_2O_2$ -mediated oxidation is L-methionine. This amino acid is one of the most prone amino acids to oxidation yielding methionine sulfoxide. Under normal physiological conditions, the oxidized amino acid is reduced by methionine sulfoxide reductase A/B (EC 1.8.4.11,

MSRA/B) (Ogawa, Sander et al. 2006; Schallreuter 2006; Schallreuter, Rübsam et al. 2006). It has been documented that the level of these enzymes is considerably lower in vitiligo (Ogawa, Sander et al. 2006; Schallreuter, Rübsam et al. 2008). It has been also shown that MSRA/B themselves are targeted by H<sub>2</sub>O<sub>2</sub>-mediated oxidation, thus affecting the repair of oxidised proteins and peptides (Schallreuter, Rübsam et al. 2008).

Furthermore, it has been demonstrated that the epidermis of patients contains oxidised pterins (Schallreuter, Wood et al. 1994). The accumulation of these fluorescent compounds in the epidermis of patients with vitiligo is due the defective *de novo* synthesis/recycling/regulation of 6BH<sub>4</sub>, the essential cofactor for melanogenesis, which subsequently leads to the accumulation of its abiogenic isomer, 7BH<sub>4</sub>. It has been shown that μM concentration of 7BH<sub>4</sub> inhibit PAH resulting to the prevention of the turnover of L-phenylalanine to L-tyrosine leading in turn to L-phenylalanine build up in the epidermis (Davis, Ribeiro et al. 1992; Schallreuter, Zschiesche et al. 1998). It has also been reported that H<sub>2</sub>O<sub>2</sub> deactivates pterin-4α-carbinolamine dehydratase (EC 4.2.1.96, PCD) in both epidermal melanocytes and keratinocytes. PCD is the rate limiting recycling enzyme in 6BH<sub>4</sub> biosynthesis (Schallreuter, Wood et al. 1994; Schallreuter, Moore et al. 2001). A low PCD enzyme activity also results in the accumulation of the isomer 7BH<sub>4</sub> (Schallreuter, Wood et al. 1994). Moreover, it has been shown that the final step of 6BH<sub>4</sub> recycling is decreased due to deactivation of dihydropteridine reductase (EC 1.6.99.7, DHPR) since H<sub>2</sub>O<sub>2</sub> oxidises L-methionine residues in the structure of this enzyme resulting in an altered NADH binding site which consequently leads to enzyme deactivation (Schallreuter, Moore et al. 2001; Hasse, Gibbons et al. 2004).

In this context, it is noteworthy that H<sub>2</sub>O<sub>2</sub>-mediated oxidation of other proteins and peptides such as calmodulin, POMC peptides, acetylcholinesterase (EC 3.1.1.7, AchE), butyrylcholinesterase (EC 3.1.1.8, BchE), affects their normal biological functionality in the epidermis of patients (Schallreuter and Pittelkow 1988; Schallreuter, Elwary et al. 2004; Schallreuter, Gibbons et al. 2006; Schallreuter and Elwary 2007; Schallreuter, Gibbons et al. 2007; Spencer, Gibbons et al. 2007; Spencer, Gibbons et al. 2008).



**Table 1**  
**H<sub>2</sub>O<sub>2</sub> Production in the Human Epidermis in the mM Range**

Sources	Epidermal sources and targets
UVA/UVB (Schallreuter, Bahadoran et al. 2008)	sunlight
Pheomelanin (Schallreuter, Bahadoran et al. 2008)	melanosomes
TNF- $\alpha$ (Schallreuter, Bahadoran et al. 2008)	physical/chemical stress to the human epidermis, acts via the mitochondrial SOD
TGF- $\beta$ PDGF EGF (Thannickal, Day et al. 2000; Thannickal and Fanburg 2000)	epidermal melanocytes and keratinocytes
TYR TH (Marles, Peters et al. 2003; Wood, Chavan et al. 2004)	epidermal melanocytes and keratinocytes
PAH (Schallreuter, Wood et al. 1994)	epidermal melanocytes and keratinocytes
MAO-A (Schallreuter, Wood et al. 1996)	catecholamine degradation in the human epidermis
Photo-oxidation of pterins (Rokos, Beazley et al. 2002)	epidermal melanocytes and keratinocytes
Aromatic steroids (estrogens, progestrones and androgens) (Thornton 2002; Schallreuter, Bahadoran et al. 2008)	Via the oxidation by cytochrome P450 in both epidermal melanocytes and keratinocytes
NADPH-oxidase (oxygen burst) (Yu 1994)	activation of epidermal leucocytes and macrophages due to inflammation
NOS (Landmesser, Dikalov et al. 2003)	epidermal melanocytes and keratinocytes
XO (Shalhaf, Gibbons et al. 2008)	epidermal melanocytes and keratinocytes

**Table 2**

**Effect of H<sub>2</sub>O<sub>2</sub>-mediated oxidation on proteins (enzymes, albumin), peptides and hormones (aromatic steroids) transcription (protein expression) in the human epidermis**

<b>Enzymes</b>	<b>mM H<sub>2</sub>O<sub>2</sub></b>	<b>μM H<sub>2</sub>O<sub>2</sub></b>
Catalase (Schallreuter, Bahadoran et al. 2008) GR (Schallreuter, Bahadoran et al. 2008) TR (Schallreuter, Pittelkow et al. 1986; Schallreuter and Wood 2001) MSRA MSRB (Schallreuter 2006) TYR (Wood, Chavan et al. 2004; Wood, Decker et al. 2009)	Oxidation of the structure yields -loss of functionality -impaired functionality and Deactivation	Up-regulation of transcription
<b>Cholinergic system</b>	Oxidation of the structure yields -loss of functionality -impaired functionality and Deactivation	Up-regulation of transcription and enzyme activity
AchE (Schallreuter, Elwary et al. 2004) BchE (Schallreuter, Gibbons et al. 2006)		
<b>Adrenergic system</b>	Oxidation of the structure yields -loss of functionality -impaired functionality and Deactivation	Up-regulation of transcription and enzyme activity
TH (Marles, Peters et al. 2003) PAH		
<b>6BH<sub>4</sub> synthesis/ recycling</b>	Oxidation of the structure yields -loss of functionality -impaired functionality and Deactivation	Up-regulation of transcription
GTP-CHI (Chavan, Beazley et al. 2009) PAH (Chavan, Gillbro et al. 2006) PCD (Schallreuter, Moore et al. 2001) DHPR (Hasse, Gibbons et al. 2004)		
6BH <sub>4</sub> (Schallreuter, Wood et al. 1994; Schallreuter, Moore et al. 2001; Rokos, Beazley et al. 2002; Hasse, Gibbons et al. 2004)	Oxidation of the cofactor	Works as a cofactor
<b>Hormones</b> (Schallreuter, Chiuchiarelli et al. 2006)	Via oxidation to ortho-quinone by cytochrome P450 which in turn causes DNA-damage	Work as hormones
Estrogen Progesterone Androgen		

<b>POMC-peptides</b> <b>PC1, PC2, Furin</b> <small>(Spencer, Gibbons et al. 2006)</small>	<b>mM H<sub>2</sub>O<sub>2</sub></b>	<b>μM H<sub>2</sub>O<sub>2</sub></b>
<b>α-MSH</b> <b>β-endorphin</b> <b>ACTH</b>	H <sub>2</sub> O <sub>2</sub> -mediated oxidation changes the structure yielding loss of the function	Up-regulation of transcription of POMC
<b>Albumin</b> <small>(Rokos, Moore et al. 2004)</small> <small>(Hasse, Kothari et al. 2005)</small>	Structural changes	Up-regulation of transcription
<b>XO</b> <small>(Shalhaf, Gibbons et al. 2008)</small>	Structural changes (minor oxidation)	Up-regulation of transcription
<b>p53</b> <small>(Salem 2009)</small>	No structural change, enhanced p53-DNA binding capacity	NO change

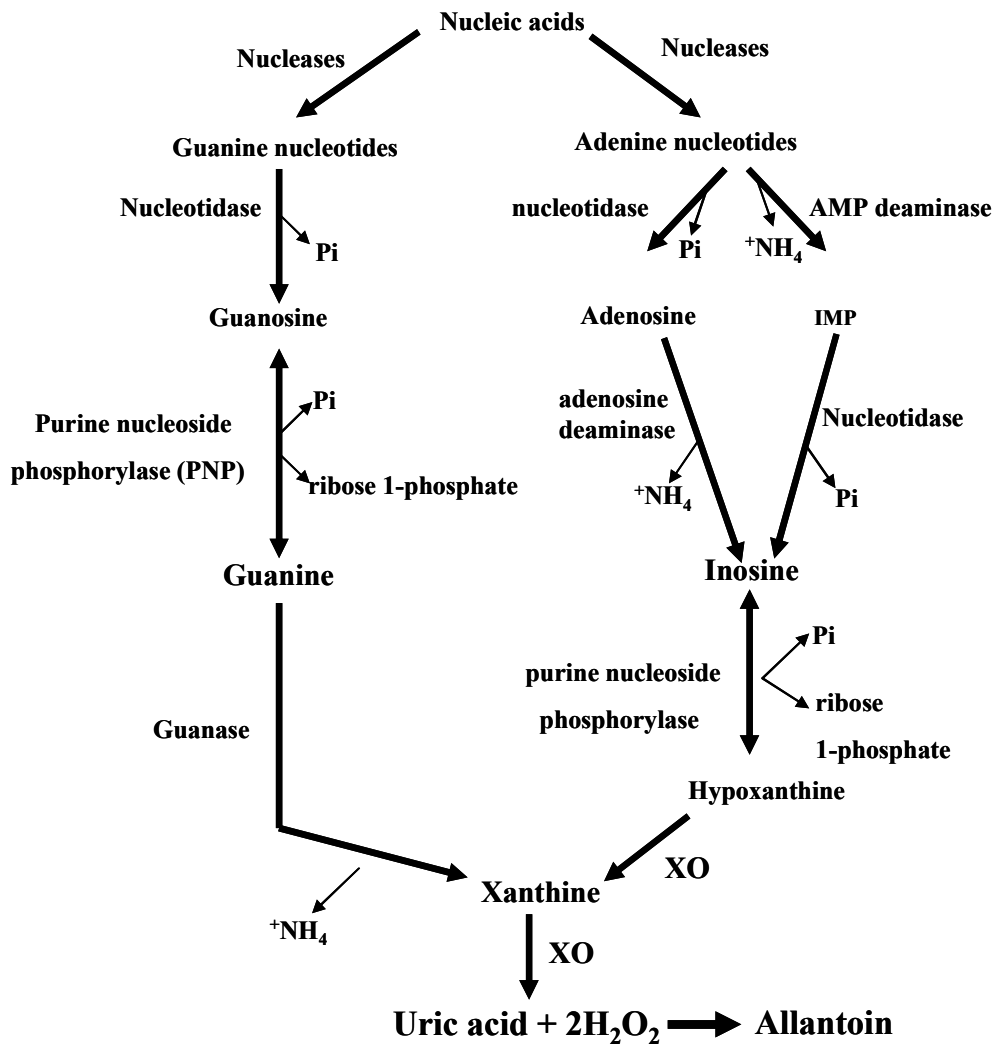
## 1.7 Xanthine oxidase

In the human epidermis  $H_2O_2$  can be generated by several mechanisms (Table 1). Xanthine oxidase (EC 1.1.3.22, XO) is one of these mechanisms which is able to produce  $H_2O_2$  from the degradation products of released purine bases (Dixon and Lemberg 1934; McCord and Fridovich 1968; Olson, Ballow et al. 1974).

### 1.7.1 Xanthine oxidoreductase enzymes (XOR)

XO belongs to the family of the mononuclear molybdopterin cofactor containing enzymes and was isolated for the first time by the Austrian biochemist Franz Schardinger in 1902 from bovine milk and was described as aldehyde reductase (Schardinger 1902). In 1920, Frederick Gowland Hopkins, isolated and reported a xanthine-oxidising enzyme from bovine milk (Hopkins 1920). However, it was Malcolm Dixon in 1926 who showed that the enzyme discovered by Schardinger was identical to the one reported by Hopkins (Dixon 1926). This enzyme exists in different species from bacteria to man (Morgan 1926; Richert and Westerfeld 1951; Al-Khalidi and Chaglassian 1965) and also in the various tissues of mammals (Roberts 1936; Parks and Granger 1986; Sarnesto, Linder et al. 1996). XO performs its role in terminal oxidation of all purine bases as the rate-limiting enzyme in purine degradation pathways (Dixon and Lemberg 1934; Parks and Granger 1986). In these pathways, XO catalyses the oxidative hydroxylation of hypoxanthine to xanthine followed by xanthine to uric acid, the last two steps in the catabolic metabolism of purine bases in mammals leading to the formation of uric acid (Figure 3). These catalytic reactions are accompanied by the generation of  $H_2O_2$  and  $O_2^{\bullet-}$  (Dixon and Lemberg 1934; Morell 1952; McCord and Fridovich 1968; Olson, Ballow et al.

1974), hence XO has been considered a major biologic source of ROS in many organs causing oxidative stress (Manson, Anthenelli et al. 1983; Im, Shen et al. 1984; Chambers, Parks et al. 1985; Parks and Granger 1986; Picard-Ami, MacKay et al. 1991; Glantzounis, Tsimoyiannis et al. 2005).



**Figure 3**

**Purine degradation pathway**

Uric acid/allantoin formation from guanine and adenine nucleotides requires XO and produces H<sub>2</sub>O<sub>2</sub> (Pi = inorganic orthophosphate, IMP = inosine monophosphate).

In humans a single gene encodes xanthine oxidoreductase enzymes (XOR) and its locus maps to chromosome 2p22 (Ichida, Amaya et al. 1993; Xu, Zhu et al. 1994; Garattini, Mendel et al. 2003). Mammalian XOR enzyme exists in two forms: xanthine dehydrogenase (EC 1.1.1.204, XDH) and XO. The two proteins have similar molecular weights and composition of redox centres (Hille and Nishino 1995). In normal physiological conditions XOR is found in the dehydrogenase form in the cell, however, under oxidising conditions, XDH can be immediately converted to the oxidase form, XO (Green 1934; McKelvey, Hollwarth et al. 1988; Hille and Nishino 1995; Nishino, Nakanishi et al. 1997; Enroth, Eger et al. 2000; Glantzounis, Tsimoyiannis et al. 2005).

### 1.7.2 The general structure of XOR enzymes

XOR enzymes all consist of approximately 1,333 amino acid residues. The basic structure of these enzymes is a monomer composed of three main domains. A small N-terminal 20 kDa domain (residues 1 to 165) contains two iron-sulfur centres, which are connected to a central 40 kDa FAD (flavin adenine dinucleotide) domain (residues 226 to 531) by a segment consisting of residues 166 to 225. The FAD centre is then connected to the large third domain, a C-terminal 85 kDa molybdopterin domain (residues 590 to 1,332) by another segment consisting of residues 532 to 589. The molybdopterin domain is close to the interfaces of the Fe/S and FAD binding domains. The three domains altogether give rise to a total molecular weight of approximately 145 kDa per monomer (Enroth, Eger et al. 2000). The Fe/S centres are coordinated to four cysteine residues, 113, 116, 148, 150, located close to the flavin ring. Thus, the active form of xanthine oxidase is a homodimer with 290kDa molecular mass (Enroth, Eger et al. 2000). The redox-active centres of XOR enzymes has therefore the same constitution: a pterin cofactor attached to the molybdenum via a dithiolene linkage, two 2Fe/2S centres and one FAD which altogether are organised in a linear fashion ideal for electron transfer (Huber, Hof et al. 1996; Kisker, Schindelin et al. 1997; Romao, Knablein et al. 1997; Kisker, Schindelin et al. 1998; Enroth, Eger et al. 2000; Nishino and Okamoto 2000; Hille and Anderson 2001). The oxidation of xanthine takes place at the molybdopterin centre, and reducing equivalents are transported via iron-sulfur centres to FAD, where physiological oxidation occurs. Therefore, the electrons from the substrate are passed into the molybdenum centre and afterwards through Fe/S clusters, and eventually into the FAD centre in the course of a reductive half reaction (Olson, Ballou et al. 1974; Hille

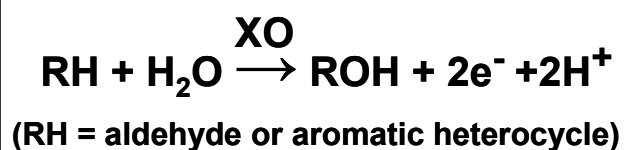
1991; Hille and Anderson 1991; Kisker, Schindelin et al. 1997; Enroth, Eger et al. 2000).

#### **1.7.2.1 The role of the molybdopterin cofactor (moco)**

The molybdopterin cofactor plays an essential role in a variety of enzymes named hydroxylases or oxotransferases (Kisker, Schindelin et al. 1997; Kisker, Schindelin et al. 1998; Hille 2002; Garattini, Mendel et al. 2003; Hille 2005). It is a non-planar tricyclic structure consisting of a pterin nucleus which is fused to a pyran ring and attached to the molybdenum via a dithiolene sulfur groups (Rajagopalan 1991; Romao, Archer et al. 1995; Kisker, Schindelin et al. 1997). The active form of XO contains a Mo=S ligand since the replacement of the sulfur with oxygen results in a catalytically inactive form of the enzyme (Massey and Edmondson 1970). In this cofactor, molybdenum catalyses an oxo-transfer reaction accompanied by electron transfer from substrates to other cofactors, such as Fe/S centres and FAD (Kisker, Schindelin et al. 1998). The cycling between the fully oxidised state of the molybdenum atom ( $\text{Mo}_{\text{VI}}$ ) and the fully reduced form ( $\text{Mo}_{\text{IV}}$ ) is common in all the reactions catalysed by molybdenum cofactor containing enzymes (Hille and Massey 1981; Hille and Sprecher 1987; Hille 1991; Hille and Anderson 1991; Hille and Massey 1991; McWhirter and Hille 1991; Hille, Kim et al. 1993; Kim and Hille 1993; Ryan, Ratnam et al. 1995; Kisker, Schindelin et al. 1997; Xia, Dempski et al. 1999; Stockert, Shinde et al. 2002; Choi, Stockert et al. 2004). It has been documented that although the molybdopterin cofactor is not directly involved in catalysis, it still has numerous hydrogen bonding to the protein which associate in transferring electrons out of the molybdenum centre. Crystallographic information of different molybdenum containing enzymes show that these hydrogen bonding from the distal amino group of



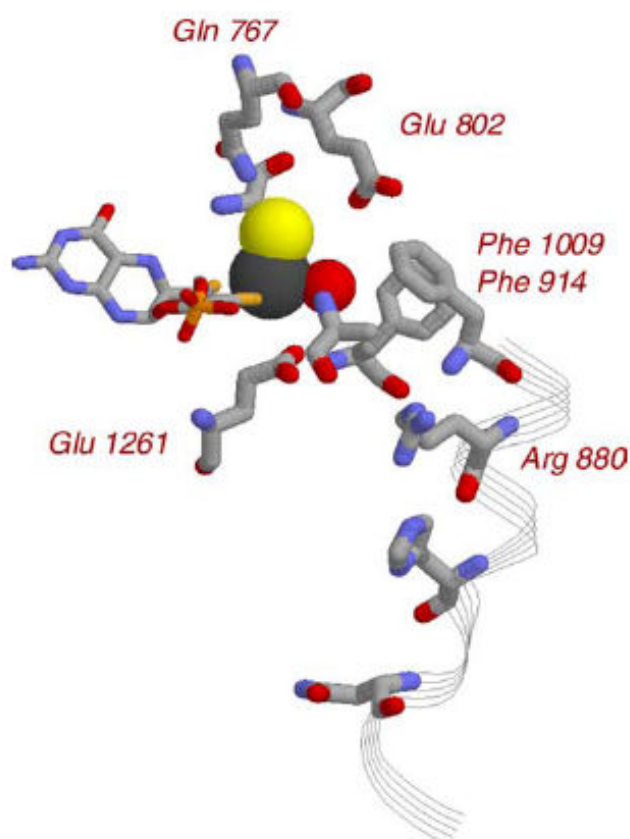
the molybdopterin cofactor to a cysteine residue of the iron-sulfur centre have been organised in line suitable for electron transfer (Hille 2002). The general reaction which is catalysed by molybdenum hydroxylases enzymes such as XO and XDH is shown below (Kisker, Schindelin et al. 1998):



Rather than O<sub>2</sub>, H<sub>2</sub>O is utilised by these enzymes as the ultimate source of oxygen atom incorporated into the product. Therefore, the hydroxyl group introduced into the substrate is derived from H<sub>2</sub>O and the electrons introduced into the enzyme may be transferred to any of the specific physiological electron acceptors such as O<sub>2</sub> (in XO) or NAD<sup>+</sup> (in XDH) (Rajagopalan and Johnson 1992; Choi, Stockert et al. 2004). Hence molybdenum hydroxylases are able to generate reducing equivalents rather than consume them in the reactions which they catalyse (Hille and Massey 1986; Choi, Stockert et al. 2004).

### 1.7.2.2 Important amino acid residues involved in catalysis

The specific amino acid residues which may play significant catalytic role have been identified. Glu<sup>1261</sup> (Glutamine) has been reported to be the foremost amino acid identified as a candidate in the active site of the enzyme which initiates the catalytic reaction. It has been also speculated that Glu<sup>802</sup> which is situated directly above the substrate binding site between Phe<sup>1009</sup> (phenylalanine) and Phe<sup>914</sup> may be also involved in catalysis (Bray and Malmstrom 1964; Choi, Stockert et al. 2004).



**Figure 4**

**Structure of the active site of XO, with amino acid residues likely to be involved in catalysis**

Glu<sup>1261</sup> has been suggested to be the most significant amino acid in the active site of XO which initiates catalysis. Glu<sup>802</sup> may also be involved in catalysis (Choi, Stockert et al. 2004).

### 1.7.3 XO and XDH

As stated earlier, XOR enzymes exist in two inter-convertible forms; XDH and XO. Under physiological conditions XOR enzyme is only transcribed as dehydrogenase form (XDH) but it is readily converted to its oxidase form (XO) either by proteolysis or oxidation of cysteine residues (Green 1934; McKelvey, Hollwarth et al. 1988; Hille and Nishino 1995; Nishino, Nakanishi et al. 1997; Enroth, Eger et al. 2000; Kuwabara, Nishino et al. 2003). The conversion of XDH to XO has been implicated in diseases characterised by oxygen-radical-induced tissue damage such as ischemia reperfusion injury (McCord 1985; Hille and Nishino 1995) and oxidative stress in the human epidermis as observed in vitiligo (Schallreuter 2005; Shalhaf, Gibbons et al. 2008). These two enzymes have similar molecular weights and composition of redox centres (Hille and Nishino 1995; Hille 1996). XDH has a greater affinity for oxidised nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) as the electron acceptor when catalysing the hydroxylation of hypoxanthine to xanthine and the latter to uric acid to produce NADH. The reduction of  $\text{NAD}^+$  occurs through the  $\text{FADH}_2$  cofactor (McKelvey, Hollwarth et al. 1988; Enroth, Eger et al. 2000; Nishino, Okamoto et al. 2005). However, it has been reported that XDH itself is capable of generating considerable amounts of  $\text{O}_2^{\bullet-}$ , although under normal conditions  $\text{NAD}^+$  would be expected to effectively compete with  $\text{O}_2$  for reduced enzyme (Hille and Nishino 1995). Furthermore, as mentioned earlier XDH uses  $\text{H}_2\text{O}$  as the source of oxygen introduced into the product (Enroth, Eger et al. 2000). XO, however, utilises  $\text{O}_2$  instead of  $\text{NAD}^+$  leading to the formation of free  $\text{O}_2^{\bullet-}$  (Olson, Ballow et al. 1974) which is subsequently converted to  $\text{H}_2\text{O}_2$  through spontaneous disproportionation or by superoxide dismutase activity (Horecker and Heppel 1949; McCord and Fridovich 1968).  $\text{H}_2\text{O}_2$  can react with iron via the Fenton reaction to produce  $\text{OH}^\bullet$  (Beauchamp

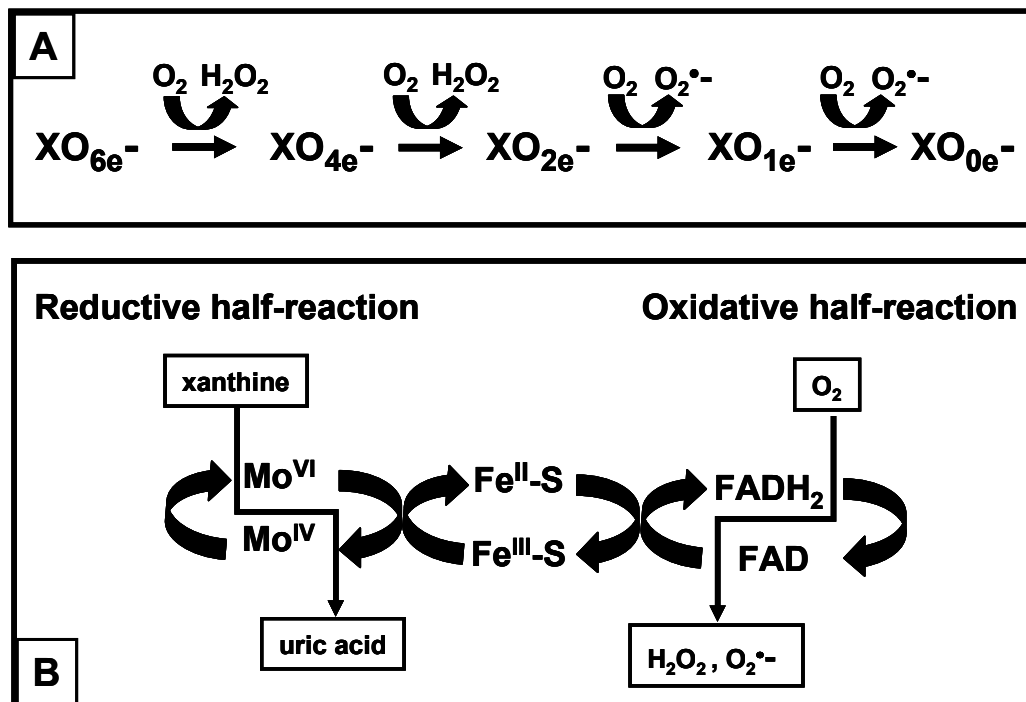
and Fridovich 1970; Bannister, Bannister et al. 1982; Parks and Granger 1983; Britigan, Pou et al. 1990; Hille and Nishino 1995; Glantzounis, Tsimoyiannis et al. 2005).

#### **1.7.4 Conversion of XDH to XO**

As stated above under certain conditions, the XDH can be readily converted to oxidase by reversible oxidation of two cysteine residues (Cys<sup>535</sup> and Cys<sup>992</sup>) and this is accompanied by a conformational change due to the formation of a disulfide bond (Kuwabara, Nishino et al. 2003). XDH can also be irreversibly converted to the oxidase form by proteolysis at lysine<sup>551</sup> by trypsin and by cleavage after leucine<sup>219</sup> and lysine<sup>569</sup> (Kuwabara, Nishino et al. 2003; Nishino, Okamoto et al. 2005). The major difference between XDH and XO exists in the FAD domain. The conversion of XDH to XO form results in a small conformational change near the FAD cofactor binding domain which affects the NAD binding site on the FAD accounting for XDH's ability to utilise NAD as an electron acceptor, whereas XO has high reactivity towards O<sub>2</sub> reducing it to O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>. However, this structural change does not affect the other cofactors (moco and Fe/S centres) or enzyme function (McKelvey, Hollwarth et al. 1988; Enroth, Eger et al. 2000; Kuwabara, Nishino et al. 2003; Nishino, Okamoto et al. 2008; Tsujii and Nishino 2008).

### 1.7.5 Mechanism of action of XO

It has been indicated that the overall reaction mechanism catalysed by XO consists of two half-reactions taking place at different sites on the enzyme. The reductive half-reaction occurs first and it takes place at the molybdenum centre by the reduction of  $\text{Mo}_{\text{VI}}$  to  $\text{Mo}_{\text{IV}}$  involving the oxidation of the substrate (McWhirter and Hille 1991; Hille, Kim et al. 1993; Kim and Hille 1993; Hille 1994; Kim, Ryan et al. 1996). In this half reaction xanthine is converted to uric acid. Then the oxidative half-reaction takes place at the FAD centre (Bray and Malmstrom 1964; Komai, Massey et al. 1969). In this half-reaction  $\text{O}_2$  is used as the final electron acceptor to generate  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\bullet-}$  (Hille and Massey 1981). It is considered that in this half-reaction, re-oxidation of the reduced XO to the fully-oxidised form of the enzyme occurs (Olson, Ballou et al. 1974; Olson, Ballou et al. 1974). The Fe/S centres facilitate the electron transfer between the molybdenum and the FAD centres (Nishino and Okamoto 2000). The crystallographic data indicates that the pterin portion of the molybdenum cofactor is pointing out towards the first Fe/S centre (Hille 2002) therefore, accordingly, XO requires an active site sulfur in order to be catalytically active (Massey and Edmondson 1970). XO needs 6 electrons to be completely reduced (two on the molybdenum centre, one in each of the two Fe/S centres, and two on the FAD centre), therefore, it requires three molecules of substrate to become fully reduced (Hille and Massey 1981). Re-oxidation of the enzyme takes place in four steps. First, the six electron reduced enzyme is oxidised to four electron reduced enzyme and then it loses another two electron in the next oxidation step.  $\text{H}_2\text{O}_2$  is produced in these two oxidation steps. In the last two steps, the enzyme loses two electrons to become fully oxidised accompanied by the production of  $\text{O}_2^{\bullet-}$ . These properties contribute to oxidative stress (Repine 1991).



**Figure 5**

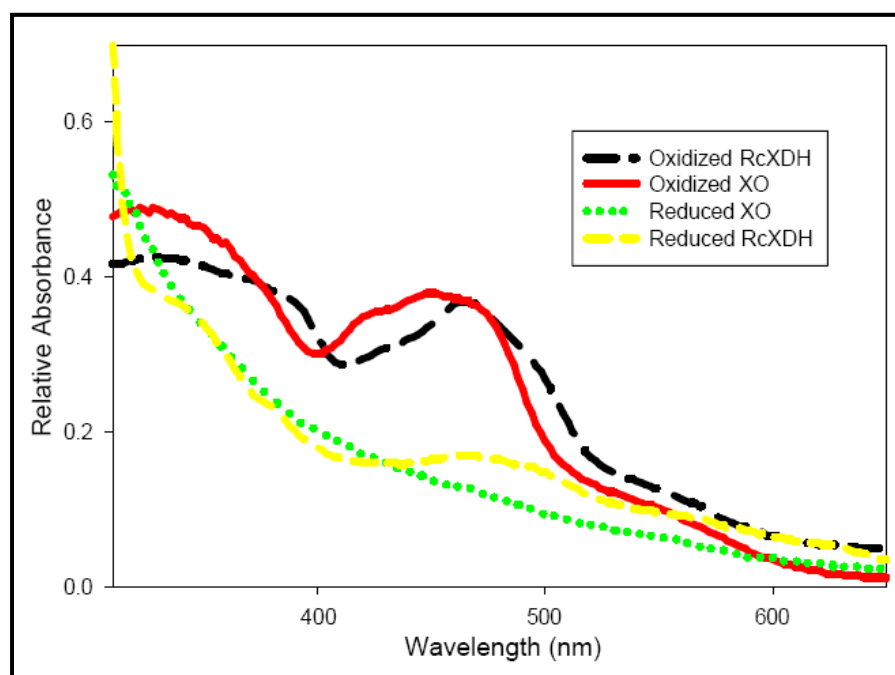
**The oxidative and reductive half-reactions of XO**

**(A):** The oxidative half-reaction. In the first two steps of this reaction, two molecules H<sub>2</sub>O<sub>2</sub> are produced while in the last two steps, two molecules O<sub>2</sub><sup>•-</sup> are the product of the reaction.

**(B):** The overall reaction of XO. The reductive half-reaction takes place at the moco centre in which Mo is reduced from Mo<sub>VI</sub> to Mo<sub>IV</sub> while xanthine is oxidised to uric acid. The oxidative half-reaction occurs at the FAD centre, where H<sub>2</sub>O<sub>2</sub> is produced (modified from (Newsholm and Leech 1984; Hille and Nishino 1995; Stockert 2004 ).

### 1.7.6 Absorbance spectroscopy of xanthine oxidoreductase enzymes

The absorption of the FAD and iron-sulfur centres dominates the UV/visible absorption spectra of both XO and XDH. It is thought that the difference between the absorbance of the two enzymes is likely due to FAD cofactor since the absorption spectra of the iron-sulfur centres in both enzymes are essentially identical. Therefore it is believed that the difference between the two absorption spectra of XO and XDH is due to the absence of the NAD binding site near the FAD centre in XO. It has been also reported that the absorbance of the molybdenum centre is virtually indistinguishable in the spectrum because of the absorbance of the FAD and Fe/S centres (Hille and Nishino 1995; Stockert 2004 ).



**Figure 6**

**The comparative absorbion spectra of both oxidised and reduced bovine milk XO and Rhodobacter capsulatus XDH.**

The maximum absorbance in XO at 450nm is due to both FAD and Fe/S centres whereas in XDH this absorbance is shifted to 465nm [adopted form (Stockert 2004 )].

## **1.8 Oxidative DNA damage and its responses**

Reactive oxygen species (ROS) are formed as by products during cellular metabolism (Halliwell 1989; Halliwell and Gutteridge 1989). These oxygen-derived reactive species are able to damage various biomolecules such as lipids, proteins and nucleic acids (Halliwell 1989; Halliwell and Gutteridge 1989; Esterbauer, Dieber-Rotheneder et al. 1990; Smith, Marks et al. 2004). It has been also reported that the attack on lipids and proteins by ROS can lead to the generation of intermediates which are able to damage DNA (Esterbauer, Eckl et al. 1990). Since integrity of the genome and cell survival is highly preserved among living organisms, cells have evolved multiple protective strategies in response to DNA damage. These responses are very complex involving a variety of signals and proteins that regulate an intricate network of pathways including cell cycle checkpoints, DNA repair, cellular senescence and apoptosis (Zhivotovsky and Kroemer 2004; Schmitt, Paquet et al. 2007).



### **1.8.1 Formation of 8-oxoGuanine (8-oxoG)**

The oxidative damage to DNA results in the hydroxylation of DNA bases (Lu, Li et al. 2001; Cadet, Douki et al. 2003; Cooke, Evans et al. 2003), mainly guanine, as it has the lowest oxidation potential amongst the four bases. The oxidation of guanine leads to the formation of a plethora of oxidised guanine products (Neeley and Essigmann 2006). 8-oxoG (the oxidised base), identified by Kasai in 1984, is the most thoroughly studied oxidised guanine product (Kasai and Nishimura 1984; Kasai and Nishimura 1984; Kasai, Crain et al. 1986; Adelman, Saul et al. 1988; Kasai 1997; Neeley and Essigmann 2006). Also, oxidation of dGTP in the nucleotide pool in the cell prepared for DNA synthesis leads to the formation of the 8-oxo-dGTP, which can be subsequently misincorporated into DNA during replication (Akiyama, Maki et al. 1989; Maki and Sekiguchi 1992; Mo, Maki et al. 1992; Nakano, Kawanishi et al. 2003). The oxidised DNA generally undergoes repair. Oxidised nucleosides and bases are almost water-soluble; therefore after the repair process they appear in the urine without further metabolism (Wu, Chiou et al. 2004). Hence 8-oxoG has been extensively used as a biological marker for oxidative stress in tissues, saliva, serum (plasma) and urine (Kasai, Crain et al. 1986; Fraga, Shigenaga et al. 1990; Loft, Vistisen et al. 1992; Loft, Fischer-Nielsen et al. 1993; Ahmed, Ueda et al. 1999; de Zwart, Meerman et al. 1999; Helbock, Beckman et al. 1999; Lovell, Gabbita et al. 1999; Perra, Maxia et al. 2006; Bahar, Feinmesser et al. 2007; Chen, Liou et al. 2007).

### **1.8.2 The mutagenic potential of 8-oxoG**

8-oxo-G potentially has the ability to mimic T functionality in the *syn* confrontation. Therefore, if not removed from the damaged DNA or from the nucleotide pool, it can form a stable 8-oxoG/A base pair during DNA replication which, although inaccurate, is efficiently bypassed by replicative DNA polymerases inducing G:C to T:A transversion. This is potentially mutagenic or even can lead to apoptosis (Floyd 1990; Wood, Dizdaroglu et al. 1990; Shibutani, Takeshita et al. 1991; Cheng, Cahill et al. 1992; Maki and Sekiguchi 1992; Grollman and Moriya 1993; Moriya 1993; Le Page, Margot et al. 1995; Johnson, Yu et al. 1996; Hussain and Harris 1998; Sunaga, Kohno et al. 2001; Bjelland and Seeberg 2003; Hussain, Hofseth et al. 2003). Therefore, oxidative damage to DNA is thought to be a common pathway leading to mutations and apoptosis. It is also believed to be one of the major causes of cancer (Hattori-Nakakuki, Nishigori et al. 1994; Johnson, Yu et al. 1996; Marnett 2000; Cooke, Evans et al. 2003; Hussain, Hofseth et al. 2003). Hence, the removal of DNA-oxidised lesions or modified bases from the nucleotide pool is extremely vital and required for genomic stability and cell survival.

### **1.8.3 Repair of oxidative DNA damage**

In order to minimise the induction of mutations following oxidative damage to DNA and to maintain genomic integrity, all cells have evolved an effective machinery to remove oxidised bases to prevent their incorporation into DNA during replication (Dempfle and Harrison 1994; Lu, Li et al. 2001; Friedberg 2003; Hazra, Das et al. 2007). DNA damage signalling pathways are very complex. In a simplified manner, in response to genotoxic stress p53 is activated and in turn it transactivates a series of its downstream effector genes involved in delaying cell cycle arrest including p21, PCNA and Gadd45 $\alpha$ , cellular senescence and also apoptosis including pro-apoptotic members of Bcl-2 family of proteins such as BAX (Kastan, Onyekwere et al. 1991; Bates and Vousden 1996; Chen, Ko et al. 1996; Harris 1996; Ko and Prives 1996; Smith and Seo 2002; Mills 2005; Rozan and El-Deiry 2007; Shu, Li et al. 2007).

### **1.8.3.1 Induction of Gadd45 $\alpha$**

Gadd45 family members, are rapidly induced following genotoxic stress and they play critical roles as stress sensors in different apoptotic and growth inhibitory pathways (Fornace, Alamo et al. 1988; Fornace, Nebert et al. 1989; Papathanasiou, Kerr et al. 1991; Fornace, Jackman et al. 1992; Beadling, Johnson et al. 1993; Hollander, Alamo et al. 1993; Zhan, Fan et al. 1996; Takekawa and Saito 1998; Smith and Mocarski 2005). These proteins are localised within the nucleus (Carrier, Smith et al. 1994; Kearsley, Coates et al. 1995; Vairapandi, Balliet et al. 1996; Zhang, Bae et al. 1999). Gadd45 $\alpha$ , is one of several growth-arrest and DNA-damage-inducible genes and it has been implicated in cell cycle checkpoint in G2/M, DNA repair and apoptosis via interaction by other cellular proteins including PCNA, p21, cdc2, cyclinB1 and the p38 and JNK stress response kinases (Fornace, Jackman et al. 1992; Kastan, Zhan et al. 1992; Smith, Chen et al. 1994; Chen, Smith et al. 1995; Hall, Kearsley et al. 1995; Kearsley, Shivji et al. 1995; Harkin, Bean et al. 1999; Wang, Zhan et al. 1999; Zhan, Antinore et al. 1999; Sheikh, Hollander et al. 2000; Maeda, Hanna et al. 2002; Zhan 2005; Liebermann and Hoffman 2007; Miyake, Takekawa et al. 2007). It has been also shown that Gadd45 $\alpha$  is involved in the induction of G2/M cell cycle checkpoint following UVB irradiation in epidermal KCs, MCs and also in melanoma cells (Maeda, Hanna et al. 2002; Pedeux, Lefort et al. 2002). Based on these results, it has been suggested that the activation of Gadd45 $\alpha$  in MCs and melanoma cells may play a crucial role for their survival due to their ability to efficiently repair UVB-induced lesions (Pedeux, Lefort et al. 2002; Fayolle, Pourchet et al. 2006; Fayolle, Pourchet et al. 2008; Rajpara, Hu et al. 2008).

### 1.8.3.2 Base-excision repair of oxidative DNA damage

In living organisms, oxidative DNA damage is predominantly repaired via base-excision repair (BER) pathways (Sancar 1994; Parikh, Mol et al. 1997; Fortini, Parlanti et al. 1999; Dianov, Souza-Pinto et al. 2001; Dogliotti, Fortini et al. 2001; Lindahl 2001; Lu, Li et al. 2001; Fromme and Verdine 2004). DNA glycosylases are the key enzymes to initiate BER processes which remove the oxidised bases by *N*-glycosylic bond hydrolysis between the bases and the deoxyribose moieties of the nucleotides leaving apurinic/apyrimidinic (AP) sites in the DNA (Michaels, Tchou et al. 1992; Boiteux 1993; van der Kemp, Thomas et al. 1996; Boiteux and Radicella 1999; Boiteux and Radicella 2000; Lu, Li et al. 2001; Fortini, Pascucci et al. 2003; Dizdaroglu 2005; David, O'Shea et al. 2007). These enzymes are responsible to specifically recognise and remove different oxidised-bases from the damaged DNA (Chetsanga and Lindahl 1979; Tchou, Kasai et al. 1991; Michaels, Tchou et al. 1992). Moreover, in order to protect cells and organisms from mutagenesis and carcinogenesis induced by 8-oxo-dGTP, there is a mechanism to remove this oxidised base from the nucleotide pool so that it cannot be misincorporated into DNA by DNA polymerases (Akiyama, Maki et al. 1989; Maki and Sekiguchi 1992; Lu, Li et al. 2001; Nakano, Kawanishi et al. 2003). BER of 8-oxoG/C pairs is initiated by hOgg1 (8-oxoGuanine-DNA glycosylase 1), which removes 8-oxoG, leaving an AP site (Boiteux and Radicella 1999; Kinoshita, Wanibuchi et al. 2002). However, if at this stage the repair of the damaged base does not occur and replication takes place, an incorrect 8-oxoG/A mismatch is formed. Therefore the second level of defence is an additional post-replication double check to recognise and correct the 8-oxoG/A mismatches produced by persistent 8-oxoG residues. In human, this is accomplished by DNA glycosylase hMYH, which is responsible to detect and excise the

inappropriate A residues misincorporated opposite the unrepaired 8-oxoG during replication (Michaels, Tchou et al. 1992; Tajiri, Maki et al. 1995; Lu and Fawcett 1998; Parlanti, Fortini et al. 2002; Russo, De Luca et al. 2007). Furthermore, hMTH in human hydrolyses 8-oxo-dGTP from nucleotide pools thus preventing its incorporation into nascent DNA (Colussi, Parlanti et al. 2002). The generated AP site is subsequently treated by a repair polymerase in the replication process to form an 8-oxoG/C substrate for the hOgg1 (Boiteux and Radicella 1999; Boiteux and Radicella 2000; Bruner, Norman et al. 2000; Shinmura and Yokota 2001; David, O'Shea et al. 2007).

Depending on the number of damaged bases, the abasic site is then processed by two alternative pathways: short-patch DNA polymerase $\beta$ -dependent BER in which only one damaged nucleotide is repaired (Kubota, Nash et al. 1996; Fortini, Parlanti et al. 1999; Dogliotti, Fortini et al. 2001) and long-patch PCNA-dependent BER involving the repair of more damaged nucleotide at the lesion site (Matsumoto, Kim et al. 1994; Frosina, Fortini et al. 1996; Klungland and Lindahl 1997; Wilson and Thompson 1997; Dogliotti, Fortini et al. 2001). The repair mode of oxidative DNA damage is predominantly via short-patch BER where DNA polymerase $\beta$  plays a critical role although there is evidence suggesting that it is implicated in the long-patch pathway as well (Dianov, Prasad et al. 1999; Prasad, Dianov et al. 2000). To complete the repair process after the glycosylase action, APE1 (apurinic/apyrimidinic endonuclease 1) and DNA polymerase $\beta$  are recruited to excise the remaining sugar moiety and to reinstall an undamaged nucleotide respectively, and the remaining nick is sealed by DNA ligaseI thereby repairing the damaged base site (Parikh, Mol et al. 1997; Gros, Saparbaev et al. 2002; Fortini, Pascucci et al. 2003; Chaudhry 2007). It should be noted that BER of the oxidised DNA is more complex and it is the result of multiple

interactions among various proteins and enzymes that may alter the choice of short- or long-patch BER modes and the detailed mechanisms which govern switching between these pathways have remained unknown (Dianov, Souza-Pinto et al. 2001; Dogliotti, Fortini et al. 2001; Lu, Li et al. 2001; Fortini, Pascucci et al. 2003; Fromme and Verdine 2004; Sung and Demple 2006).

#### **1.8.4 Apoptosis regulators**

If the damage to DNA is so extensive and DNA is irreparable, apoptosis (programmed cell death) would be the major control mechanism by which cells are eliminated. The induction and execution of apoptosis are complex, including very well controlled processes and involving many proteins (Steller 1995; White 1996; Song and Steller 1999; Teraki and Shiohara 1999; Zhivotovsky and Kroemer 2004). The Bcl-2 family of proteins play crucial roles in the regulation of apoptosis (Kroemer 1997). This family of proteins are divided into two subgroups: anti-apoptotic (such as Bcl-2, Bcl-XL) and pro-apoptotic proteins (such as BAX, BAD and BAK) (Chao and Korsmeyer 1998). It has been reported that the ratio of Bcl-2 and Bax is important in order to study cellular responses to apoptosis (van den Wijngaard, Aten et al. 2000). Gene expression of Bcl-2 and BAX proteins can be regulated by p53 (Miyashita, Krajewski et al. 1994).

It has been documented that the activation of Bcl-2 is an essential cellular response to suppress the initiation of apoptosis (Kroemer 1997). After receiving the apoptotic stimulus, pro-apoptotic members of the Bcl-2 family of proteins which reside on the outer membrane of the mitochondrion, are activated. The activation of these proteins leads to the release of cytochrome c from the mitochondrion into the cytoplasm which in turn leads to the activation of caspase 9 and this functions in the activation of caspase 3 where it is a key effector of apoptosis (Boldin, Varfolomeev et al. 1995; Steller 1995; Tewari, Quan et al. 1995; Kuida, Zheng et al. 1996; Liu, Kim et al. 1996; White 1996; Cai, Yang et al. 1998; Skulachev 1998; Song and Steller 1999; Abu-Qare and Abou-Donia 2001; Schmitt, Paquet et al. 2007). Therefore cytochrome c and caspase 3 have been considered as important biomarkers for apoptosis (Abu-Qare and Abou-Donia 2001).



## 2.0 AIM

Nowdays, there is accumulating evidence that the entire epidermis of patients with vitiligo is under oxidative stress, due to the presence of mM H<sub>2</sub>O<sub>2</sub> concentrations (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008). XO is one of the several mechanisms generating H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>, from catalysing the last two steps of the purine degradation pathway (Parks and Granger 1986). Therefore, this enzyme is considered as a major biologic source of oxygen-derived free radicals (ROS) in many organs (Manson, Anthenelli et al. 1983; Im, Shen et al. 1984; Parks and Granger 1986; Picard-Ami, MacKay et al. 1991).

To the best of our knowledge, the presence of XO in the human epidermis has not been demonstrated so far. Therefore the aim of this research was to show the presence and function of this enzyme in normal and healthy epidermal melanocytes and keratinocytes. Moreover, we wanted to know whether this system differs in patients with vitiligo compared to controls.

Due to the presence of severe oxidative stress in the epidermis of this patient group, oxidative DNA damage and / or apoptosis would be highly expected. Consequently, this study aimed to address this senario in vitiligo in more detail.

## **3.0 MATERIALS AND METHODS**

### **3.1 Cell culture**

#### **3.1.1 Establishment of epidermal primary cell cultures**

Epidermal melanocytes and keratinocytes were primarily cultured from full thickness of normal human skin (skin phototype III, Fitzpatrick classification) (Kiistala 1968) obtained as surgical waste after cosmetic or non-cosmetic operations such as facelifts, breast and abdomen reductions. Briefly, fat was removed from the skin following a quick wash in a PBS solution containing 5% penicillin/streptomycin (P/S) (Gibco Invitrogen, Paisley, UK) and 5% fungisone (Gibco Invitrogen, Paisley, UK). To facilitate the separation of the epidermis from the dermis, incisions were made on the epidermis using a scalpel. The tissue samples were then incubated in dispase (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, USA) containing, 5% P/S and 5% fungisone for approximately 12 hours at 4°C.

The next day the epidermal sheets were separated from the underlying dermis and placed in a solution of 1x RPMI media (RPMI 1640, Gibco Invitrogen, Paisley, UK) containing 5% P/S and 5% fungisone until the separation was completed. In order to prepare a cell suspension solution, the separated sheets of epidermis were incubated in x1 (0.05%) trypsin/EDTA (Gibco Invitrogen, Paisley, UK) at 37°C for 10 minutes. Trypsinisation was neutralised by the addition of either FBS (Gibco Invitrogen, Paisley, UK) or RPMI media containing 5% P/S, with the volume of almost 4 times of the trypsin volume. The mixture was gently pipetted up and down a few times in order to get a single cell solution followed by centrifugation at 200 x g for 10 minutes at

4°C in a Heraeus Sepatech bench-top centrifuge (Heraeus Instruments, Hannover, Hamburg, Germany).

In order to obtain primary co-cultures of both melanocytes and keratinocytes, the cell pellets were re-suspended in a 50/50 mixture of the medium for each cell type. i.e. for MCs, MC Medium 254 (Cascade Biologics, Mansfield, Nottinghamshire, UK) containing 1% P/S and 1% supplied growth factor (HMGS, Human Melanocyte Growth Supplement), (Cascade Biologics, Mansfield, Nottinghamshire, UK), and for KCs, Defined K-SFM keratinocyte medium (Gibco, Invitrogen, Paisley, UK) containing 1% P/S and growth supplements provided by the same company. After re-suspension, the cells were seeded into 25cm<sup>2</sup> cell culture flasks containing 5ml of the mixed media (Scientific Laboratory Supplies, Nottingham, UK). Cells were allowed to attach at 37°C, 5% CO<sub>2</sub> and 95% humidity. The medium was changed every 2-3 days.

### **3.1.2 Separation of MC and KC cell cultures and their maintenance**

Once the co-cultured cells were confluent around 80%, keratinocytes and melanocytes were separated via selective trypsinisation to obtain single cell populations. Briefly, the medium was aspirated and the cells were washed gently with PBS to remove any medium proteins that could otherwise inhibit the trypsin. Cells were then incubated with 1x trypsin at 37°C. This process was carefully monitored under phase microscopy using a Leica DMIRB inverted microscope (Leica Microsystems Ltd, Milton Keynes, Buckinghamshire, UK), until the cells were rounding up and detached. MCs detach from the flask in approximately 2 minutes whereas KCs need 5 to 15 minutes. The separated cell types were transferred into their specific media and allowed to attach and proliferate. Once confluent by 80%, both cell types were split

and passaged 1:3 into 75cm<sup>2</sup> flasks. This step repeated up to passage 3-4. In order to promote differentiation of KCs, 2mM calcium chloride was added into the medium.

### **3.1.3 Preparation of MC and MC cell extracts**

In order to prepare whole cell extracts, the cells were harvested via trypsinisation and/or scraping followed by centrifugation as described above. After removing the supernatant, the pellet was re-suspended in reaction buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.05 M, pH 7.5) (Sigma, Pool, Dorset, UK). Extracts were made by grinding the suspension with sand using a pestle and mortar. Once completed, the solution was centrifuged at 14000 x g for 10 minutes using a Heraeus Kendro Biofuge fresco microcentrifuge (Heraeus Instruments, Hannover, Hamburg, Germany). The supernatant was aliquoted and stored at -80°C until further use.

### **3.1.4 Preparation of whole cell extracts from epidermal suction blister tissues**

Epidermal suction blister tissues were obtained from patient's lesional and non-lesional skin and also healthy controls after written consent using the method of Kiistala (Kalb and Bernlohr 1977). All probands had skin phototype III, Fitzpatrick classification. This study was approved by the local Ethics Committees and was in agreement with the declaration of Helsinki. In order to prepare whole cell extracts from the epidermis, deep frozen tissues were ground with sand in a pestle and mortar containing 100 to 200µl of Tris-buffer (Sigma, Pool, Dorset, UK) (0.05M, pH7.5). The rest of the procedure was as described above. This method allows a very gentle extraction without the danger of any protein denaturation. Samples were aliquoted and stored at -80°C.

### **3.1.5 Determination of protein content**

The protein content of cell extracts was determined either spectrophotometrically at 280 nm with a Beckman DU-64 UV spectrophotometer (Beckman Coulter Ltd, High Wycombe, Buckinghamshire, UK) as described by Kalb and Bernlohr (Xu, Huecksteadt et al. 1996) or using the Bradford assay as described in the manufacturer's protocol (BioRad Laboratories Ltd., Hemel Hempstead, Herts., UK) with a standard curve of BSA 0.2-1.6 mg/ml (Sigma, Poole, Dorset, UK).

## **3.2 Reverse transcriptase polymerase chain reaction (RT-PCR)**

### **3.2.1 Isolation of total RNA**

Following the manufacturer's protocol, total RNA was extracted from cultured epidermal melanocytes and keratinocytes before the fourth passage using the Ambion total RNA isolation kit (AMS Biotechnology, Oxon, UK).

### **3.2.2 cDNA formation**

Using the above obtained RNA, reverse transcription was carried out using the reverse transcription system (Promega, Southampton, UK). cDNA was synthesised in 20µl aliquots containing 1µg of isolated RNA, 5mM MgCl<sub>2</sub>, 1x reverse transcription buffer, 1mM dNTP mixture, 1U/µl RNasinribonuclease inhibitor, 0.5µg of random primer and 15U of AMV Reverse Transcriptase per µg of RNA. The conditions used to synthesis the cDNA were as follows; 1 hour at 42°C, 5 minutes at 95°C and 5 minutes at 4°C. The resulting cDNA was stored at -20°C until required.

### **3.2.3 PCR of XDH**

For PCR amplification of XDH we used the primers reported earlier (Wilson and Walker 2005). All primers for the study were obtained from Genosys Biotechnologies, Europe Ltd, Pampisford, UK. Primer sequences and thermal parameters are described below:

Forward primer: 5'-CTCCGCACAGATATTGTCATGGAT-3'

Reverse primer: 5'-AAATGCCGGGATCTTGTAGGTGCT-3'

The amplification conditions for XDH were 5 minutes denaturing at 95°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 59.4°C and 1 minute at 67°C (decrease

of 0.5°C each cycle). Once the PCR cycles were completed, the resulting cDNA strands were separated in a 1.2% agarose gel containing ethidium bromide for 90 minutes. Bands were visualised using a Uvitec camera (Uvitec, Cambridge, UK).

### **3.3 Immunofluorescence labelling**

Immunofluorescence allows detecting the location and relative abundance of any protein or cellular antigen expressed in cells or tissues by using specific antibodies. Therefore the high sensitivity of the technique is based on the specificity of the antibody-antigen interaction.

#### **3.3.1 Indirect immunofluorescence staining**

In this method a primary unlabelled antibody binds to a specific protein. The primary antibody is then bound to a secondary fluorochrome-conjugated antibody that can be visualised using a fluorescence microscope. Immuno-reactivity can be documented by photographing.

#### **3.3.2 Preparation of cryosections**

3 mm punch biopsies of normal skin from consented volunteers and from lesional, non-lesional and repigmenting skin of patients with vitiligo (before and after treatment with a narrowband UVB-activated pseudocatalase, PC-KUS) were obtained under local anaesthesia taken from inner forearm. Samples were embedded in optimal cutting temperature compound (O.C.T) (Sakura, RA Lamb, Eastbourne, UK) and kept at -80°C. Once the skin samples were frozen, 3-5 µm thicknesses cryosections were cut using a Leica CM3050 S cryostat (Leica Microsystems Ltd, Milton Keynes, Buckinghamshire, UK) and placed onto slides coated with poly-L-Lysine (Sigma, Pool, Dorset, UK) and stored at -80°C until required for immuno-staining.



### **3.3.3 Preparation of chamber slides from epidermal cells**

For *in vitro* immuno-reactivity studies, keratinocytes or melanocytes were seeded into 8-well chamber slides (Lab-Tek<sup>®</sup> II, Chamber Slide<sup>™</sup> System, Nalge Nunc International Corp, Naperville, IL, USA) and allowed to attach for 48 hours. This was followed by changing the medium until each well was almost 60-70% confluent. Each well contained 100µl cell suspension (approximately  $1 \times 10^4$  cells) and 400µl medium. Cells were fixed in ice-cold methanol for 5 minutes in -20°C and kept at -20°C until required.

### **3.3.4 *In situ* single immuno-fluorescence staining**

Frozen slides were taken out of -80°C freezer and allowed to defrost at RT for 5 minutes. After being fixed in ice-cold methanol for 5 minutes at -20°C, slides were rehydrated in PBS for 5 minutes and blocked with 10% normal donkey serum (NDS) (Jackson Immuno Research Laboratories, Cambridgeshire, UK) blocking solution diluted in PBS for 90 minutes at RT. After a quick wash in PBS for 5 minutes, the primary antibody (already diluted to the desired concentration in 1% NDS/PBS) was added to the sections and slides were incubated overnight 4°C. The following day, slides were washed twice in PBS for 5 minutes and once in PBS containing 0.05% Tween-20 (Bio-Rad Laboratories, Life Science Group, CA, USA) in between for 5 minutes. Slides were then air-dried and incubated with a fluorescent secondary antibody in a dilution of 1:100 in 1% NDS/PBS for 1 hour. The choice of secondary fluorescein antibody, depends on the colour preferred [green = FITC (fluorescein isothiocyanate) or red = TRITC (tetramethyl rhodamine isothiocyanate)] and also in which animal the primary antibody was raised (donkey anti- mouse, rabbit, sheep or goat, depending on the primary antibody). Secondary antibodies were from Jackson

Immuno Research Laboratories, Cambridgeshire, UK. After a washing process, slides were finally dried and mounted in Vectashield Mounting Medium with 4,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and covered with a coverslip. Sections were viewed using a Leica DMIRB/E fluorescence microscope (Leica Microsystems Ltd, Milton Keynes, Buckinghamshire, UK) and photodocumented using a computer-assisted 3-CCD colour video camera and the Image Grabber PCI graphics program (both from Optivision, Osset, West Yorkshire, UK). In addition, some pictures were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture program (Nikon UK Limited, Kingston upon Thames, Surrey, UK).

### **3.3.5 Clarification of acute vitiligo**

To characterise acute vitiligo we determined catalase levels in the epidermis of the same patients. Epidermal catalase levels are too low in the presence of  $10^{-3}$ M H<sub>2</sub>O<sub>2</sub> compared to healthy controls. Therefore this protein provides a useful marker for oxidative stress in vitiligo (Schallreuter, Wood et al. 1991; Maresca, Roccella et al. 1997; Maresca, Flori et al. 2008; Schallreuter, Bahadoran et al. 2008).

### **3.3.6 *In situ* double immuno-fluorescent labelling**

*In situ* double immuno-fluorescence allows the detection of two specific markers within one section. The protocol was used as described above with some slight modifications. After incubation with the secondary antibody and a brief rinse in PBS, the sections were blocked again and the described procedure was followed again step by step. Paint Shop Pro™ 9 (Jasc Software, Inc, Eden Prairie, Minnesota, USA) was

utilized to merge the two different fluorochromes (FITC and TRITC) in order to follow possible co-localization.

The primary antibodies used for *in situ* staining were mouse monoclonal to XDH/XO (Abcam, Cambridge, UK), in a dilution of 1:10, goat polyclonal to tyrosinase (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A), mouse monoclonal anti 8-oxoG (Alpha Diagnostic International, San Antonio, TX, USA) in a dilution of 1:20, sheep polyclonal to cytochrome c (Abcam, Cambridge, UK) in a dilution of 1:50, mouse monoclonal to caspase 3 (Abcam, Cambridge, UK), in a dilution of 1:10, mouse monoclonal anti-gadd45 $\alpha$  (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A) in a dilution of 1:25. In all negative control studies the primary antibody was omitted from the staining procedure and substituted with 1% NDS/PBS.

### **3.3.7 *In vitro* immuno-fluorescence labelling of KCs and MCs**

To perform *in vitro* staining of cultured cells, apart from fixation with methanol, the above-described procedure was followed. The washing processes were shortened as prolonged washing led to bursting of cells. Both keratinocytes and melanocytes were again incubated overnight with mouse monoclonal anti XDH/XO antibody (Abcam, Cambridge, UK) at the dilution of 1:20 at 4°C. In order to follow possible expression in melanosomes, melanocytes were incubated with tyrosinase antibody (goat polyclonal, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A) overnight at 4°C in the dilution of 1:20.

### **3.3.8 Quantification of fluorescence intensity**

For quantification of XO fluorescence intensity, ImageJ version 1.37 was used (supplied online by NIH at <http://rsb.info.nih.gov/ij/index.html>). For quantification, the target region was highlighted, and the mean intensity was calculated. This was repeated for all sections. The calculated mean values were plotted. The fluorescence was expressed in arbitrary units.

### **3.4 Western blot analysis**

Western blotting is an analytical technique which is often used to detect expression of proteins in cells, tissues or a complex mixture of biomolecules using specific probes such as labelled antibodies. The proteins are first denatured via SDS and then separated according to their molecular weight.

#### **3.4.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

A 5, 10 or 13% polyacrylamide resolving gel of 1mm in thickness was prepared and allowed to polymerise in a mini cassette (NC2010 cassettes 1mm, Invitrogen, Carlsbad, CA, USA) for about 90 minutes at RT. Then a 3% stacking gel was prepared (see the appendix) and poured over the resolving gel to which a 10 or 15-well comb was inserted and allowed to set for 30 minutes at RT. The cassette was then placed in a NOVEX EI 900-XCELL II<sup>TM</sup> Mini cell gel system (NOVEX<sup>®</sup> NuPAGE, San Diego, CA, USA). The chamber was filled with running buffer (see the appendix) and the comb was removed. Sample buffer (see the appendix) was added to all protein samples prior to loading onto the gel in order to visualise the separation while running the gel. Then 10µl biotinylated protein ladder (Cell Signalling Technologies) and 15µl of each sample containing 5-15µg protein (depending on the protein of interest) were loaded onto the gel. A power supply (Gibco, Invitrogen, Paisley, UK) was connected to the apparatus and set at 100 volts. The gel was allowed to run for approximately 120 minutes until the separation completed when the dye reached to the bottom of the gel.

### **3.4.2 Western blotting**

Once the separation of proteins was completed, the power was disconnected. In order to expose the protein of interest to the probe, using a NOVEX blot module the proteins were transferred by electro-blotting to a 0.45 $\mu$ m pore size polyvinylidene difluoride membrane (PVDF) (Immobilon-P, Millipore, MA, USA). The transferring process was started by opening the gel cassette and removing the stacking gel. A blotting sandwich was assembled consisting of transfer buffer-soaked blotting pads, filter paper in transfer buffer (see the appendix), filter paper in methanol, pre-soaked PVDF membrane in methanol and transfer buffer, the polyacrylamide gel, filter paper in methanol, filter paper in transfer buffer and finally transfer buffer-soaked blotting pads. The blotting sandwich was placed into the transfer chamber (Novex<sup>®</sup>, San Diego, CA, USA) filled with transfer buffer. A voltage of 25 volts was applied using the same power supply. The transfer was allowed to take place for around 2 hours. During the electro-blotting, the chamber was placed on ice to avoid the heat produced by the transfer.

### **3.4.3 Blocking**

Once the transfer was completed, the acrylamide gel was stained with coomassie blue, and de-stained with a 40% methanol / 7% acetic acid solution to visualise any bands present. At the same time, the PVDF membrane containing all transferred proteins was blocked in order to prevent non-specific protein interactions between the membrane and the antibody protein. This was done by placing the membrane in a solution of 5% non-fat dried milk in PBS/0.4% Tween 20, or 0.5% gelatine (Sigma, Pool, Dorset, UK) dissolved in Tris buffered saline / 0.2% Tween 20 (TBS/Tween)

for around 2 hours at RT. N.B without blocking, the probe would bind non-specifically to the membrane.

#### **3.4.4 Immuno- reactivity detection**

After blocking, the membrane was incubated with the primary antibody overnight at 4°C. The primary antibody was diluted in 2.5% non-fat dried milk in PBS/0.4% Tween 20 in the case of XDH/XO (rabbit polyclonal, Abcam, Cambridge, UK) (1:10000) and caspase 3 (mouse monoclonal, Abcam, Cambridge, UK) (1:100), and in 0.05% gelatine in TBS/0.2% Tween 20 in the case of Gadd45 $\alpha$  (mouse monoclonal, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A) (1:300), APE1 (mouse monoclonal, Abcam, Cambridge, UK) (1:2000), hOgg1 (rabbit polyclonal, Abcam, Cambridge, UK) (1:500), DNA polymerase  $\beta$  (rabbit polyclonal, Abcam, Cambridge, UK) (1:250) and FEN1 (rabbit polyclonal, Abcam, Cambridge, UK) (1:500). The following day the membrane was washed 6 times each time 10 minutes for a total of 60 minutes in either TBS/0.2% Tween 20 or 2.5% milk in PBS / 0.4% Tween 20. Horseradish peroxidase conjugated anti-biotin (Cell Signaling Technology Inc, Danvers, MA, USA) (1:4000) was used to detect the biotinylated ladder. Horseradish peroxidase conjugated anti-mouse secondary antibody (Sigma, Pool, Dorset, UK) (1:5000) was used for the detection of primary antibody for Gadd45 $\alpha$ , Caspase 3 and APE1. Anti-rabbit (Cell Signaling Technology Inc, Danvers, MA, USA) (1:5000) was used for detection of the primary antibody for XDH/XO, hOgg1, DNA polymerase  $\beta$  and FEN1. 5 $\mu$ l of anti-biotin (1:4000) and 4 $\mu$ l (1:5000) of anti-mouse or anti-rabbit (secondary antibody) was added to 20ml of the relevant solution used to dilute primary antibody in each case and the membrane was incubated with the secondary antibodies for 60 minutes at RT.

After incubation with the secondary antibodies, the same washing procedure was repeated as described above. To visualize the bands, the PVDF membrane was placed into an enhanced chemiluminescent solution containing ECL-1 and ECL-2 solutions in equal quantities and shaken for 2 minutes at RT. These solutions react with the labeled probe and makes visualization of the protein possible. Finally the membrane was placed into a developer cassette (Sigma, Poole, Dorset, UK) and taken to a dark room where it was exposed to a film sheet (Kodak, Sigma, Pool, Dorset, UK) for 30 seconds in the case of XDH/XO and around 10 to 20 minutes for other proteins. After developing the film in developer and fixer, the protein of interest can be seen as bands.

#### **3.4.5 Western blot and statistical analysis**

For evaluation of the bands we utilised Image J version 1.37 (supplied online by NIH at <http://rsb.info.nih.gov/ij/index.html>). Each band was highlighted, and the intensity was calculated. This was repeated for each band. Statistical analysis was performed using Graph pad prism version 4 and 1-way ANOVA with Bonferroni analysis. The mean of the calculated values were used for demonstration in the figures.



### **3.5 Dot blot analysis**

Dot blotting is a technique for detecting, analysing and identifying proteins, similar to the Western blot technique but differing in that protein samples are not separated electrophoretically but are spotted directly onto the membrane. The dissolved samples are pulled through the membrane by absorption. Proteins bind to the membrane while the other sample components pass through. The proteins on the membrane are then available for analysis.

#### **3.5.1 Dot blotting for XO**

The aim of using this technique was to study whether H<sub>2</sub>O<sub>2</sub> (30%, Fluka, Sigma, Pool, Dorset, UK) can affect XO binding ability to the antibody. For this purpose, 0.4µg XO (Sigma, Pool, Dorset, UK) was exposed to different concentration of H<sub>2</sub>O<sub>2</sub> (1, 5, 10, and 100mM) and the samples were incubated for 60 minutes at RT. Then they were spotted in a row onto a previously methanol soaked PVDF membrane and allowed to dry for 15 minutes at RT. Thereafter, the membrane was incubated in blocking solution containing 3% BSA (Bovine Serum Albumin) (Sigma, Pool, Dorset, UK) in TBS/Tween 20 and left on a shaker overnight at 4°C. The next day the membrane was incubated with the primary antibody (rabbit polyclonal to XDH/XO, Abcam, Cambridge, UK) at the dilution of 1:10000 for 4 hours. The rest of the experiment including the washing procedures and incubation with the secondary antibody and finally the developing process were all exactly the same as Western blotting as described above.

## 3.6 XO protein purification

Gel filtration chromatography is a technique for the separation of proteins on the basis of their molecular size. It involves passing the sample which contains the analyte in the mobile phase, often in a stream of solvent through the stationary phase. Smaller molecules enter a porous media and take longer to exit the column; whereas larger particles leave the column first (Wilson and Walker 2005).

### 3.6.1 Purification of XO

Due to some impurities of XO purchased from Sigma which affected spectrophotometric enzyme oxidation analysis, it was decided to re-purify the enzyme. In order to do so, an S-200 column (Pharmacia Biotech, Amersham, Buckinghamshire, UK) was used. The flow rate was adjusted to 0.4ml/min and 1.5ml eluent was collected in each fraction of a total of 96 fractions. The chart speed (pen recorder) was set at 1 whereas absorbance sensitivity on the detector was adjusted to 0.5 absorbance unit. The column was washed extensively and then equilibrated with buffer containing 0.1M pyrophosphate (Sigma, Pool, Dorset, UK) and 0.3mM EDTA (Sigma, Pool, Dorset, UK) in which pH was adjusted to 8.5 (Stockert 2004 ). Then 60 mg XO was dissolved in 800 $\mu$ l dH<sub>2</sub>O and loaded onto the column which was allowed to run overnight. The next day the suspected fractions were collected according to their absorbance on the chromatograph and the UV-absorbance of suspected fractions was measured at 276 and 450nm spectrophotometrically. The ratio of  $A_{276}$  over  $A_{450}$  was calculated. Enzyme with a ratio < 6.0 was considered pure (Stockert 2004 ). It was aliquoted and kept in -80°C until required for further experiments.

### 3.7 Spectrophotometric studies of XO

XO activity was determined under different conditions by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 290nm. The compounds used in following assays were all purchased from Sigma (Sigma, Pool, Dorset, UK). All reactions were started by addition of enzyme (XO) and were performed at RT ( $25 \pm 0.2^\circ\text{C}$ ). The reaction mixtures were placed in a cuvette, mixed thoroughly and the absorbance of uric acid formation was measured over time. The total volume of reaction mixture in all experiments was 1ml. All the reactions were done in duplicates.

#### 3.7.1 Standard assays

Since XO activity generates  $\text{H}_2\text{O}_2$ , standard assays were run in the presence and absence of catalase. The reaction mixture without catalase contained 970 $\mu\text{l}$  potassium phosphate buffer ( $\text{KH}_2\text{PO}_4$ , 0.05M, pH 7.5), 10 $\mu\text{l}$  of 10mM xanthine solution (100 $\mu\text{M}$ ) and 20 $\mu\text{l}$  of 6mg/ml XO (120 $\mu\text{g}$ ).

Next, we ran the same reaction in the presence of catalase. The reaction mixture contained 965 $\mu\text{l}$  buffer, 100 $\mu\text{M}$  xanthine, 120 $\mu\text{g}$  XO and 5 $\mu\text{l}$  of 5mg/ml catalase (25 $\mu\text{g}$ ). The two reactions were studied over 7 minutes at 290nm using a UV/visible spectrophotometer (Pharmacia Biotech, Ultrospec 2000, Amersham, Buckinghamshire, UK) and the absorption was recorded every minute.

### **3.7.2 Inhibition of XO by its substrate**

In order to study the rate of the reaction between a constant enzyme concentration (purified and not-purified XO) with varying concentrations of xanthine (substrate), the reaction product (uric acid) was determined in the presence and absence of catalase. In the case of not-purified XO, the reactions contained 10 $\mu$ l of the 6mg/ml XO solution (60 $\mu$ g) and 25, 50, 100, 200, 300 and 400 $\mu$ M final concentrations of xanthine, in a total volume of 1ml reaction mixture. Uric acid formation was spectrophotometrically followed at 290nm where the absorptions were recorded every two minutes.

In the case of purified XO (0.5mg/ml), 20 $\mu$ l of the enzyme (10 $\mu$ g) was used in all reactions. Substrate (xanthine) concentrations were: 25, 50, 100 and 200 $\mu$ M in the total volume of 1ml reaction mixtures. 5 $\mu$ l of 10mg/ml solution of catalase was added to all reaction mixtures in order to remove H<sub>2</sub>O<sub>2</sub> produced during the reactions. Buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.05M pH 7.5) was used to make the final volume to 1ml. The reactions were followed over 10 minutes and the rate of the reaction/min was calculated.

### **3.7.3 Effects of H<sub>2</sub>O<sub>2</sub> on XO activity**

Due to the presence of mM concentrations of H<sub>2</sub>O<sub>2</sub> in the epidermis of patients with vitiligo (Wilson and Walker 2005), it was decided to study the effects of this ROS on XO activity. In order to do this, a series of spectrophotometric experiments were carried out and the effects of different H<sub>2</sub>O<sub>2</sub> concentrations from μM to mM on XO activity were studied. All the concentrations have been calculated in the total volume of 1ml reaction mixture. Buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.05M pH 7.5) was used to make the final volume to 1ml.

#### **3.7.3.1 XO activity in the presence of low H<sub>2</sub>O<sub>2</sub> concentrations**

In order to study the effects of low concentrations of H<sub>2</sub>O<sub>2</sub> on XO activity, the enzyme reaction containing 20μl of 6mg/ml XO (120μg) and 100μM substrate was followed over 10 minutes in the presence of different H<sub>2</sub>O<sub>2</sub> concentrations (0, 25, 50, 75, 100, 150 and 200μM) which were directly added into the reaction. Uric acid formation was recorded every minute and the rate/min for all reactions was calculated from the linear region.

#### **3.7.3.2 XO activity in the presence of high H<sub>2</sub>O<sub>2</sub> concentrations**

In order to study XO functionality under severe oxidative conditions, two different sets of experiments were designed in the presence of mM H<sub>2</sub>O<sub>2</sub> concentrations.

Firstly, XO activity was spectrophotometrically determined when mM H<sub>2</sub>O<sub>2</sub> concentrations (0, 1.25, 2.5, 5, 10 and 20mM in total volume of 1ml reaction mixture) were directly added into the reaction mixture as described above. All reactions were carried out over 10 minutes and the rate/2min was calculated from the linear region.

Secondly, functionality of oxidised XO was followed after the enzyme incubated with H<sub>2</sub>O<sub>2</sub> in the mM range for 2 hours prior to the experiment. In order to do so, 25µg of purified XO (50µl of 0.5mg/ml) was incubated with different H<sub>2</sub>O<sub>2</sub> concentrations (0, 5, 10, 20 and 30 mM) in a total volume of 200µl at RT for 2 hours. In this set of experiments, buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.05M pH 7.5) was used to dilute H<sub>2</sub>O<sub>2</sub> to the desirable concentrations. After incubation, the residual H<sub>2</sub>O<sub>2</sub> was removed from the mixture utilising Zeba™ mini Desalt Spin chromatography Column (Pierce Biotechnology Inc, Rockford, IL, USA). The mixture was placed into the column and the column was placed in a lidless 1ml microtube followed by centrifugation using a Heraeus Kendro Biofuge fresco microcentrifuge (Heraeus Instruments, Hannover, Hamburg, Germany) at 14000 x g for 15min at 4°C. After the removal of H<sub>2</sub>O<sub>2</sub>, the activity of the oxidised enzyme was measured spectrophotometrically at 290nm over 10 minutes. 100µg catalase (10µl of 10mg/ml solution) was added to all the reaction mixtures to remove the produced H<sub>2</sub>O<sub>2</sub> during the reaction. A 200µl mixture of the purified XO (25µg) with buffer (150µl) was incubated at RT as control. Again the absorption was recorded every minute and the rate/min for all the reactions was calculated from the linear region.

### **3.7.4 Computer modelling of native and oxidised XO**

The crystal structure of XO was obtained from the protein data bank which is available online at: <http://www.rcsb.org/pdb/home/home.do>. Using the molecular modelling program “HyperChem” (Hypercube, Inc, Gainesville, FL, USA), Met and Trp residues in the FAD binding domain, molybdopterin co-factor binding domain and the active site were oxidised to methionine sulfoxide (MetSO) and 5-hydroxy-tryptophan (5-OH Trp) respectively. Minimisation was performed to obtain the most thermodynamically stable structure. DeepView analysis was used to compare and analyse changes between the native and oxidised states of the protein (with acknowledgement to Dr. Nicholas Gibbons for his molecular modelling).

### **3.7.5 Determination of H<sub>2</sub>O<sub>2</sub>-mediated oxidation of XO**

Furthermore, in order to find out more about the alterations in the structure of the enzyme’s main domains after oxidation by H<sub>2</sub>O<sub>2</sub>, 250µg purified XO (500µl of 0.5mg/ml) was incubated with a very high concentration of H<sub>2</sub>O<sub>2</sub> (1.5M in a total volume of 600µl) and the oxidised enzyme was scanned spectrophotometrically every 15 minutes in a range from 200nm to 600nm to see the ongoing alterations in the main domains via the spectra while the enzyme is being oxidised. This of course was compared with the UV-absorbance spectrum of the reduced enzyme.

### **3.8 Determination of XO activity using TLC**

Thin layer chromatography (TLC) is a method for separation and identifying compounds. Using this technique, it is also possible to determine the purity of a compound and to follow the progress of a reaction. This technique involves spotting the samples to be analysed on a sheet of glass or plastic that is coated with a thin layer of a special adsorbent (0.25-2mm) which is stationary phase. Depending on the size of the molecule of interest components of the samples will separate in this phase. The sheet is placed in a covered tank containing a reservoir of solvent (mobile phase) which travels up the stationary phase, carrying and separating the samples with it (Shalhaf, Gibbons et al. 2008). Once the separation is completed, in order to visualise the separated spots the plate can be either exposed to UV light (350nm) or they can be developed by heat or special agents.

#### **3.8.1 Separation of xanthine, uric acid and allantoin**

20x20cm cellulose coated TLC plates were purchased from Fluka (Fluka, Sigma, Pool, Dorset, UK). Due to the UV absorption of xanthine, uric acid and allantoin, a saturated solution (approximately 5-10mg/ml) of these compounds (all from Sigma, Pool, Dorset, UK) in  $\text{NH}_4\text{OH}$  (Sigma, Pool, Dorset, UK) was prepared for identification of these compounds. Using a glass pipette one spot xanthine, one spot uric acid (5 times applied as it had a weaker UV absorption compared to xanthine) and one spot xanthine and uric acid on top of each other (together) were spotted on the TLC plate to optimise the separation and the solvent system. To study the separation of allantoin, the same procedure was repeated but since allantoin has a very



weak UV absorption compared to the other two compounds, it was applied in higher concentrations in each spot to increase its visualisation under UV light (350nm).

After optimisation, a solvent was chosen containing 66.5% 1-propanol (Sigma, Pool, Dorset, UK), 20% of 8N NH<sub>4</sub>OH and 13.5% dH<sub>2</sub>O. After spotting the samples, the TLC plate was air-dried and placed in a covered tank containing the solvent system. It was allowed to run for around 3-4 hours at RT until the solvent reached the top of the plate. Then the plate was removed from the tank, air-dried and the separated components were visualized using a UV lamp (350nm). *R<sub>f</sub>* values of these compounds were  $0.31 \pm 0.02$  for xanthine,  $0.25 \pm 0.02$  for allantoin and  $0.2 \pm 0.02$  for uric acid.

### **3.8.2 Determination of XO activity**

The XO activity was determined via a modified TLC method using [<sup>14</sup>C]xanthine (47.5μCi/500μl activity in original stock) (Sigma, Pool, Dorset, UK) where the production of [<sup>14</sup>C]uric acid by the activity of XO was measured. A [<sup>14</sup>C]xanthine working solution was made up of 20μl [<sup>14</sup>C]xanthine (from the original stock) diluted in 1ml of 10mM cold xanthine solution in NH<sub>4</sub>OH. This working solution contained 0.2μCi/1ml.

#### **3.8.2.1 Standard enzyme assay**

According to the method described above, the plate was spotted separately from the saturated solutions of xanthine and uric acid on top of each other as a series of 7 spots for each time point. The standard reaction solutions contained 5μl of radioactive working solution (0.5mM cold xanthine and 1nCi [<sup>14</sup>C]xanthine), 25μg XO (5μl of 5mg/ml) with or without 25μg catalase (5μl of 5mg/ml) made up to a final volume of 100μl with reaction buffer (KH<sub>2</sub>PO<sub>4</sub> 0.05M pH 7.5) Immediately after the reaction

was started by the addition of XO, using a timer and a UV lamp, at various time points (2, 5, 10, 15, 20, 30 and 40min) an aliquot of 2 $\mu$ l out of the reaction mixture was applied on top of each spot. After spotting the last time point at 40min, the plate was developed twice at RT as described before up to a distance of 16cm. Once the separation was completed, under a UV lamp the separated uric acid and allantoin spots were visualised, marked with a pencil, cut and placed in scintillation vials containing 3ml scintillation liquid (Ready Safe<sup>TM</sup>, Beckman Coulter Ltd, Fullerton, CA, USA). Then the rack containing the vials was placed in [<sup>14</sup>C] channel in a scintillation analyser (Packard, Tri-Carb 2100TR, Liquid Scintillation Analyser, Packard Instrument Co., Meriden, CT, USA) in order to count the radioactivity present in each spot which represent <sup>14</sup>[C]uric acid or <sup>14</sup>[C]allantoin production in the reaction that depends on XO activity.

#### **3.8.2.2 Determination of XO activity in normal KC and MC cell extracts**

XO activity of epidermal KC and MC cell extracts was determined via the above described method. The reaction mixture contained 50 $\mu$ l of KC cell extract (5mg/ml) or 90 $\mu$ l of MC cell extract (0.4mg/ml) cell extract instead of 5 $\mu$ l XO in the above standard assay and 5 $\mu$ l of radioactive working solution (0.5mM cold xanthine as well as 1nCi activity) in the final volume of 100 $\mu$ l using reaction buffer KH<sub>2</sub>PO<sub>4</sub>. The rest of the experiment was the same as described above. Due to differences in protein contents of cell extracts and in order to compare XO activity in them, each value was standardised per mg protein related cell extract.

### **3.9 HPLC analysis of allantoin**

H<sub>2</sub>O<sub>2</sub> oxidises uric acid to allantoin (Newsholm and Leech 1984) which is considered as a biomarker for oxidative stress (Grootveld and Halliwell 1987; Kaur and Halliwell 1990; Benzie, Chung et al. 1999; Yardim-Akaydin, Sepici et al. 2004; Yardim-Akaydin, Sepici et al. 2006). Allantoin was determined in whole cell extracts from epidermal suction blister tissue materials from both healthy controls and vitiligo patients using high performance liquid chromatography (HPLC) described by George et al. (George, Dipu et al. 2006).

#### **3.9.1 Principle of detection**

Allantoin was purchased from Sigma (Sigma, Pool, Dorset, UK). Analysis was conducted using a Waters 510 pump (Waters Ltd, Elstree Hertfordshire, UK) with a Spherclone 250x4.60mm C-18 reverse-phase column (Phenomenex, Macclesfield, Cheshire, UK) maintained at 22°C coupled to a Waters 486 UV detector (Waters Ltd, Elstree, Hertfordshire, UK) set at a wavelength of 220nm. The mobile phase was 10 mM potassium dihydrogen phosphate buffer (KH<sub>2</sub>PO<sub>4</sub> pH 4.7) in HPLC grade water (Fisher Chemicals, Loughborough, Leics, UK). The flow rate was maintained at 1.0 ml/min with a pressure of 1900 ± 200 psi. To identify the allantoin peak, 20µl of 0.04µg allantoin (standard solution prepared 1mg/ml) was injected into the instrument. The allantoin peak appeared at a retention time of 3.5 minutes.

### **3.9.2 Detection of allantoin in epidermal suction blister tissue material**

#### **3.9.2.1 Sample preparation**

60µl of each epidermal suction blister cell extracts from acute lesional (n=10) and non-lesional (n=10) vitiligo as well as controls (n=5) were de-proteinated by the addition of 10µl 2N perchloric acid (HClO<sub>4</sub>) on ice for 15 minutes followed by centrifugation at 14000g for 10 minutes at 4°C.

#### **3.9.2.2 Detection of allantoin**

20µl of the obtained supernatant from each sample was diluted 1:10 in mobile phase buffer and the final volume of 200µl was injected into the HPLC. Fractions covering the retention time range 3-4 min, where allantoin elutes, were collected for each sample. 200µl of each fraction was re-analysed via HPLC to see any suspected peak for allantoin. In order to confirm the presence of allantoin in any positive samples, another aliquot of 200µl of each collected fraction was re-analysed in the presence of 0.04µg allantoin. Between each run, the column was washed for 30 minutes. All experiments were carried out in duplicates (Shalhaf, Gibbons et al. 2008). The presence of allantoin in positive samples was re-confirmed by mass spectroscopy using a Quattro Ultima mass spectrometer (Waters, Elstree, Hertsfordshire, UK) (with acknowledgement to Dr. Derek J. Maitland for his co-operation on mass spectroscopy).

### 3.10 Inhibition of XO by pterins

Pteridine derivatives [neopterin, 6-biopterin, xanthopterin, leukopterin and tetrahydrobiopterin (6BH<sub>4</sub>)] inhibit XO activity in a concentration-dependent manner (Kalckar, Kjeldgaard et al. 1948; Kalckar and Klenow 1948; Hofstee 1949; Lowry, Bessey et al. 1949; Kalckar, Kjeldgaard et al. 1950; Isaka 1952; Wede, Altindag et al. 1998; Oetl and Reibnegger 1999). So far there has been no report of inhibiting XO activity by 7BH<sub>4</sub> or 7-biopterin. The aim of this study was to see whether these compounds have inhibitory effects on the activity of this enzyme.

In order to do this, a set of experiments were designed where uric acid production from the reaction between xanthine and XO was spectrophotometrically measured at 290nm in the presence of different concentrations of pterins. All the pterins used in this study were purchased from Dr. Schirck's Laboratory (Schirck's Laboratories, Jona, Switzerland).

Firstly, inhibition of XO activity by different 6BH<sub>4</sub> concentrations (0, 10, 20, 50, 100 and 200µM) was studied by calculating the rate/min for each pterin concentration and the results were compared to the inhibition by the same concentrations of 7BH<sub>4</sub>. The reaction mixture contained 120µg XO, 100µM xanthine, 100µg catalase and either 6BH<sub>4</sub> or 7BH<sub>4</sub>. The final volume of the reaction mixtures was made up to 1ml using reaction buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.05M pH 7.5).

Secondly, the inhibitory effects of different concentrations of 6-biopterin (0, 10, 20, 50, 100, 200 and 500µM) on XO activity was compared to XO inhibition by the same concentration of 7-biopterin. The reaction mixtures used the above concentrations of enzymes and substrate but 6 & 7-biopterin instead of 6 & 7BH<sub>4</sub>.

### 3.11 [<sup>3</sup>H]6BH<sub>4</sub> binding to XO

The aim of this experiment was to confirm 6BH<sub>4</sub> binding to XO. To do this experiment radio-labelled 6BH<sub>4</sub> ([<sup>3</sup>H]6BH<sub>4</sub>) (a generous gift from E. Werner, University of Innsbruck, Austria) was employed. First 10mg XO (Sigma, Pool, Dorset, UK) was dissolved in 2.5ml dH<sub>2</sub>O with 200μM final concentration of cold 6BH<sub>4</sub> (Schirck's Laboratories, Jona, r,Switzerland). Then 5μl of [<sup>3</sup>H]6BH<sub>4</sub> containing approximately 5nCi activity (250000 counts) was added into the mixture. This mixture was then placed in the dark at RT for 1 hour to allow binding of the labelled 6BH<sub>4</sub> to XO after which it was added to a G-25 sephadex column (GE Healthcare Ltd, Amersham, Buckinghamshire, UK) equilibrated with distilled water. The total of 40 fractions (500μl) were collected and 100μl of each fraction was counted in 3ml scintillation fluid (Ready Safe<sup>TM</sup>, Beckman Coulter Ltd, Fullerton, CA, USA) in the [<sup>3</sup>H]-channel of the scintillation analyser (Packard, Tri-Carb 2100TR, Liquid Scintillation Analyser, Packard Instrument Co., Meriden, CT, USA). The remaining 400μl was used for protein determination via Bradford assay as described in the manufacturer's protocol (BioRad Laboratories Ltd., Hemel Hempstead, Herts., UK).

### 3.12 Reactivation of inhibited tyrosinase by XO

The aim of this study was to see whether XO is able to reactivate 6BH<sub>4</sub> / 7BH<sub>4</sub>-inhibited tyrosinase. All pteridines used in this study were purchased from Dr. Schirck's Laboratory (Schirck's Laboratories, Jona, Switzerland).

The standard tyrosinase reaction mixture contained 1mM (100µl of 0.01M solution pH 9) L-tyrosine (Sigma, Pool, Dorset, UK) and 40µl (containing 84 units) mushroom tyrosinase (Sigma, Pool, Dorset, UK) in the total volume of 1ml using reaction buffer (KH<sub>2</sub>PO<sub>4</sub>, 0.05M pH 7.5) where L-dopachrome formation was followed spectrophotometrically at 475nm.

In order to see the capability of XO for reactivation of the 6BH<sub>4</sub> / or 7BH<sub>4</sub> / tyrosinase inhibitor complex, the standard assay was repeated in the presence of final concentrations of 200µM either 6BH<sub>4</sub> (19µl of 10.8mM stock solution) or 7BH<sub>4</sub> (26µl of 7.8mM stock solution) in 1ml reaction mixture to inhibit the reaction (Wood, Schallreuter-Wood et al. 1995; Spencer, Chavan et al. 2005; Spencer 2007). After 15 minutes, when the inhibition was observed, 100µg XO (100µl of 1mg/ml stock) was added into the reaction and formation of dopachrome was followed at 475nm (reaction volume now increased to 1.1ml).

### **3.13 Enzyme-linked immunosorbent assay (ELISA)**

ELISA is used for rapid detection and quantification of a protein or an antigen in a sample (Wilson and Walker 2005). We utilised this technique to detect plasma levels of 8-oxoG using OxiSelect™ Oxidative DNA Damage ELISA kit obtained from Cell Biolabs (Cell Biolabs, Inc, Cambridge Bioscience Ltd., Cambridge, UK). The experiment was carried out according to the manufacturer's protocol.

#### **3.13.1 Preparation of the microtiter plate**

All wells of a 96-well plate were first coated with 100µl of the 10µg/ml 8-oxoG conjugate and the plate was incubated overnight at 4°C. The next day 8-oxoG coating solution was removed from the wells and the plate was washed once using dH<sub>2</sub>O. The plate was then blocked using 200µl assay diluent (provided in the kit) in each well for 1 hour at 37°C after which the plate was transferred to 4°C until used.

#### **3.13.2 Preparation of the samples**

To obtain a standard curve, a series of 8-oxoG dilutions (0, 0.1, 0.3125, 0.625, 1.25, 2.5, 5 and 10ng/ml) in assay diluent were prepared. Plasma samples of 10 healthy individuals and 10 patients with acute vitiligo were centrifuged at 3000g for 10 minutes prior to analysis. All the samples including unknown and standard were assayed in duplicate.



### **3.13.3 Assay protocol**

Assay diluent (blocking solution) was removed and 50µl of 8-oxoG standards as well as controls and acute patients were pipetted to the wells in order. The plate was incubated on a shaker at RT for 10 minutes. Followed by addition of 50µl anti- 8-oxoG (at a dilution of 1:500 with assay diluent) to each well and incubation at RT for 1 hour. Since 8-oxoG in the sample or standard competes with 8-oxoG bound on the plate for 8-oxoG monoclonal antibody binding sites, higher 8-oxoG concentrations in the sample cause reduced binding of the antibody to the 8-oxoG on the plate.

After incubation, the plate was washed 3 times for 20 minutes using washing solution provided in the kit. Antibodies bound to the 8-oxoG in the sample are washed out of the well, while those bound to the 8-oxoG coated on the plate remain.

After the last wash, 100µl of the enzyme-labelled secondary antibody (at a dilution of 1:1000 with assay diluent) was added to each well and the plate was incubated for 1 hour at RT in order to allow the secondary binding to the primary antibody that remained on the plate. The washing process was repeated in order to remove the unbound enzyme-labelled secondary antibody.

Next 100µl of pre-warmed (at RT) substrate solution (which is a chromogen) was added to each well and the plate was incubated on a shaker at RT for approximately 15 minutes. During this time, the colour developed in proportion to the amount of antibody bound to the plate. The enzyme reaction was stopped by the addition of 100µl stop solution (provided in the kit) to each well. Using a plate reader spectrophotometer, the absorbance of each well was read at 450nm and the graph was plotted. The sensitivity of the kit was 0.1 to 10ng/ml.

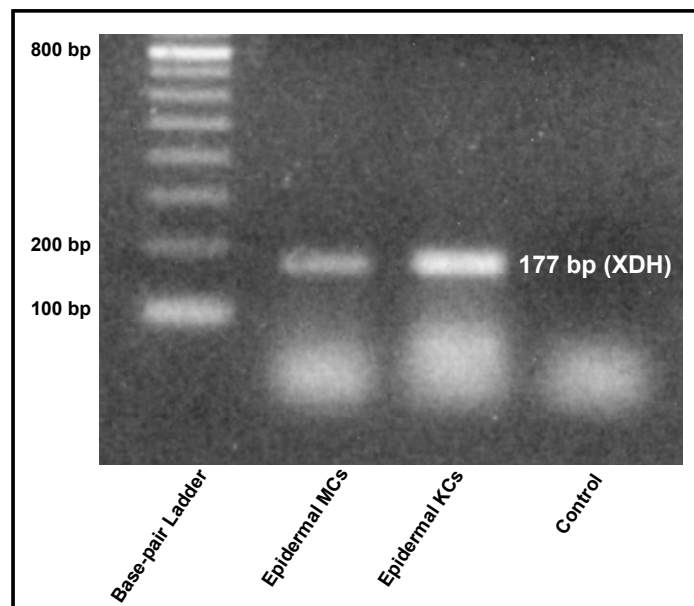
## 4.0 RESULTS

### 4.1 The presence of XDH/XO protein in the human epidermis

(Shalhaf, Gibbons et al. 2008)

#### 4.1.1 Epidermal mRNA of XDH is expressed in human MCs and KCs

PCR is a technique used to demonstrate whether a gene is transcribed into mRNA. Using this technique we showed that XDH mRNA is expressed in human epidermal MCS and KCs (Shalhaf, Gibbons et al. 2008). **Figure 7** shows a strong band at 177bp corresponding to the presence of the XDH mRNA in human epidermal MCs and KCs.



**Figure 7**

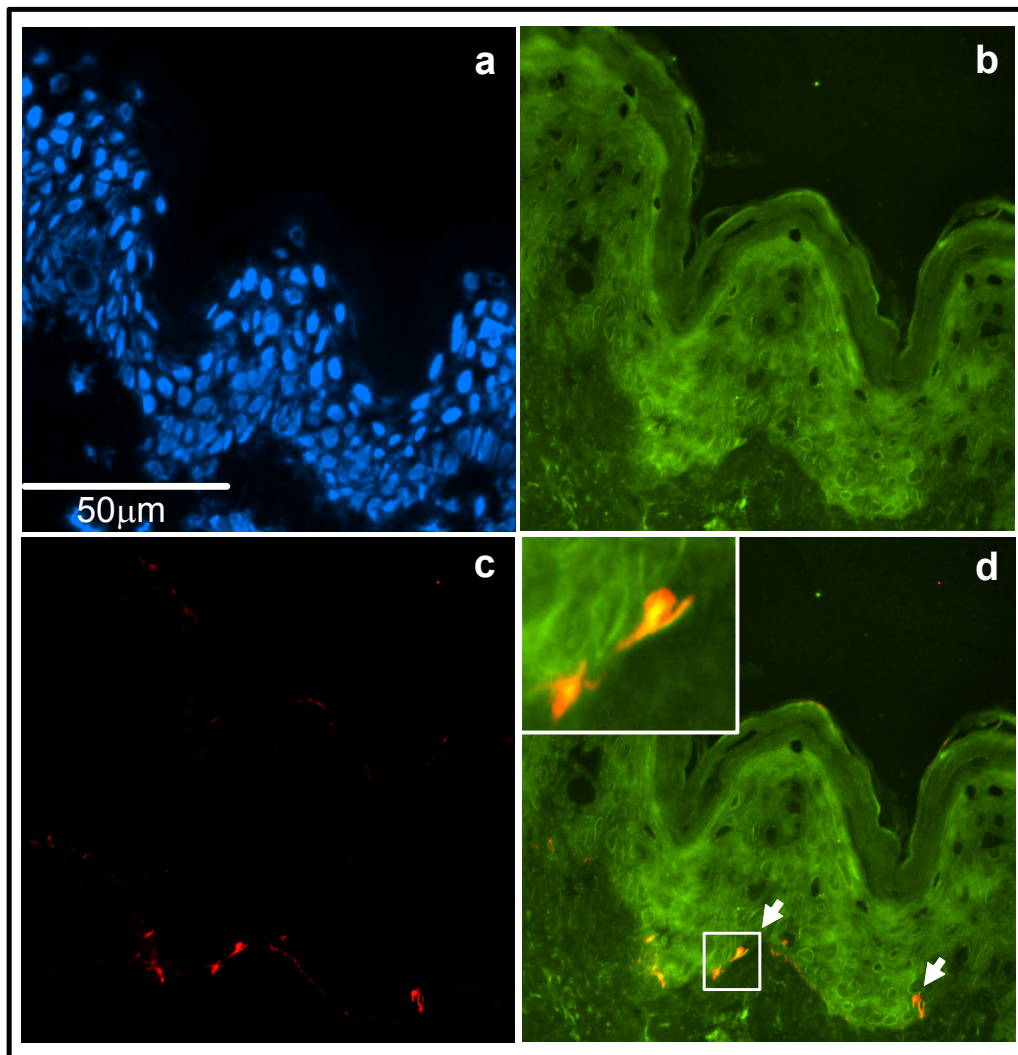
#### **XDH mRNA expression in the human epidermis**

PCR amplification of epidermal cell cDNA gave rise to a specific 177 base pair product confirming the expression of XDH transcript in both epidermal MCs and KCs. This experiment was done in duplicates (n=2) using primary cell cultures (passages 1-3).

#### **4.1.2 XDH/XO protein is expressed *in situ* in the human epidermal cells**

In order to show the presence of XDH/XO protein expression in the human epidermis *in situ*, immunofluorescence staining of full skin biopsies from normal healthy control skin phototype III (Fitzpatrick classification) was performed using a specific XDH/XO antibody. The results indicate that this enzyme is highly expressed throughout the entire epidermis of normal skin; in both the stratum basale and in suprabasal layers (**Figure 8b**).

In order to test whether this enzyme is present within melanocytes, XDH/XO was double stained with a specific tyrosinase antibody. Tyrosinase is the key enzyme in melanogenesis which is present in the melanosomes. The result showed that XDH/XO enzyme is present in melanocytes and within melanosomes (**Figure 8; c&d**).



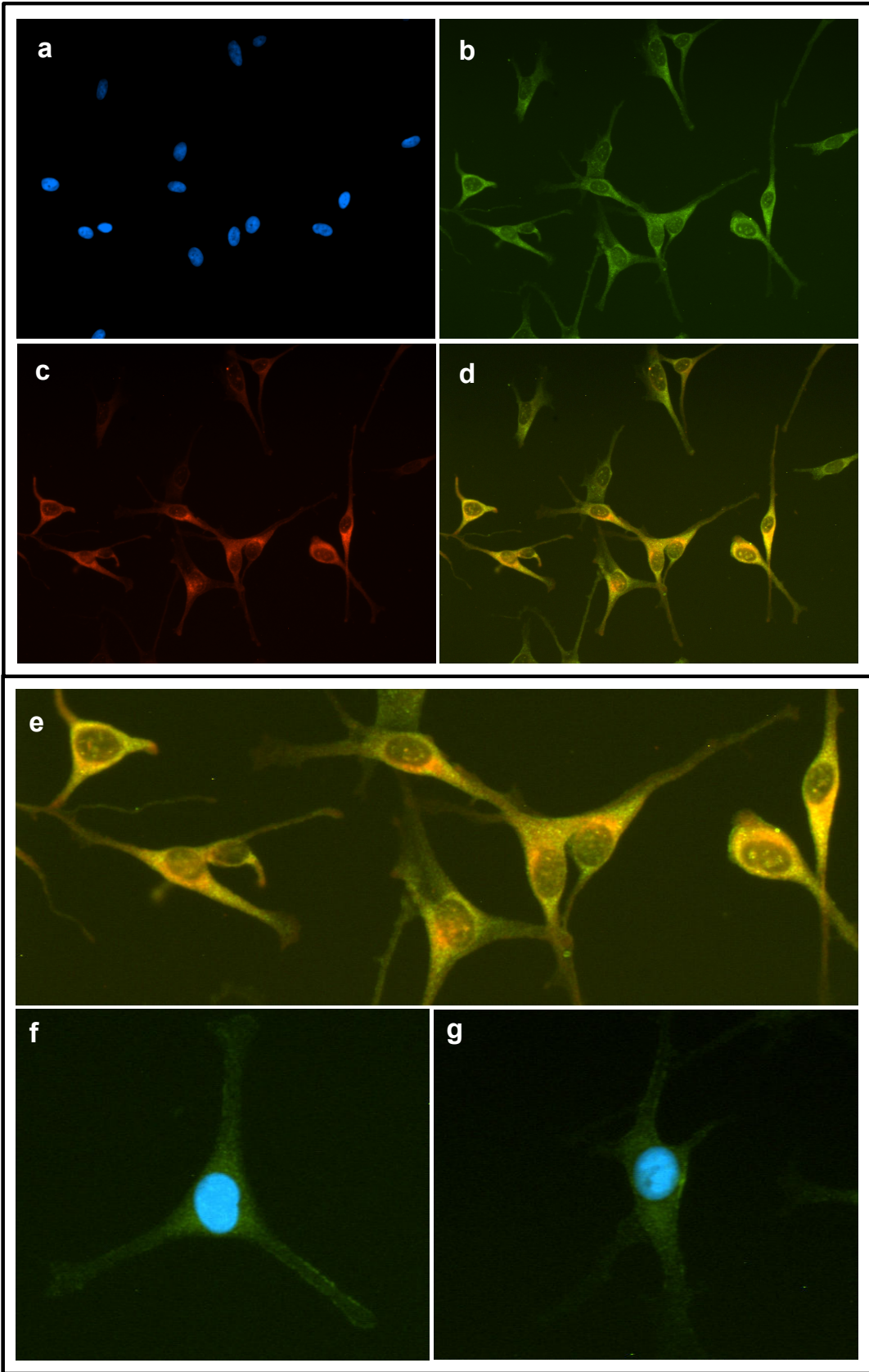
**Figure 8**

***In situ* distribution of XDH/XO in the human epidermis and its localisation in MCs and melanosomes**

(a) DAPI, (b) FITC labelling of XDH/XO; the positive immuno-reactivity demonstrates that the enzyme is expressed throughout the entire epidermis in both the stratum basale and suprabasal layers, (c) TRITC labelling of tyrosinase identifies melanosomes (d) XDH/XO was double stained with tyrosinase where co-localisation was observed in a sub-population of melanocytes suggesting its presence within this pigment producing cell (x 400 magnification). Labelling was carried out in three different healthy individuals and was done in duplicates (n=2).

#### **4.1.3 XDH/XO protein is expressed *in vitro* in human epidermal MCs and KCs**

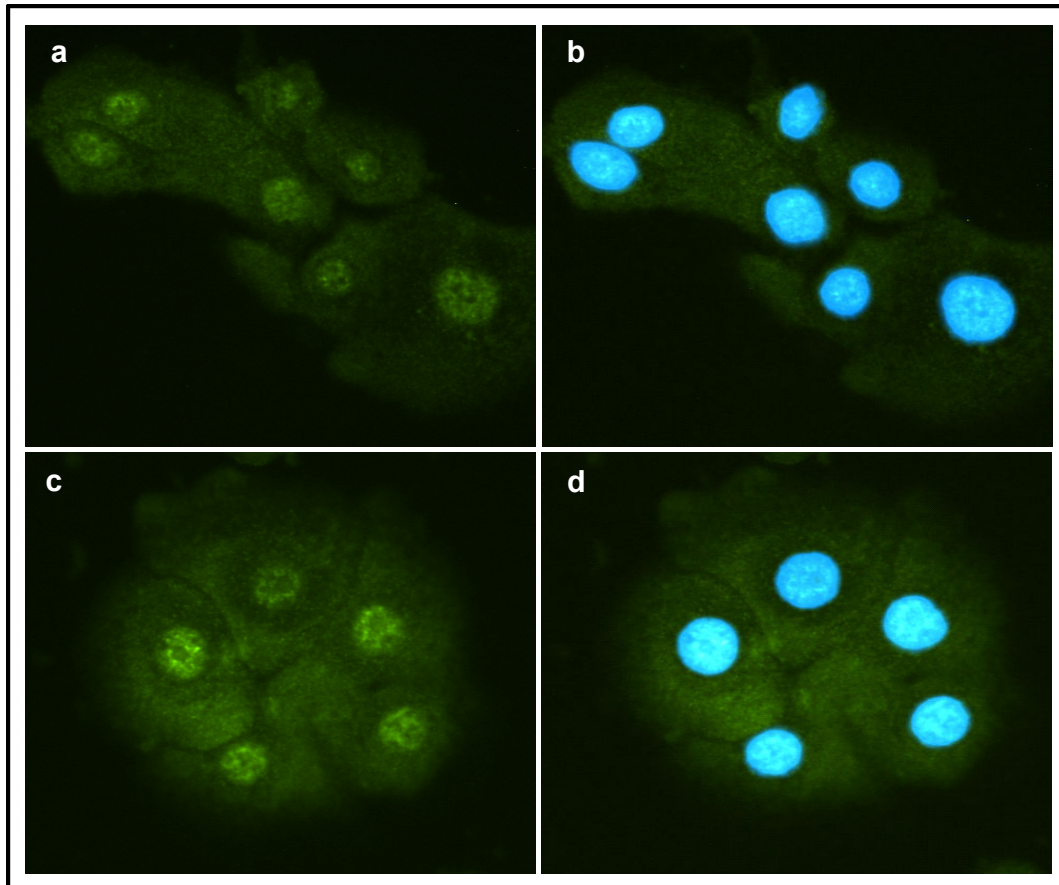
After confirmation of XDH/XO protein expression throughout the entire epidermis *in situ*, the presence of this enzyme was investigated in epidermal derived cells under *in vitro* conditions. The results showed that this enzyme is present in both epidermal MCs and KCs in a granular pattern (**Figure 9b**). Moreover, co-localisation with tyrosinase was detected indicating the presence of the enzyme within melanosomes in a perinuclear granular pattern (**Figure 9; d&e**). Another interesting observation was XO localisation in the nucleus of both MCs and KCs, while is shown by merging of XDH/XO and DAPI (**Figure 9; f&g and Figure 10**).



## Figure 9

### *In vitro* distribution of XO in human epidermal MCs

The staining results show that XO is expressed throughout MCs derived from normal human skin phototype III (Fitzpatrick classification), where **(a)** is DAPI, **(b)** is FITC-labelled XO **(c)** is TRITC-labelled tyrosinase **(d&e)** merging of b and c depicts the predominant perinuclear co-localisation of XO and tyrosinase in melanosomes suggesting the presence of XO within this organelle, while less co-localisation is observed in the dendrites and **(f&g)** close up of two MCs suggesting the possible localisation of XO in the nucleus (a, b, c, d, & e x 200 and f, & g x 400 magnification respectively). Two different cell lines were used in this experiment and labelling was done in duplicates (n=2).



**Figure 10**

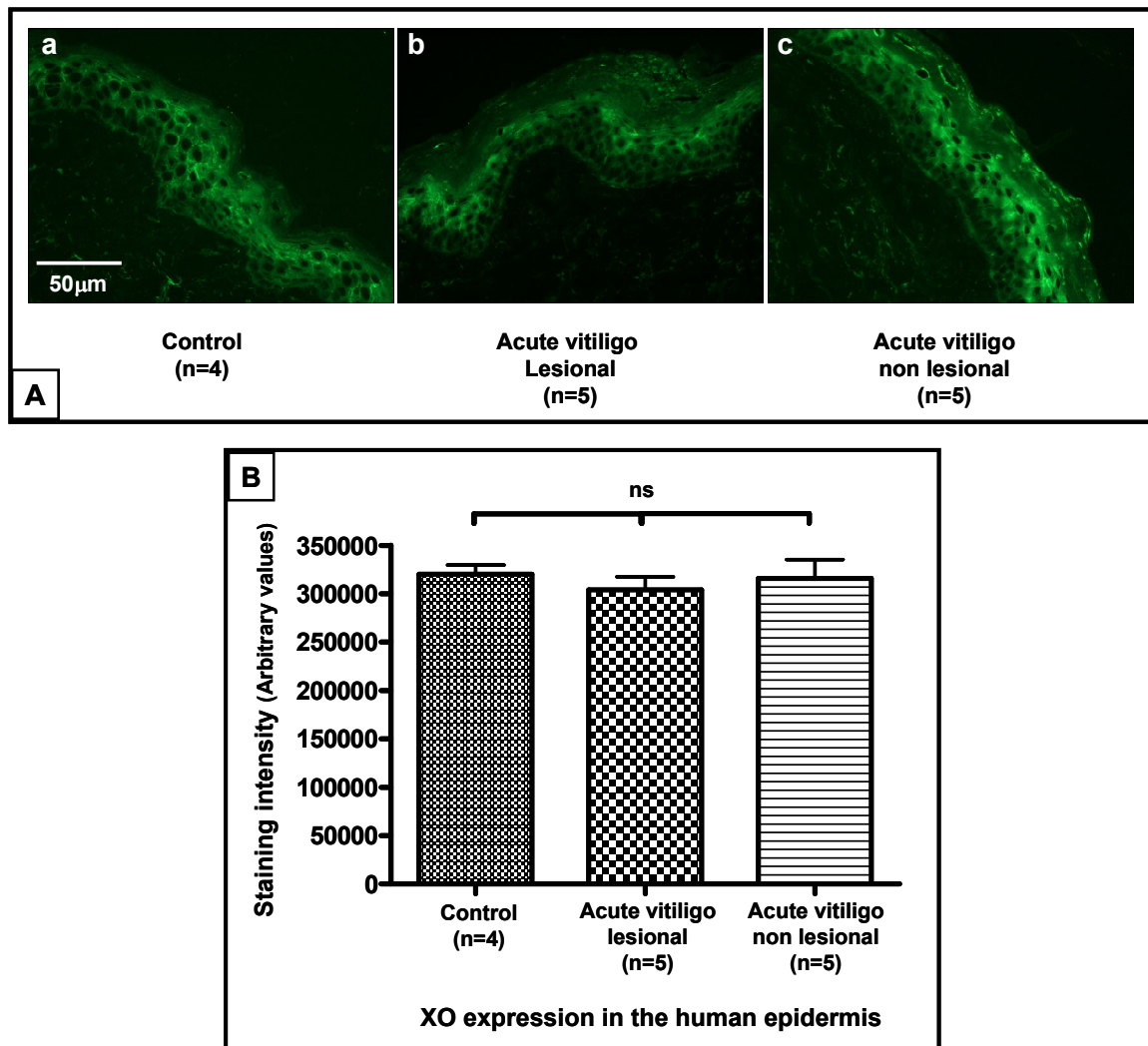
***In vitro* expression of XO in human epidermal KCs**

Undifferentiated epidermal KCs derived from normal healthy human skin phototype III (Fitzpatrick classification) where **(a&c)** show a pronounced granular expression of FITC-labelled XO throughout the cell and **(b&d)** suggest that similar to MCs, XO resides in the nucleus (magnification of x 400). Two different cell lines were used in this experiment and labelling was done in duplicates (n=2).



#### **4.1.4 *in situ* protein expression of XO in vitiligo**

In order to show the presence of XDH/XO protein expression in the epidermis of vitiligo patients, we utilised *in situ* immunofluorescence staining of full skin biopsies from normal healthy control as well as patients with vitiligo all skin phototype III (Fitzpatrick classification). The results revealed that XO protein expression in the epidermis of patients with vitiligo is not affected compared to healthy controls. This result is presented in **Figure 11 A&B**.



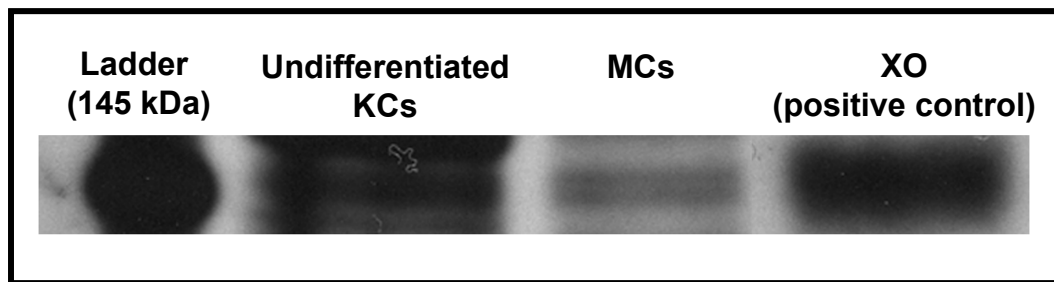
**Figure 11**

***In situ* protein expression of XO is not affected in vitiligo**

(A) FITC labelling of XO; positive immuno-reactivity demonstrates that XO protein expression in the epidermis of patients with vitiligo is not affected compared to control (x 400 magnification). (B) Image analysis of staining intensities. The graph shows XO protein expression in vitiligo is significantly different (ns) compared to control. This experiment was done in duplicates (n=2).

#### 4.1.5 Confirmation of XDH/XO in epidermal MCs and KCs by Western blot analysis

In order to substantiate the presence of XDH protein in epidermal cells, Western blot analysis was employed. The protein was detected in cell extracts from MCs and KCs. Bands at the 145 kDa molecular weight marker corresponded to the monomer of XDH/XO (Figure 12).



**Figure 12**

#### Western blot analysis of XO in human epidermal MCs and KCs

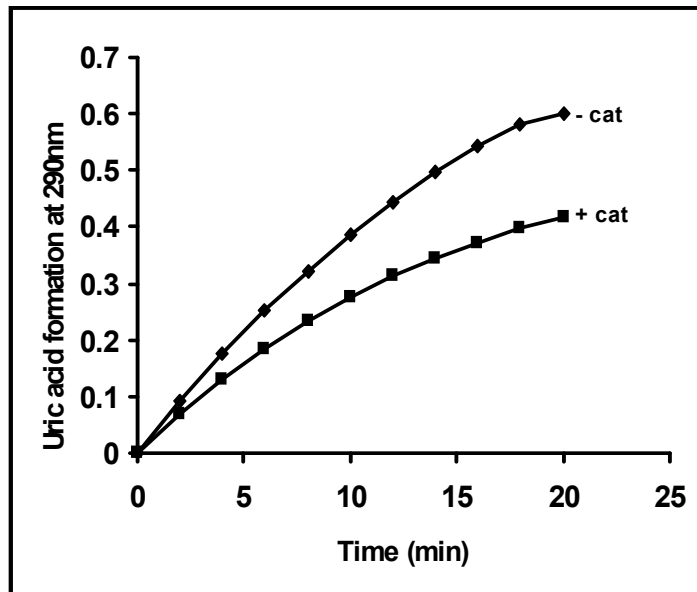
**A):** The Western blot confirms the presence of XO in epidermal MCs and KCs. Cell cultures were in passage 1-3 and the experiment was done in duplicates (n=2).

## 4.2 XO activity

### 4.2.1 Uric acid is produced from the oxidation of xanthine by XO

Purine nucleotide catabolism yields uric acid. In the last two steps of its degradation pathways, hypoxanthine is oxidized to xanthine and then further, xanthine to uric acid, by XO.  $\text{H}_2\text{O}_2$  is formed as a byproduct in these two oxidation reactions (McCord and Fridovich 1968; Granger, Rutili et al. 1981; Granger and Parks 1983; Parks and Granger 1983; McCord 1985; McCord, Roy et al. 1985). In the human epidermis, DNA damage after UVR exposure leads to release of purine bases (Sancar 1994; Sancar 1996; Friedberg 2003; Fromme and Verdine 2004; Friedberg, Aguilera et al. 2006; O'Neil and Rose 2006) which in turn are degraded by XO (Dixon and Lemberg 1934; Parks and Granger 1986). As previously described, this enzyme contains two  $\text{Fe}^{2+}$  atoms in its structure which under oxidative conditions such as oxidative stress in vitiligo, can react with  $\text{H}_2\text{O}_2$  via the Fenton reaction to produce  $\text{OH}^\bullet$  (Haber and Weiss 1932). This is the most potent ROS reacting with nucleotide bases resulting in turn in irreversible damage of DNA, peptides, proteins and lipids (Halliwell 1989; Halliwell and Gutteridge 1989; Esterbauer, Dieber-Rotheneder et al. 1990; Smith, Marks et al. 2004; Schallreuter, Bahadoran et al. 2008). Both  $\text{H}_2\text{O}_2$  and  $\text{OH}^\bullet$  have been shown to be able to oxidise amino acids in the structure of peptides and proteins (for review see (Schallreuter, Bahadoran et al. 2008)).

In order to study XO activity, the standard assay was performed spectrophotometrically both in the presence and absence of catalase over 20 minutes at 290nm as outlined in the method section.



**Figure 13**

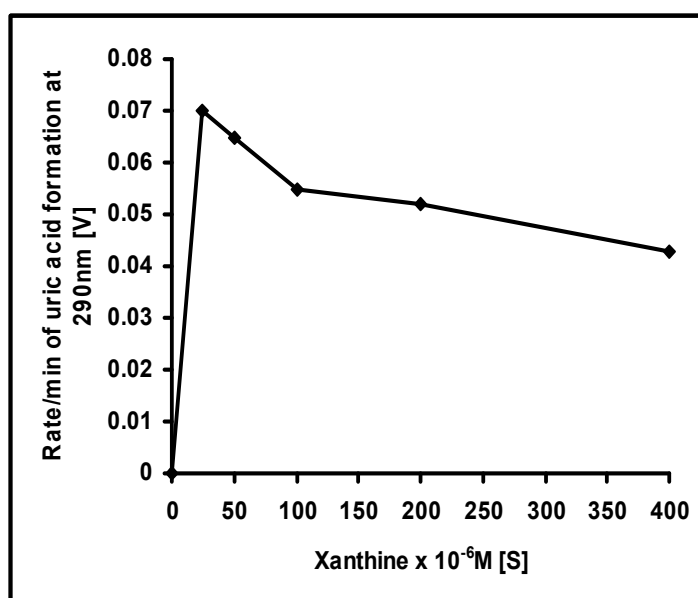
**Uric acid formation from the oxidation of xanthine by XO at 290nm in the absence (-cat) and presence (+cat) of catalase**

Catalase converts  $H_2O_2$  to  $H_2O$  and harmless oxygen. This result shows that  $H_2O_2$  produced during the reaction (-cat) increases XO activity. This assumption is supported because in the presence of catalase the reaction is slower (+cat). The assay was carried out in triplicate and the results show the mean values taken from three independent experiments.

## 4.2.2 XO activity depends on the availability of its substrate (xanthine)

### 4.2.2.1 Substrate inhibition of XO

In order to follow whether the enzyme was influenced by its own substrate, we repeated the assay with different concentrations of the substrate (xanthine) in the absence of catalase. Given that 1 molecule of xanthine produces 2 molecules of  $H_2O_2$ , the result obtained, suggested the oxidation of the reaction product (uric acid) or inhibition of the enzyme or both. The result is shown in **Figure 13**.

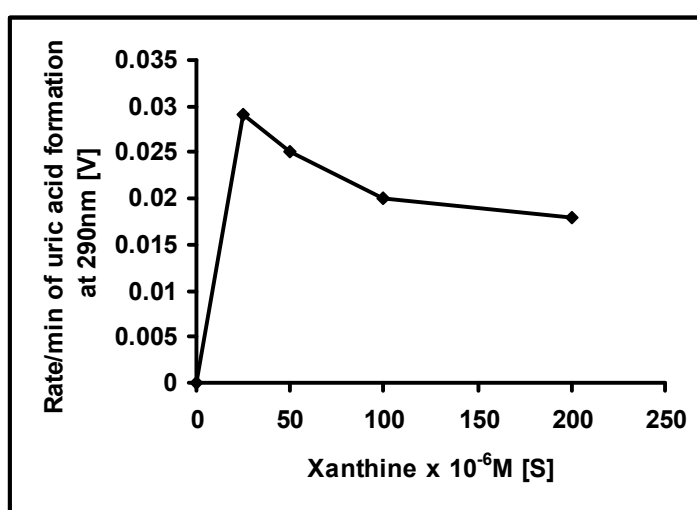


**Figure 14**

### [V] (velocity) versus [S] analysis of XO

The result shows that in the absence of catalase, the increase in concentration of substrate (xanthine) decreases the reaction rate. First it was proposed that the decrease in uric acid production was due to its oxidation to allantoin by  $H_2O_2$  as this ROS is able to oxidise uric acid to allantoin (Newsholm and Leech 1984). Therefore it was decided to repeat the experiment in the presence of catalase (see **Figure 14**). The results shown in the figure are the values obtained from a single determination in one experiment. They are representative of one other experiment.

In order to explore the causes for the decrease in uric acid formation in the reaction between XO and different concentrations of xanthine in more detail, the above experiment was repeated, but this time under different experimental conditions in the presence of catalase to remove  $H_2O_2$  produced in the reaction. The result showed that the increase in the concentration of the substrate has an inhibitory effect on enzyme activity. This proves substrate inhibition of XO activity (**Figure 14**).



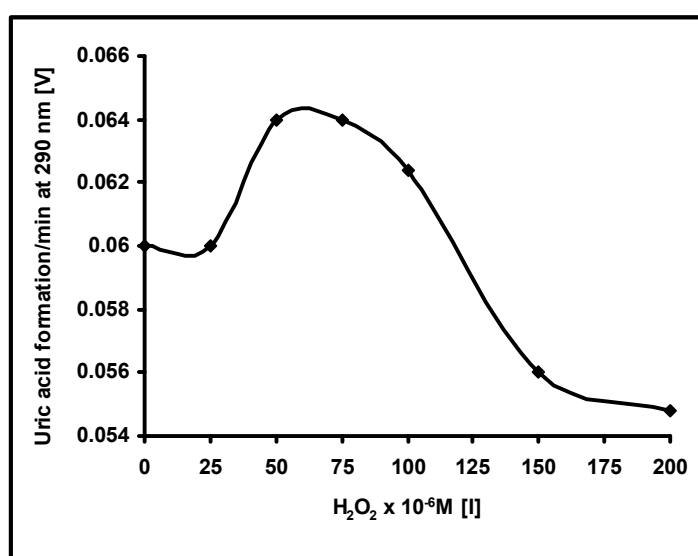
**Figure 15**

### **Substrate inhibition of XO**

The plot shows that with the increase in the concentration of substrate (xanthine), the rate of uric acid formation decreases. Catalase was included in all the reaction mixtures to remove  $H_2O_2$  produced during the reaction. Therefore the decrease in uric acid formation cannot be attributed to its oxidation to allantoin by  $H_2O_2$ . Hence this result indicates that XO activity is inhibited by its own substrate since the enzyme is more active at lower concentrations xanthine (Shalbaf, Gibbons et al. 2008). The results shown in the figure are the values obtained from a single determination in one experiment. They are representative of one other experiment.

### 4.2.3 XO is activated by H<sub>2</sub>O<sub>2</sub> in a concentration dependent manner

In order to further elucidate the effect of low H<sub>2</sub>O<sub>2</sub> concentrations on the reaction between XO and xanthine, we repeated the assay in the presence of  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> concentrations. The result showed activation of the enzyme by low H<sub>2</sub>O<sub>2</sub> concentrations (25-100 $\mu\text{M}$ ). As suggested in **Figure 15**, XO activity decreases in the presence of 25-100 $\mu\text{M}$  concentrations of H<sub>2</sub>O<sub>2</sub>.



**Figure 16**

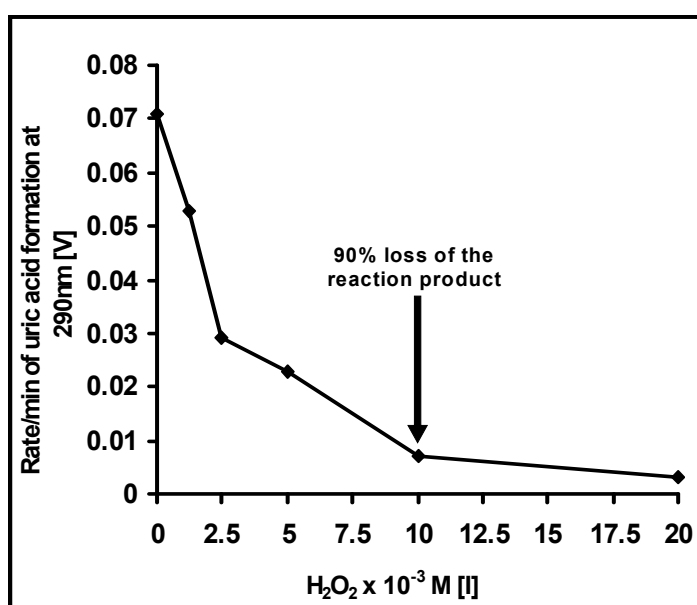
#### [V] (velocity) versus [I] (inhibitor) analysis of XO

The graph shows in the presence 25-100 $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> concentrations, XO activity is higher than <25 $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub> concentration, indicating XO activation in the presence of low H<sub>2</sub>O<sub>2</sub> concentrations (within the range of 25-100 $\mu\text{M}$ ). This result is supported by the data presented in **Figure 12**, where uric acid formation was decreased upon the addition catalase to remove H<sub>2</sub>O<sub>2</sub> produced during the reaction. The results shown in the figure are the values obtained from a single determination in one experiment. They are representative of one other experiment.



#### 4.2.4 Inhibition of XO activity by high H<sub>2</sub>O<sub>2</sub> concentrations

In order to further substantiate the effect of high concentrations of H<sub>2</sub>O<sub>2</sub> on the reaction, we repeated the assay in the presence of mM H<sub>2</sub>O<sub>2</sub> where was directly added into the reaction mixture. Importantly, this assay could not be performed in the presence of catalase because H<sub>2</sub>O<sub>2</sub> was used as the inhibitor. The assay was performed in the presence of saturating substrate (100 $\mu$ M xanthine in 1ml reaction mixture). The result shown in **Figure 16** indicates decrease of enzyme activity in the presence of H<sub>2</sub>O<sub>2</sub>. This decrease is dose dependent.



**Figure 17**

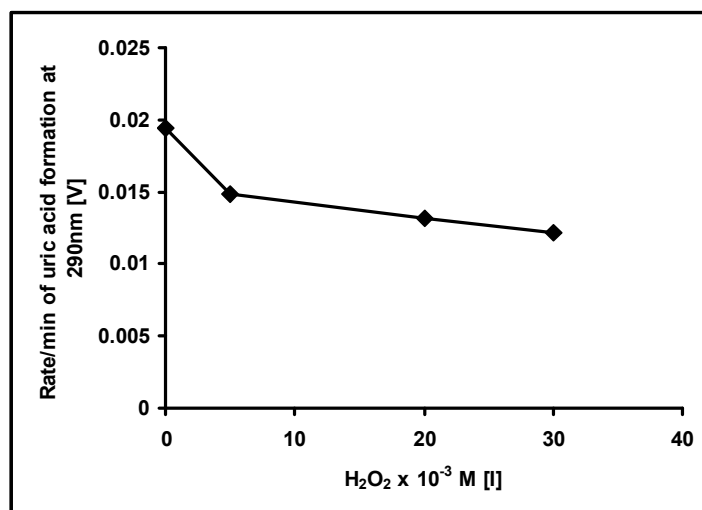
#### [V] versus [I] analysis of XO when H<sub>2</sub>O<sub>2</sub> was directly added into the reaction

The effects of 10<sup>-3</sup>M H<sub>2</sub>O<sub>2</sub> concentrations on XO activity. The graph shows with the increase in the concentration of H<sub>2</sub>O<sub>2</sub>, the rate of uric acid formation decreases. It was first assumed that H<sub>2</sub>O<sub>2</sub> directly affects XO activity. Later during this study this assumption was dismissed, since it was revealed that the decrease in uric acid formation is not based on XO inhibition by H<sub>2</sub>O<sub>2</sub>. It is indeed due its oxidation to allantoin by this ROS (Newsholm and Leech 1984). The assay was carried out in duplicate. The results shown in the figure are the values obtained

from a single determination in one experiment. They are representative of one other experiment.

#### **4.2.5 H<sub>2</sub>O<sub>2</sub> concentrations in the 10<sup>-3</sup>M range decrease enzyme activity only 37%**

The previous result demonstrated that XO (crude enzyme) is activated by 25-100µM concentrations of H<sub>2</sub>O<sub>2</sub>, whereas the enzyme seems to be inhibited by >100µM H<sub>2</sub>O<sub>2</sub> concentrations. To find out more about the activity of this enzyme under severe oxidative conditions, it was decided to incubate different concentrations of purified XO with different H<sub>2</sub>O<sub>2</sub> concentrations in 10<sup>-3</sup>M range. According to the method described before, 25µg of purified enzyme was incubated with 0, 5, 20 and 30mM H<sub>2</sub>O<sub>2</sub> concentrations for 2 hours at RT while 10µl catalase (10mg/ml) was added to all reaction mixtures to stop further oxidation. Free H<sub>2</sub>O<sub>2</sub> was separated from the oxidised enzyme using Zeba<sup>™</sup> Desalt Spin chromatography Column as described in methods. Activity of the enzyme (uric acid formation) was assessed at 290nm over 10 minutes. The results showed that XO is still functioning after 2 hours exposure to H<sub>2</sub>O<sub>2</sub> in the mM range. However, there is a loss of activity after pre-incubation with 5-10mM H<sub>2</sub>O<sub>2</sub> suggesting a direct effect on the enzyme structure. The rates/min were calculated from the linear plots for each minute over 10 minutes of all assays.



**Figures 18**

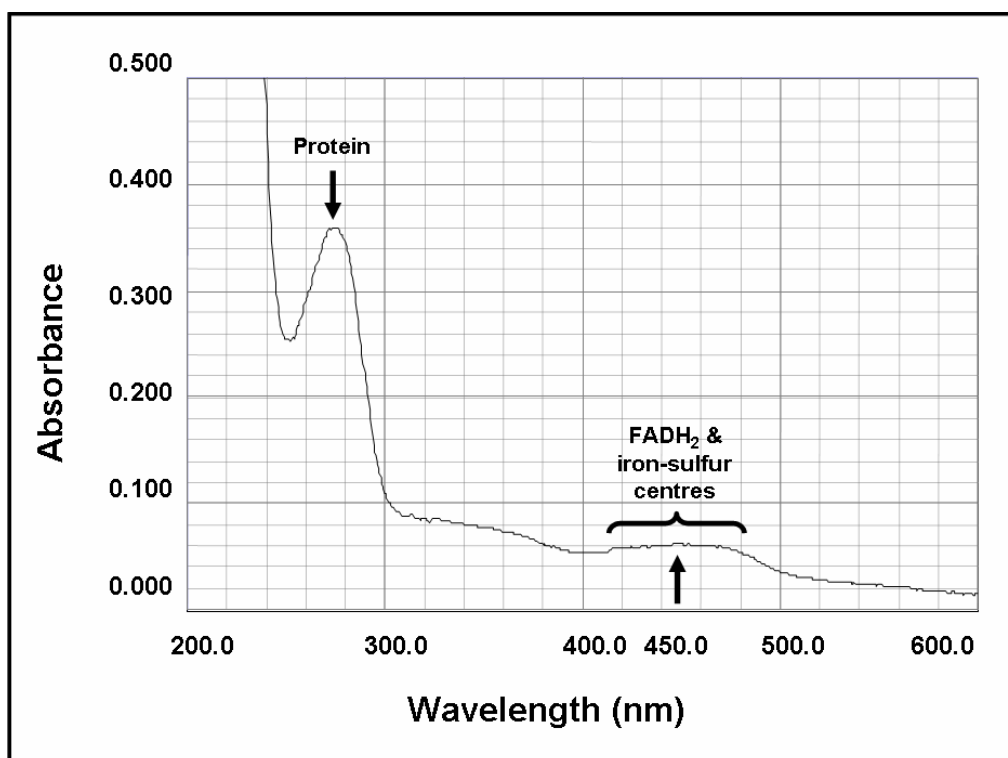
**The activity of XO after pre-incubation with 10<sup>-3</sup>M range of H<sub>2</sub>O<sub>2</sub> concentrations**

The results showed that after pre-incubation of XO with different 10<sup>-3</sup>M H<sub>2</sub>O<sub>2</sub> concentrations, the H<sub>2</sub>O<sub>2</sub>-oxidised enzyme maintains almost 75% of its original activity. As described in the methods, 25µg of purified XO was first oxidised with different 10<sup>-3</sup>M H<sub>2</sub>O<sub>2</sub> concentrations and then free H<sub>2</sub>O<sub>2</sub> was removed from the oxidised enzyme and catalase was included in all the reactions to prevent further XO oxidation/inhibition and also to prevent the oxidation of the reaction product (uric acid) to allantoin by H<sub>2</sub>O<sub>2</sub>. This allowed measurement of the real activity of oxidised XO. The results shown in the figure are the values obtained from a single determination in one experiment. They are representative of one other experiment.

## 4.3 H<sub>2</sub>O<sub>2</sub> directly affects the co-factor FADH<sub>2</sub>

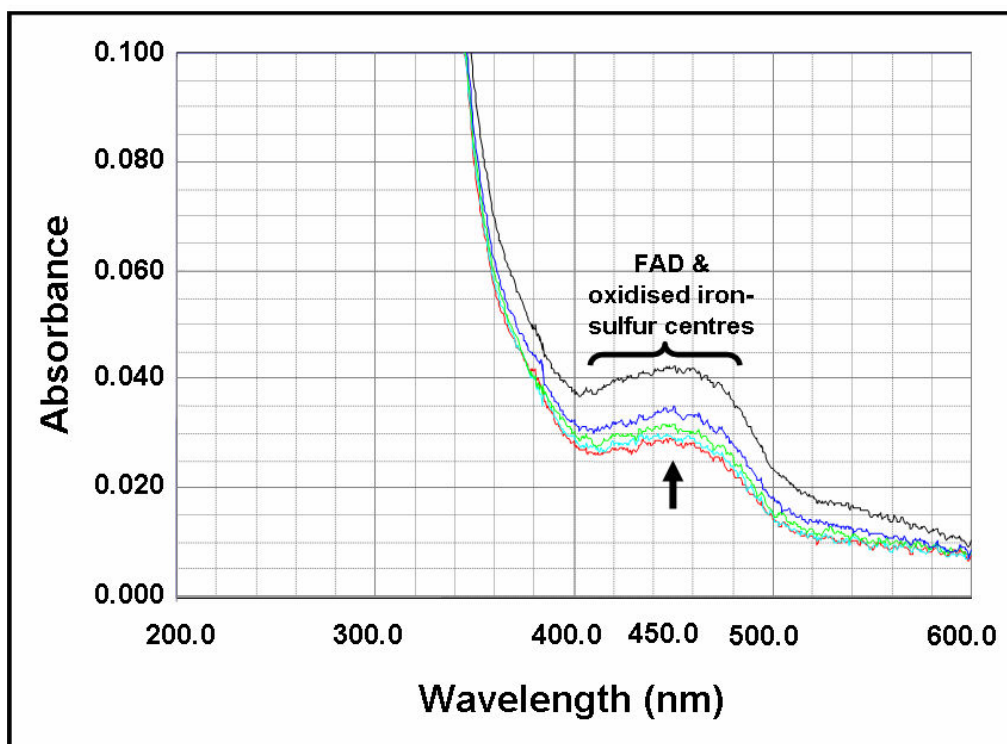
### 4.3.1 Absorbance spectroscopy of reduced and oxidised XO

In the above results we showed that high concentrations of H<sub>2</sub>O<sub>2</sub> can directly affect XO activity. In order to support these results, we decided to study the effects of high H<sub>2</sub>O<sub>2</sub> concentrations on the co-factor binding sites of the enzyme spectrophotometrically. As it was explained before, XO consists of three domains; the molybdopterin cofactor domain, the FADH<sub>2</sub> binding domain and the iron-sulfur domain which have all different UV absorbance spectra. **Figures 19&20** demonstrate the spectra of both native and oxidised XO, revealing that FADH<sub>2</sub> is indeed oxidised to FAD as indicated by the increase in absorbance at 450nm. However, this is also the region of the iron-sulfur centres, which might also come into the game. Unfortunately, the absorbance from the moco-binding domain is small and difficult to observe on the spectrum due to the large absorption of the FAD and iron-sulfur centers. These results are in agreement with the results of the computer modelling (**Figure 20**).



**Figure 19**

**The UV/visible spectrum of native purified XO (0.45mg/ml)**



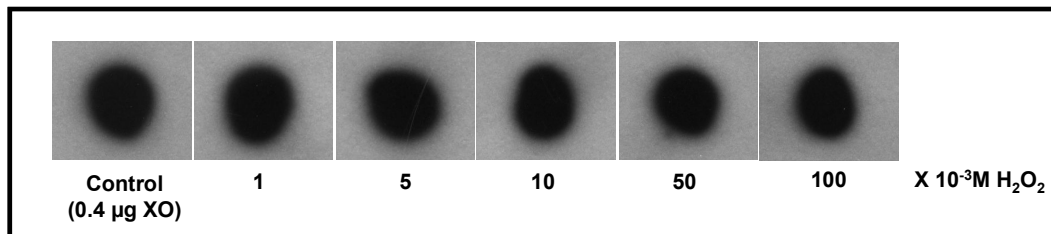
**Figure 20**

**H<sub>2</sub>O<sub>2</sub>-oxidised XO spectrum**

225µg purified XO (500µl, 0.45mg/ml) was incubated with 100µl 30% H<sub>2</sub>O<sub>2</sub> (1.5M final concentration of H<sub>2</sub>O<sub>2</sub>) and the mixture was scanned every 15min for 1 hour. The maximum absorbance at 450nm is due both to the H<sub>2</sub>O<sub>2</sub>-oxidised FAD and iron-sulfur centres whereas changes in moco domain's absorbance (approximately between 400-500nm) are very small and difficult to be identified on the spectra (Ryan, Ratnam et al. 1995; Maiti, Tomita et al. 2003). This result shows that FADH<sub>2</sub> is oxidised to FAD indicating that H<sub>2</sub>O<sub>2</sub> does indeed affect the cofactor on XO.

#### 4.4 H<sub>2</sub>O<sub>2</sub> does not affect the antibody binding site of XO

The spectrophotometric spectra of oxidised XO revealed that H<sub>2</sub>O<sub>2</sub> directly affects the oxidation of FADH<sub>2</sub> to FAD and also oxidises the iron-sulfur centres. In order to ensure that our antibody was recognising the oxidised protein, we investigated the antigen recognition of the oxidised XO using dot blotting. In order to do this, 0.4µg purified XO was exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> for 1 hour. The result showed that the epitope binding sites for the antibody are not affected by H<sub>2</sub>O<sub>2</sub>.



**Figure 21**

##### **The effects of H<sub>2</sub>O<sub>2</sub>- mediated oxidation on the epitope binding site of XO**

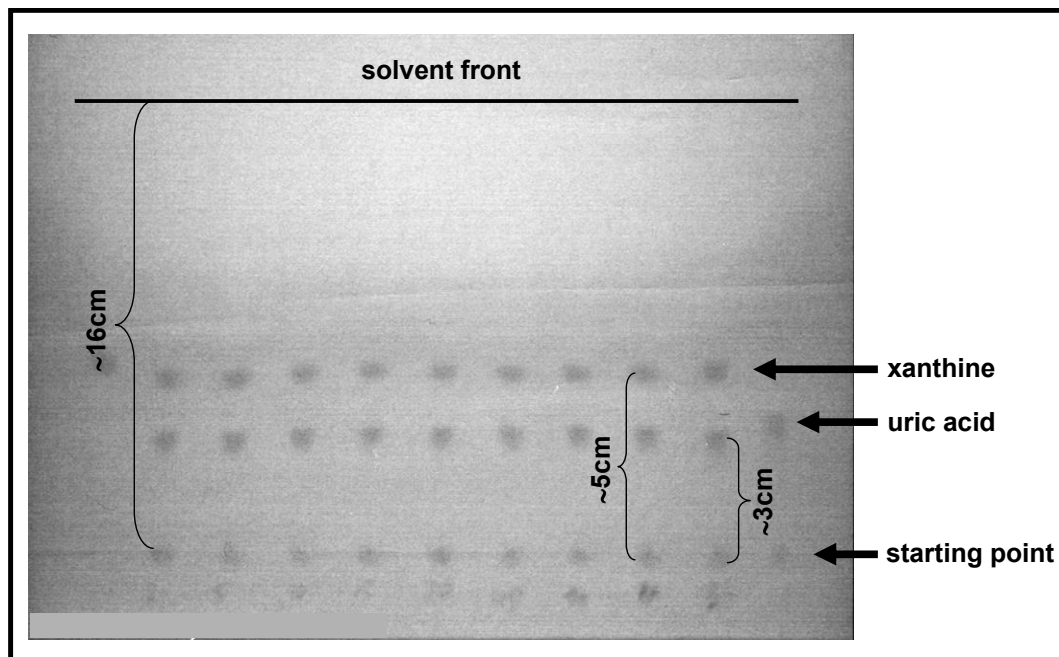
H<sub>2</sub>O<sub>2</sub>-mediated oxidation does not affect XO epitope binding site for the antibody (Dot blot analysis 0.4 µg XO/dot). This result indicates that the antibody is able to detect the oxidised XO as well as the native enzyme. The results shown are representation of one other experiment.



## 4.5 Determination of XO activity by TLC

### 4.5.1 Separation of xanthine and uric acid

In purine nucleotide catabolism XO catalyses the oxidative hydroxylation of hypoxanthine to xanthine and finally to uric acid. Under normal condition this reaction yields two molecules  $H_2O_2$  which are converted by catalase to  $H_2O$  and  $O_2$ . In the case of oxidative stress as observed in the epidermis of patients with vitiligo (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008), due to the presence of  $H_2O_2$  accumulation in the mM range in association with defective antioxidant defence (Schallreuter, Wood et al. 1991; Schallreuter 2005; Schallreuter, Bahadoran et al. 2008), it was tempting to postulate that XO is oxidised by  $H_2O_2$ . Hence uric acid production should be altered. Moreover, due to the presence of an iron-sulfur domain in the enzyme's active site,  $OH^\bullet$  formation via the Fenton reaction is expected (Haber and Weiss 1932; Beauchamp and Fridovich 1970; Bannister, Bannister et al. 1982; Britigan, Pou et al. 1990; Schallreuter 2005). This in turn would increase the oxidative stress. To further substantiate uric acid formation under oxidative stress condition, we used TLC and [ $^{14}C$ ]xanthine.



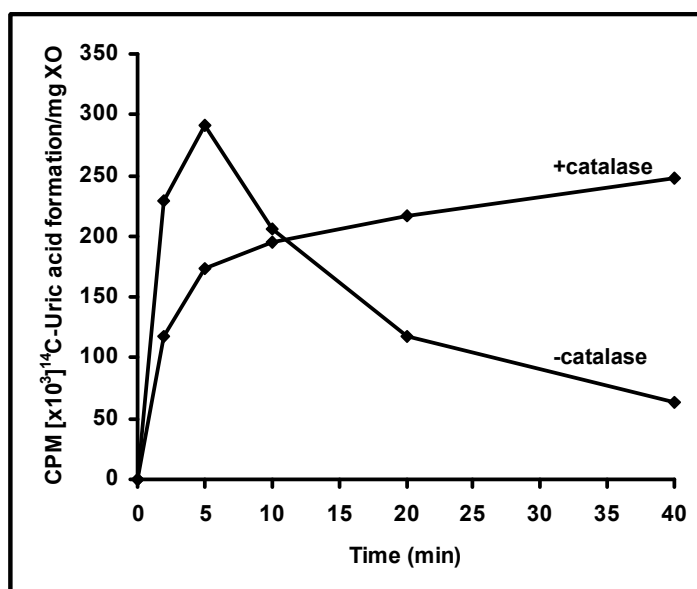
**Figure 22**

**Separation of xanthine and uric acid utilising TLC**

The figure demonstrates separation of xanthine and uric acid in a solvent system containing 66.5 % 1-propanol, 20%  $\text{NH}_4\text{OH}$  (8N), and 13.5%  $\text{dH}_2\text{O}$ . In this solvent system xanthine and uric acid are well separated.  $R_f$  values for both xanthine and uric acid were 0.31 and 0.19 respectively. The results shown are representation of one other experiment.

#### 4.5.2 Standard assay to measure uric acid production over time

In order to follow whether  $H_2O_2$  produced during the reaction could interfere with uric acid formation, we studied enzyme activity using XO and [ $^{14}C$ ]xanthine by following product formation over time in the presence and absence of catalase via TLC. The spots were visualised with a UV lamp (350nm), marked and the marked area was cut and transferred into scintillation liquid followed by counting [ $^{14}C$ ]uric acid. In the absence of catalase the results showed that uric acid formation increased up to 5min followed by a significant decrease.



**Figure 23**

#### Time dependent [ $^{14}C$ ]uric acid formation (0-40 minutes)

The graph shows in the absence of catalase, there is an initial increase in uric acid formation up to 5 minutes, which then drops significantly over time, whereas in the presence of catalase uric acid production increases over time. In **Figure 18**, it was demonstrated that under severe oxidative conditions, XO activity is only partially affected. Therefore the decrease in uric acid formation in the absence of catalase in this Figure is not due to XO inhibition by  $H_2O_2$  and is based on the oxidation of uric acid to allantoin by  $H_2O_2$  produced during the reaction as it was

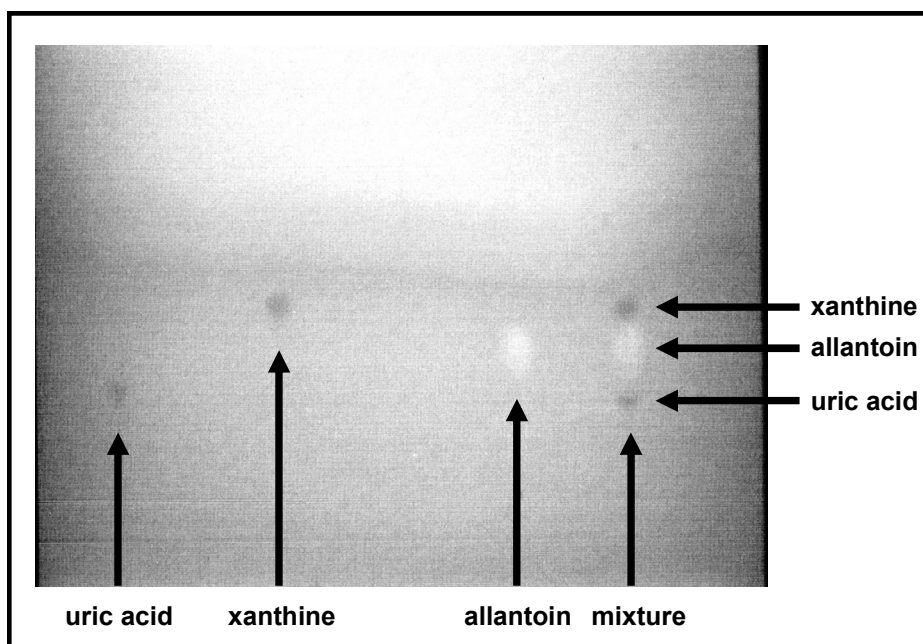
shown and described in **Figure 17**. The results shown in the figure are from a single determination in one experiment and are representative of the two others.

### **4.5.3 The question of interest was what happened to the uric acid formation under oxidising conditions?**

Purine metabolism in primates is ended by the production of uric acid which is then excreted in urine. However, in other mammals, turtles and molluscs uric acid is further oxidised by urate oxidase (uricase) to allantoin which is then excreted (Newsholm and Leech 1984). Allantoin is oxidised to allantoate by allantoinase in some fishes but in other fishes and in amphibian allantoate is oxidised to glyoxylate and urea by allantoinase (Newsholm and Leech 1984). Primates lack this enzyme which oxidises uric acid. In fact, uricase is not the only factor to oxidise uric acid. In humans, allantoin is formed non-enzymatically by the oxidation of uric acid under oxidative conditions, thus, it is considered as a biomarker for oxidative stress (Grootveld and Halliwell 1987; Kaur and Halliwell 1990; Benzie, Chung et al. 1999; Yardim-Akaydin, Sepici et al. 2004; Yardim-Akaydin, Sepici et al. 2006). Under oxidative condition, as observed in vitiligo (Schallreuter, Moore et al. 1999), due to accumulation of mM range of  $H_2O_2$  produced from the oxidation of hypoxanthine to xanthine and xanthine to uric acid by the enzyme XO in association with  $H_2O_2$  from other sources, allantoin production was expected (Grootveld and Halliwell 1987; Kaur and Halliwell 1990; Benzie, Chung et al. 1999; Simoyi, Falkenstein et al. 2003).

#### 4.5.4 Separation of xanthine, uric acid and allantoin

To test whether uric acid is oxidised to allantoin by H<sub>2</sub>O<sub>2</sub> in our system, we followed the separation of xanthine, uric acid and allantoin via TLC. The technique revealed a good separation of these three compounds on one plate as shown in **Figure 23**.



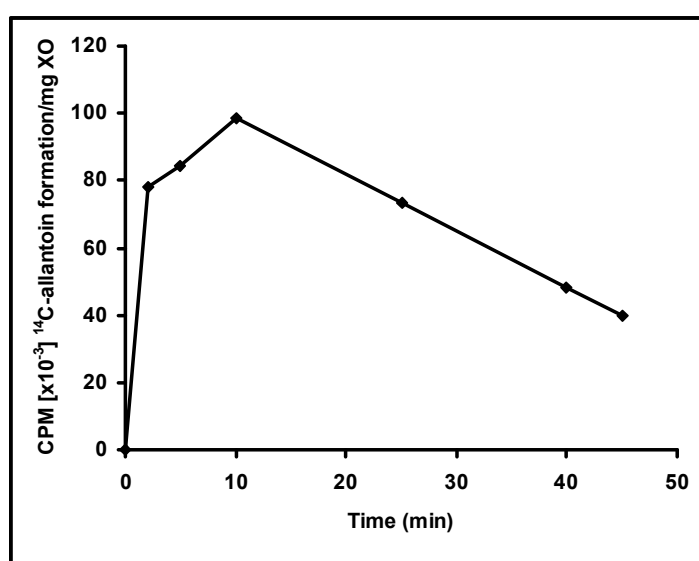
**Figure 24**

#### Separation of xanthine, uric acid and allantoin by TLC

To separate allantoin from xanthine and uric acid we utilised cellulose-coated TLC plate and the same solvent system as described in **Figure 22**. R<sub>f</sub> value for allantoin was 0.25. The results shown are representative of three experiments.

#### 4.5.5 H<sub>2</sub>O<sub>2</sub> yields oxidation of uric acid to allantoin

In order to test the formation of uric acid and its possible oxidation/degradation to allantoin by H<sub>2</sub>O<sub>2</sub> over time, we used TLC and [<sup>14</sup>C]xanthine in the presence and absence of catalase. The result showed uric acid formation is time dependent. In the presence of catalase uric acid formation increases constantly (**Figure 23**), whereas in the absence of catalase after an initial increase, uric acid production decreases due to its oxidation to allantoin as shown in here in **Figure 25**.



**Figure 25**

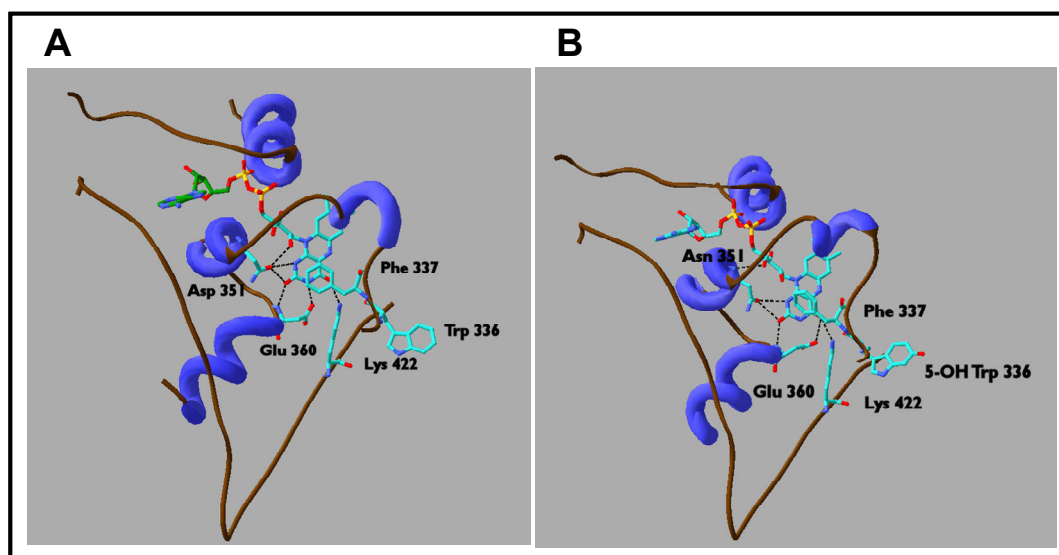
#### H<sub>2</sub>O<sub>2</sub>-mediated oxidation of uric acid to allantoin

As it was shown in **Figure 23**, in the absence of catalase uric acid is oxidised to allantoin by H<sub>2</sub>O<sub>2</sub> produced during the reaction between xanthine and XO. Now within this graph we show that [<sup>14</sup>C]allantoin formation increases over time, followed by a decrease. Allantoin is hydrolysed to glyoxylate and thereafter to urea. These two compounds do not have UV absorption thus they are not detectable under UV light. The results shown in the figure are from a single determination in one experiment and are representative of one other.

## 4.6 Computer stimulation of native and oxidised XO

### 4.6.1 H<sub>2</sub>O<sub>2</sub>-mediated oxidation of the binding domain for the flavin ring of FADH<sub>2</sub> suggests oxidation of the co-factor

In order to further substantiate our results on XO oxidation we employed computer stimulation. The result indicated that oxidation very likely affects the FADH<sub>2</sub> binding site of XO which in turn supports our FADH<sub>2</sub> binding experiments (**Figure 26**).



**Figure 26**

**(A): Native**

Flavin ring of FADH<sub>2</sub> is bound via Hydrogen bonds from Asp<sup>351</sup>, Glu<sup>360</sup>, and Lys<sup>422</sup> and via  $\pi$ - $\pi$  stacking interactions with the phenyl ring of Phe<sup>337</sup>

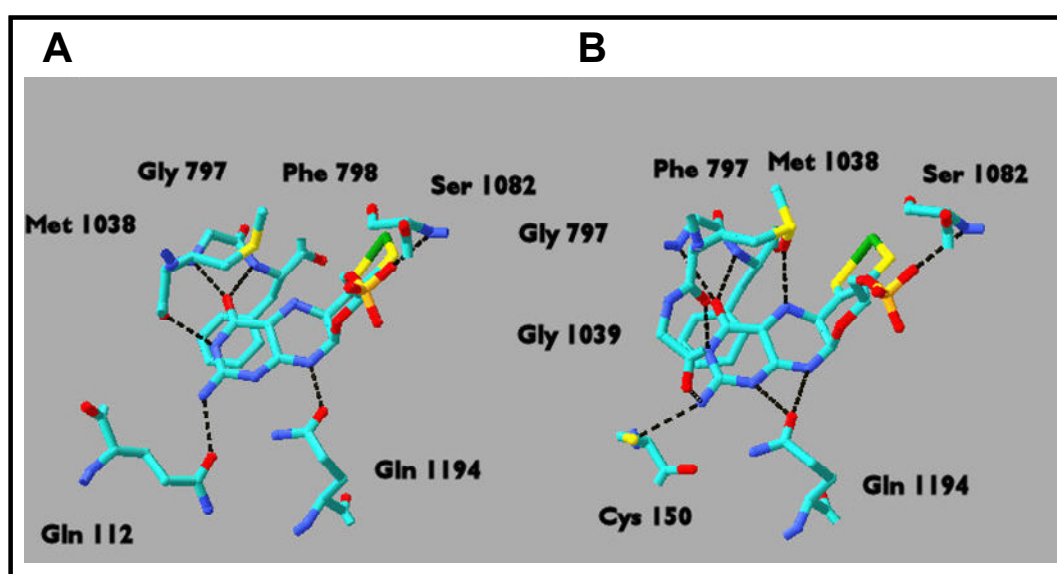
**(B): Oxidised**

oxidation of Met and Trp residues around the FADH<sub>2</sub> binding (most noticeably Trp<sup>336</sup> shown here) disrupts  $\pi$ - $\pi$  stacking of Phe<sup>337</sup> and the H-bonding network to a small extent. While this is not enough to significantly affect the affinity of XO for FAD, it may affect the vulnerability of FAD to oxidation (with kind permission from Dr. Gibbons).



#### 4.6.2 H<sub>2</sub>O<sub>2</sub>-mediated oxidation of the molybdopterin binding domain does not alter co-factor binding

Since XO is a Molybdopterin containing enzyme, we wanted to know whether this cofactor is possibly affected by H<sub>2</sub>O<sub>2</sub>-mediated oxidation. Computer stimulation reveals that despite oxidation of Met and Trp residues, the cofactor binding is not affected. The result is shown in **Figure 27**.



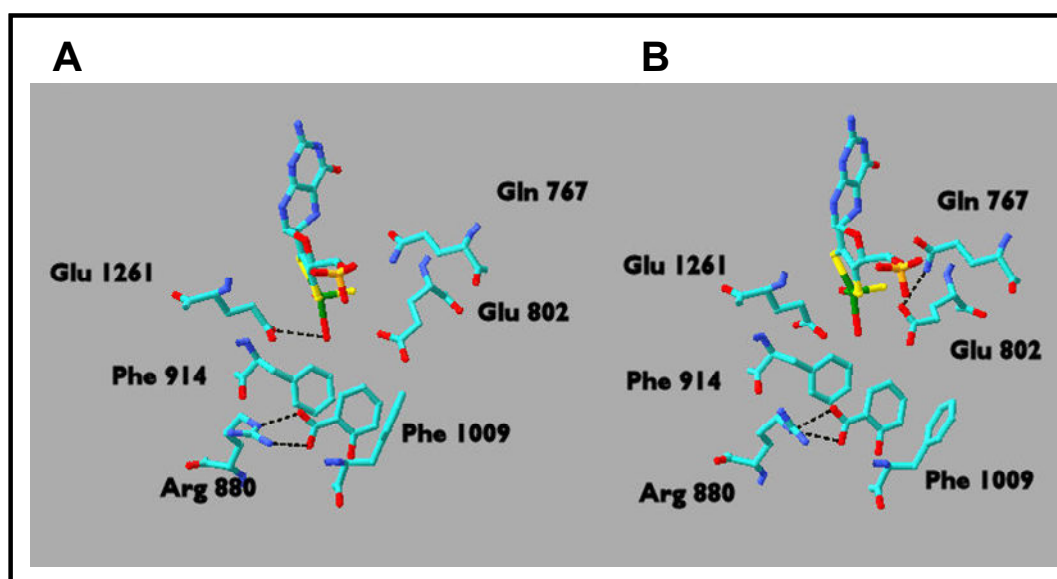
**Figure 27**

**(A):** Molybdopterin is bound by H-bonds from the residues Gln<sup>112</sup>, Gly<sup>797</sup>, Phe<sup>798</sup>, Met<sup>1038</sup>, Ser<sup>1082</sup> & Gln<sup>1194</sup>.

**(B):** Oxidation of Met and Trp residues does not adversely affect binding, with only 1 H-bond being lost from Gln<sup>112</sup> and several new ones being formed: one addition bond is formed from both Met<sup>1038</sup> & Gln<sup>1194</sup> and two completely new H-bonds form Cys<sup>150</sup> and Gly<sup>1039</sup> (with kind permission from Dr.Nicholas Gibbons).

#### 4.6.3 H<sub>2</sub>O<sub>2</sub>-mediated oxidation of the active site of XO affects the enzyme kinetics

Next we wanted to explore whether the active site of XO could be affected by H<sub>2</sub>O<sub>2</sub>. While both normal Phe<sup>914</sup> and Phe<sup>1009</sup> are not affected Gln<sup>767</sup> and Glu<sup>802</sup> are shifted, favouring possibly a stronger binding of the cofactor. The shift of Arg<sup>880</sup> and Glu<sup>1261</sup> could explain the change in the enzyme rate and catalysis as shown in our experiment (Figure 28).



**Figure 28**

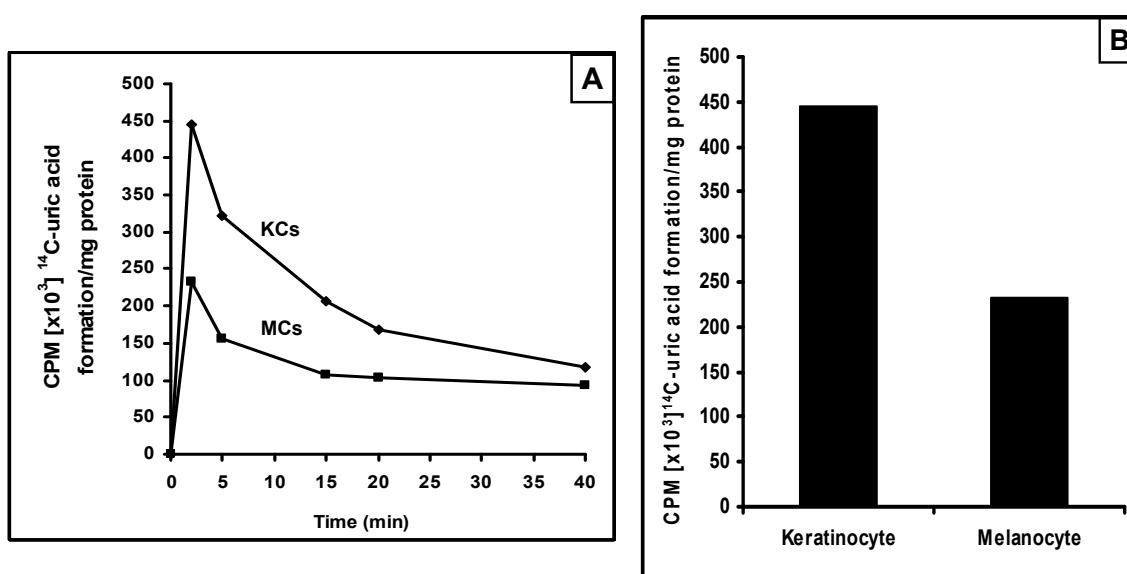
**(A) Native** - 6 residues are involved in the active site of XO. Glu<sup>1261</sup> is the crucial catalytic residue, while Gln<sup>767</sup> and Glu<sup>802</sup> are involved in aiding in catalysis. Arg<sup>880</sup>, Phe<sup>914</sup> and Phe<sup>1009</sup> are involved in forming a binding site for the substrates or inhibitors. Arg<sup>880</sup> acts to form H-bonds/salt bridges while Phe<sup>914</sup> and Phe<sup>1009</sup> are involved in  $\pi$ - $\pi$  stacking interaction with the aromatic rings of substrates/inhibitors.

**(B) Oxidised** - oxidation of Met and Trp residues around the active site causes shifts in the positions of several residues. Phe<sup>914</sup> & Phe<sup>1009</sup> do not seem to be affected at all, while Gln<sup>767</sup> & Glu<sup>802</sup> appear to be shifted into positions that may enable them to interact more strongly with the Moco-factor. Arg<sup>880</sup> and Glu<sup>1261</sup> are shifted away from their positions, affecting reaction rate and catalysis (with permission from Dr. Gibbons).

## 4.7 XO activity in epidermal MCs and KCs

### 4.7.1 XO activity is significantly higher in epidermal KCs compared to MCs

XO activity was determined in cell extracts from epidermal MCs and KCs by following uric acid formation in the absence of catalase. A maximum in [ $^{14}\text{C}$ ]uric acid production was reached at 2 minutes. The results showed that KCs have a significantly higher XO activity compared to MCs (**Figure 28**).



**Figure 29**

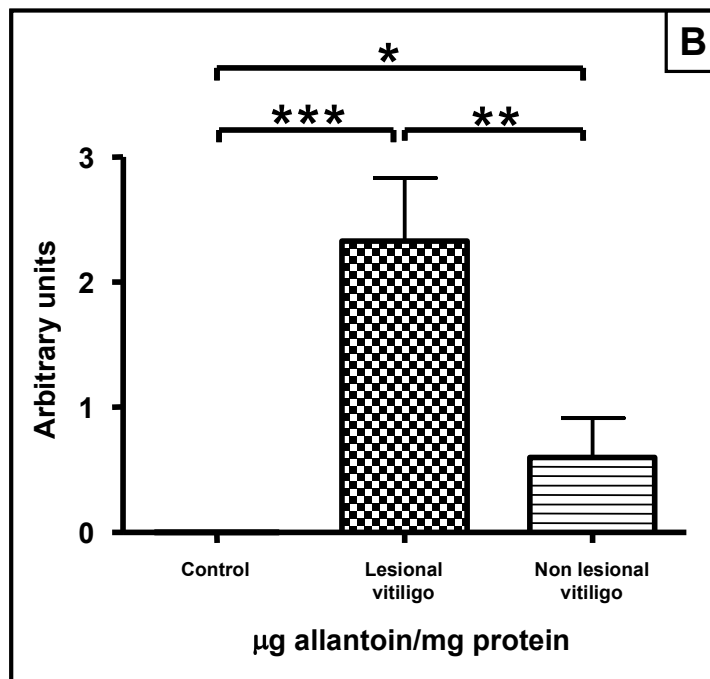
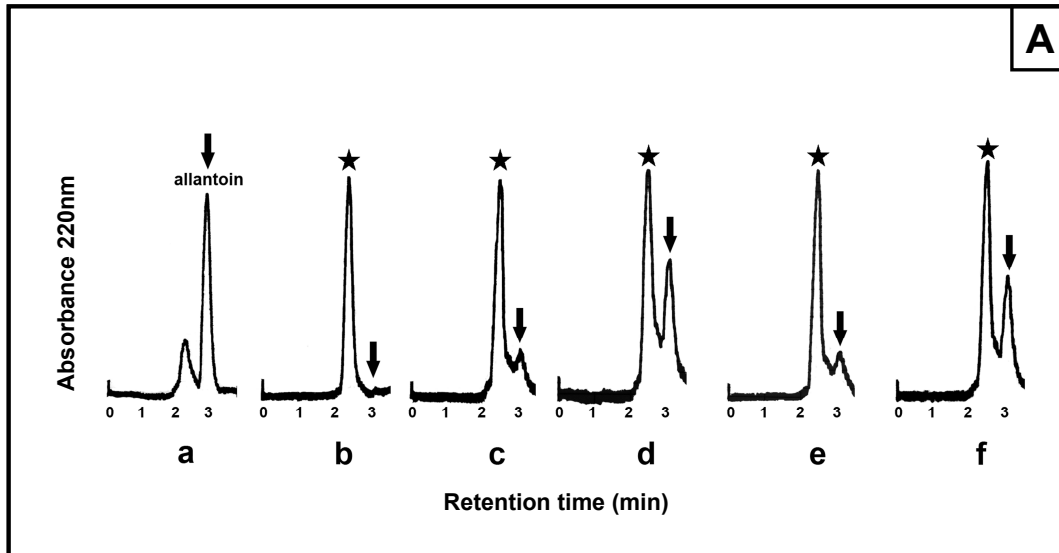
### [ $^{14}\text{C}$ ]uric acid formation in human epidermal KCs and MCs

(A): Time dependent uric acid formation in the absence of catalase. Uric acid production increases up to 2 minutes thereafter decreases due to its oxidation to allantoin by  $\text{H}_2\text{O}_2$  produced during the reaction. (B): Comparison of [ $^{14}\text{C}$ ]uric acid formation in human epidermal KCs and MCs after 2 minutes. The result indicates that both epidermal cells have XO activity. It appears that KCs show double of XO activity. This experiment was done in triplicates ( $n=3$ ) using three different primary cell cultures (passage 1-3).

## 4.8 Presence of allantoin in vitiligo

### 4.8.1 Allantoin is present in the epidermis of patients with acute vitiligo

Since allantoin is a marker for H<sub>2</sub>O<sub>2</sub>-mediated oxidation of uric acid and since epidermal H<sub>2</sub>O<sub>2</sub>-concentrations in patients with vitiligo exceed mM concentrations of this ROS, we decided to test the formation of allantoin in epidermal cell extracts from suction blister tissue originating from acute vitiligo (n=10). Allantoin was determined via high performance liquid chromatography (HPLC) and the correct assignment was confirmed by mass spectrometry (Dr. Derek Maitland, data not shown). The results of this study confirmed that allantoin is indeed present in both lesional and non lesional epidermis of acute vitiligo (n=10), whereas healthy controls (n=5) do not show this metabolite (**Figure 30**).



**Figure 30**

**(A): HPLC proves the presence of allantoin in the epidermis of vitiligo**

**(a)** Standard, 20µl allantoin solution (0.04µg allantoin) was injected and allantoin eluted at a retention time of 3min. **(b)** Healthy control extract does not show any allantoin. **(c)** The presence of allantoin in lesional epidermis of acute vitiligo was detected at 3 minutes. **(d)** Sample spiked with allantoin standard confirmed the correct retention time. **(e)** The presence of allantoin in non-lesional epidermis of acute vitiligo was detected at 3 minutes). **(f)** Sample

spiked with allantoin standard confirmed the correct retention time. (✱)Uncharacterized peak (under investigation), arrows indicate allantoin peak.

**(B)** Allantoin levels are absent in healthy controls. Levels in lesional skin of patients with vitiligo are significantly higher compared to non lesional skin (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , mean  $\pm$  SD).

#### **4.9 XO can re-activate pterin-inhibited tyrosinase**

Pterins have been reported to bind to XO and inhibit its activity in a concentration-dependent manner (Kalckar, Kjeldgaard et al. 1948; Kalckar and Klenow 1948; Hofstee 1949; Lowry, Bessey et al. 1949; Kalckar, Kjeldgaard et al. 1950; Isaka 1952; Wede, Altindag et al. 1998; Oetl and Reibnegger 1999). As shown by immunostaining, co-localisation of XO with tyrosinase was detected, indicating the presence of this enzyme within melanosomes (Shalbaf, Gibbons et al. 2008). Tyrosinase is the key enzyme in melanogenesis (Lerner, Fitzpatrick et al. 1948) and 6BH<sub>4</sub> forms an inhibitor complex with this enzyme (Wood, Schallreuter-Wood et al. 1995; Wood, Chavan et al. 2004). In vitiligo, due to oxidative stress, 6BH<sub>4</sub> is oxidised to 6-biopterin (Schallreuter, Wood et al. 1994). Therefore this question was raised, what is the function of XO in melanosome? Could XO play a role in pigmentation? To answer these questions a set of experiments were designed, where uric acid production from the reaction between xanthine and XO was spectrophotometrically monitored at 290nm in the presence of different concentrations of pterins.

#### 4.9.1 Inhibition of XO by 7BH<sub>4</sub> and 7-biopterin

As shown earlier, pterins are able to inhibit XO activity but so far there has been no report of inhibiting XO activity by 7BH<sub>4</sub> or by its oxidised form, 7-biopterin. Therefore it was of interest to test whether these pterins have also an inhibitory effect on the activity of XO. The results showed that both pterins are able to inhibit XO activity in a concentration dependent manner. In all reactions catalase was used to remove H<sub>2</sub>O<sub>2</sub> produced during the reaction to avoid allantoin formation (Shalhaf, Gibbons et al. 2008).

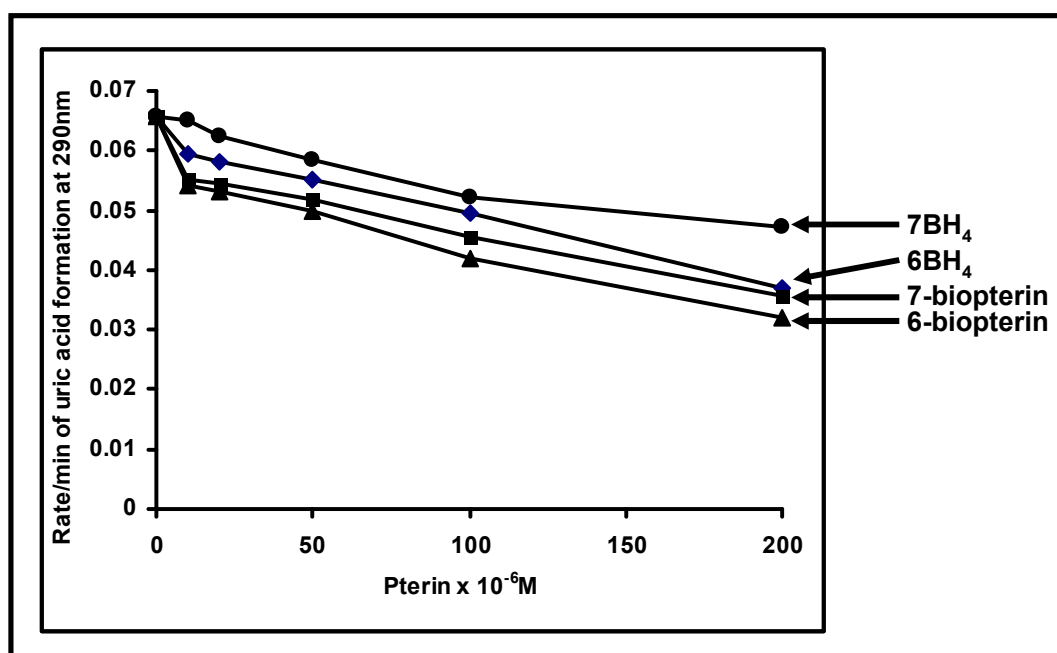


Figure 31

#### Inhibition of XO activity by 6&7BH<sub>4</sub> and their oxidised forms 6 & 7-biopterin

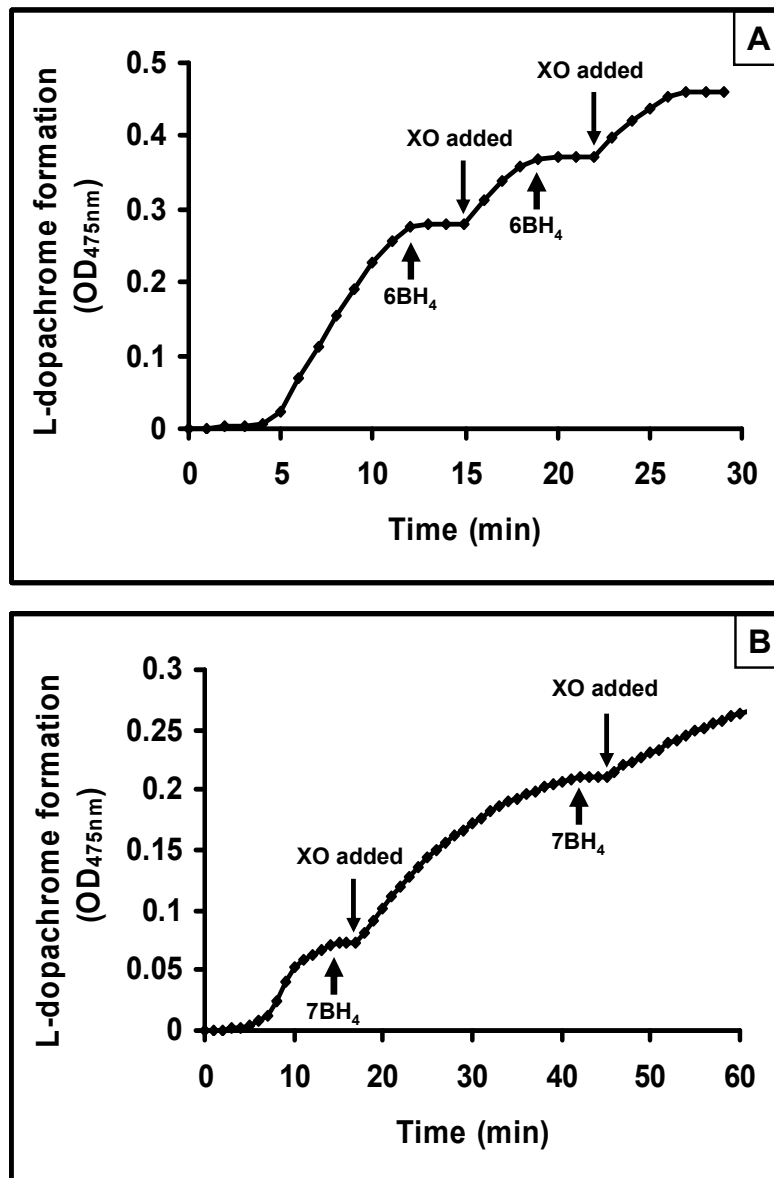
The graph shows that 6-biopterin at 200μM is the strongest inhibitor of XO activity compared to 7-biopterin, 6BH<sub>4</sub> and 7BH<sub>4</sub> (51.3%, 45.8%, 40.5% and 28.3% inhibition, respectively).

For more details please see Materials and Methods' section.



#### **4.9.2 Reactivation of 6&7BH<sub>4</sub>-inhibited tyrosinase by XO**

Since 6BH<sub>4</sub> acts as an uncompetitive inhibitor for the rate limiting enzyme in melanogenesis, tyrosinase (Wood, Schallreuter-Wood et al. 1995; Wood, Chavan et al. 2004) and since XO is present in melanosomes, it was tempting to test whether this protein could possibly reactivate the 6&7BH<sub>4</sub>/tyrosinase inhibitor complex. Therefore we followed dopachrome formation of tyrosinase at 475nm in the presence and absence of XO. The results showed that XO binds 6&7BH<sub>4</sub> from the inhibitor complex leading to reactivation of tyrosinase (**Figures 32; A&B**).



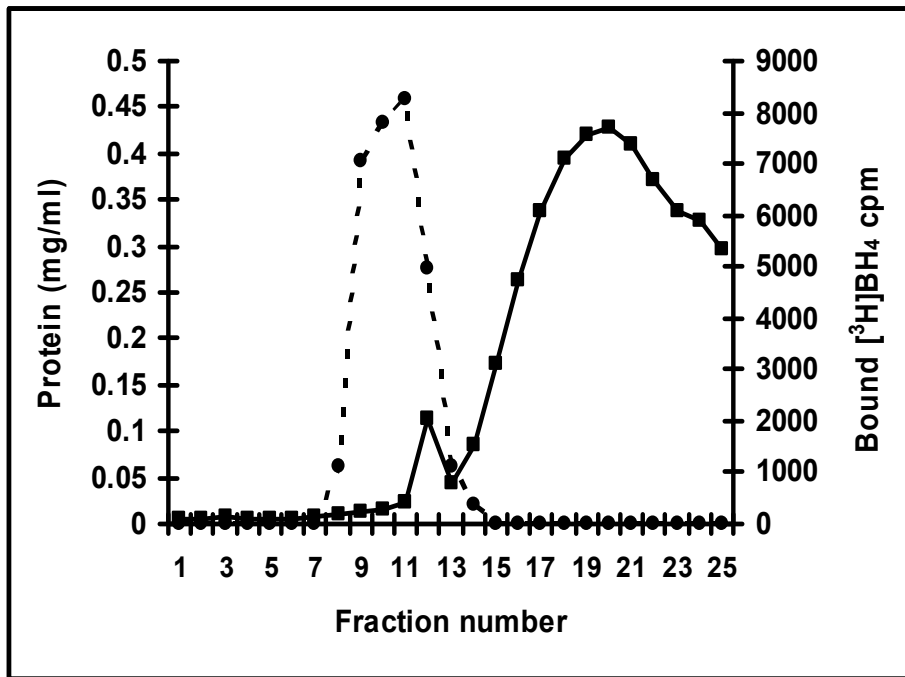
**Figure 32**

**Reactivation of the 6&7BH<sub>4</sub>/tyrosinase inhibitor complex by XO**

(A) XO can to re-activate tyrosinase inhibited by 6BH<sub>4</sub> and (B) 7BH<sub>4</sub>. 84U of tyrosinase and 1mM of L-tyrosine was added together with either 200μM of 6BH<sub>4</sub> (a) or 7BH<sub>4</sub> (b) in a total volume of 1ml 0.05M potassium phosphate buffer pH 7.5. L-dopachrome formation was measured at OD<sub>475nm</sub>. When L-dopachrome formation was inhibited, 100μl of XO (1mg/ml) was added. The figure demonstrates that after addition of XO, tyrosinase activity was recovered (n=2). XO does not react with L-tyrosine (data not shown).

### 4.9.3 XO binds to [<sup>3</sup>H]6BH<sub>4</sub>

The above results imply that XO is able to bind to and remove both 6BH<sub>4</sub> and 7BH<sub>4</sub> from the tyrosinase inhibitory complex. In order to further substantiate our observation we incubated XO with [<sup>3</sup>H]6BH<sub>4</sub>. Our result showed that XO has the ability to bind to the radio-labelled 6BH<sub>4</sub> (**Figure 33**). Importantly, this result is in agreement with earlier data (Kalckar, Kjeldgaard et al. 1948; Kalckar and Klenow 1948; Hofstee 1949; Lowry, Bessey et al. 1949; Kalckar, Kjeldgaard et al. 1950; Isaka 1952; Wede, Altindag et al. 1998; Oetl and Reibnegger 1999). However, at that time there was no radio-labelled 6BH<sub>4</sub> available. This observation is very interesting because it offers an additional mechanism to  $\alpha$ -MSH in control of the 6BH<sub>4</sub>-tyrosinase inhibitor complex (Moore, Wood et al. 1999; Schallreuter, Moore et al. 1999).



**Figure 33**

**Binding of [<sup>3</sup>H]6BH<sub>4</sub> to XO**

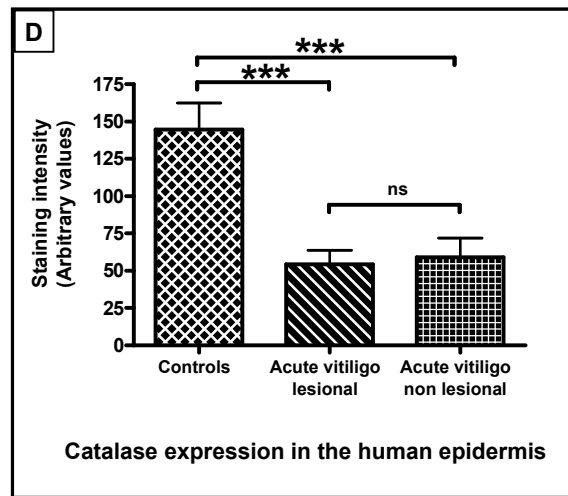
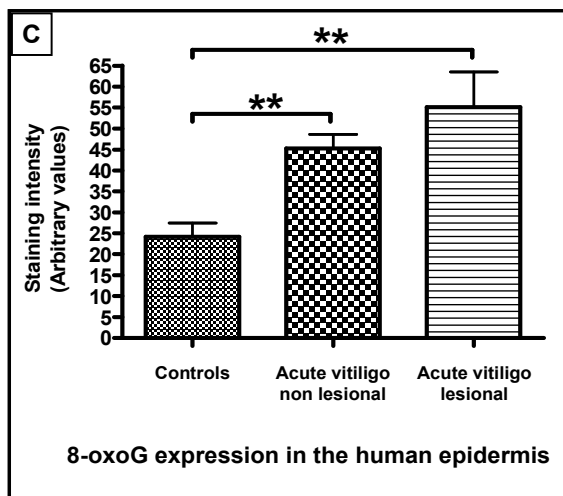
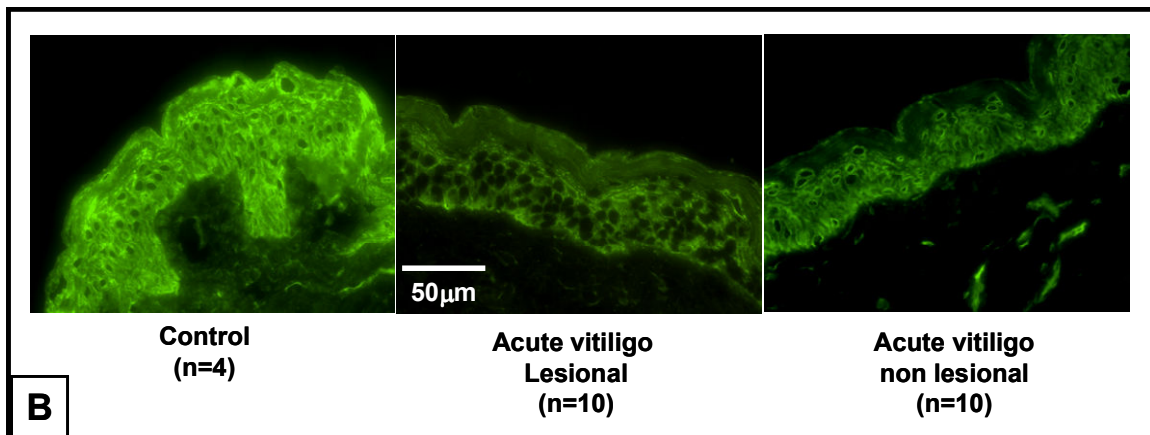
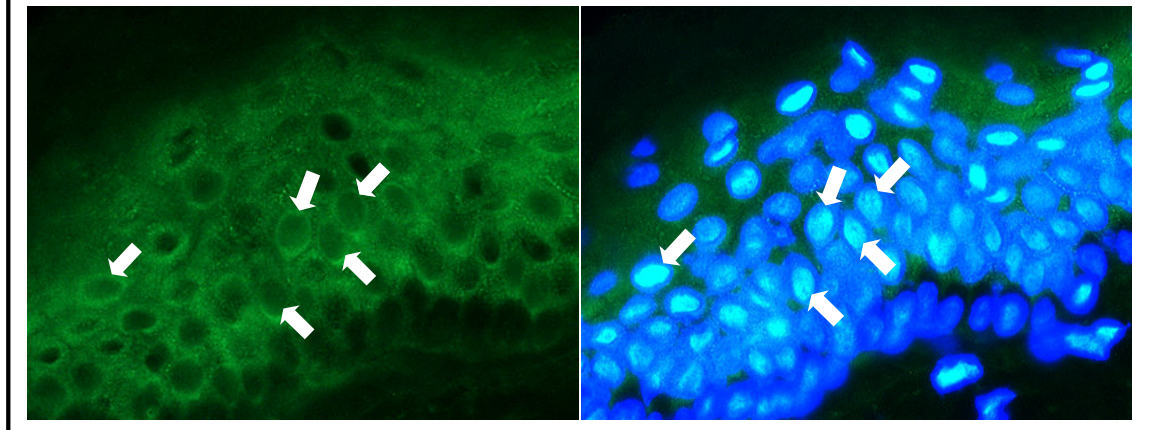
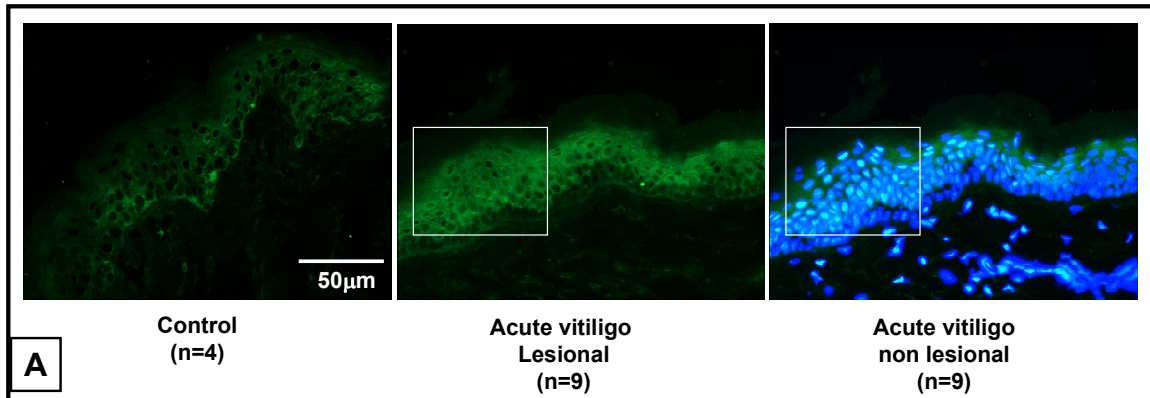
2.5ml of XO solution (4mg/ml) was incubated with 200 $\mu$ M final concentration of cold 6BH<sub>4</sub> containing 5nCi of [<sup>3</sup>H]6BH<sub>4</sub>. The mixture was separated on a G25 sephadex column equilibrated with distilled water. 500 $\mu$ l fractions were collected of which 100 $\mu$ l was counted (—■—) while the remaining fraction was used for protein determination (—●—). The result proves that XO is able to bind 6BH<sub>4</sub> (n=1).

#### **4.10 DNA damage in vitiligo**

Reactive oxygen species (ROS) are formed as by-products of the normal cellular metabolism. Oxygen-derived free radicals have been shown to be a major source for nuclear and mitochondrial DNA damage as they are able to attack DNA leading to the hydroxylation of DNA bases. The most prominent one is 8-oxoGuanine (8-oxoG) (Kasai, Crain et al. 1986; Adelman, Saul et al. 1988; Wagner, Hu et al. 1992; Smith, Marks et al. 2004). Under normal physiological conditions ROS are eliminated by various antioxidants (Smith, Marks et al. 2004). Since the entire epidermis of patients with vitiligo is under oxidative stress due to accumulation of mM H<sub>2</sub>O<sub>2</sub> concentrations (Schallreuter, Moore et al. 1999; Schallreuter 2005; Schallreuter, Bahadoran et al. 2008), DNA damage in epidermal cells would be highly expected in these patients.

#### 4.10.1 Increased *in situ* expression of 8-oxoG in vitiligo

As mentioned earlier, 8-oxoG is the most readily oxidised of the four native deoxyribonucleotides. Therefore it is regarded a ubiquitous and stable biomarker for evaluation of oxidative DNA damage in individuals (Kasai and Nishimura 1984; Loft, Fischer-Nielsen et al. 1993; Steenken and Jovanovic 1997; Bruner, Norman et al. 2000; Kasai 2002; Ichihashi, Ueda et al. 2003; Perra, Maxia et al. 2006). Since the entire epidermis of patients with vitiligo is under constant oxidative stress (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008), increased levels of 8-oxoG was postulated. Our results showed that 8-oxoG levels are indeed increased *in situ* in the epidermis of patients with acute vitiligo compared to healthy controls. Because catalase can be considered as a representative biomarker for H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress (Schallreuter, Bahadoran et al. 2008) individual expression of this protein was used as an internal standard for determining the level of H<sub>2</sub>O<sub>2</sub> in all the patients and controls. The results showed that low catalase levels in lesional and non lesional epidermis of vitiligo are correlating with increased epidermal 8-oxoG expression (**Figure 34; A,B&C**).



**Figure 34:**

**Increased *in situ* expression of 8-oxoG in patients with vitiligo**

**(A):** The figure shows that 8-oxoG levels are increased in acute vitiligo in both lesional and non lesional epidermis compared to controls (skin phototype III, Fitzpatrick classification).

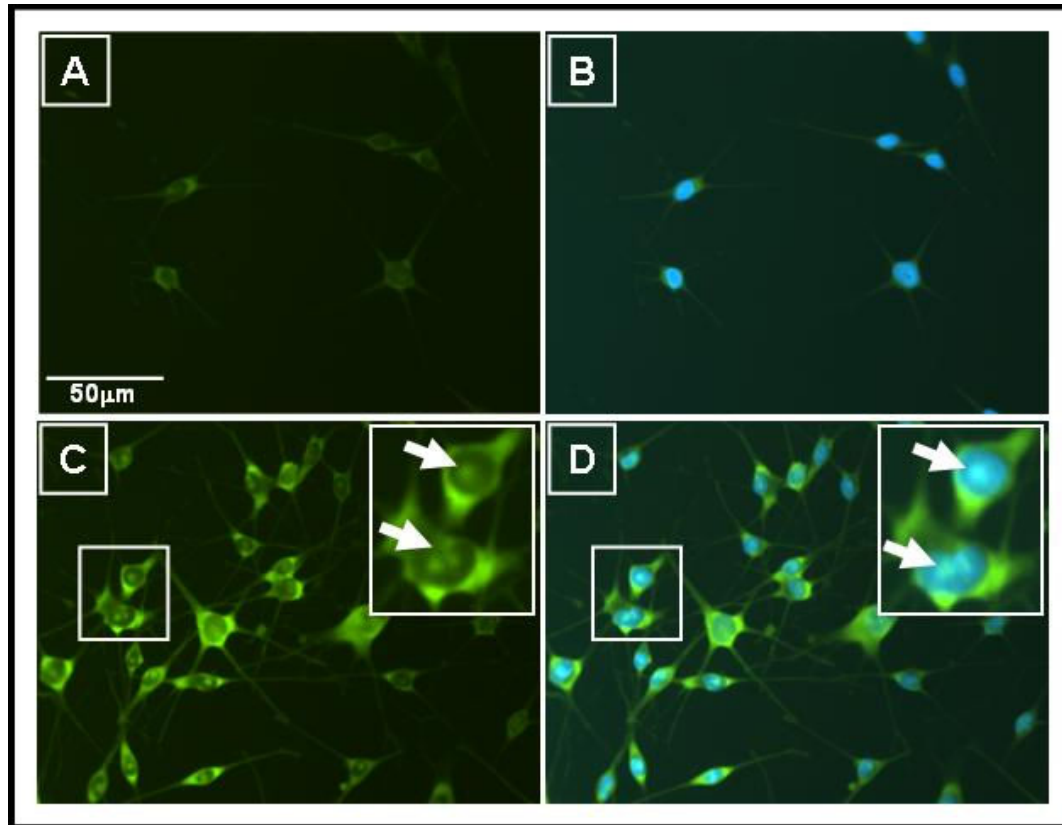
N.B. there is hardly any 8-oxoG present in the controls.

**(C):** Image analysis of staining intensities. The graph shows that 8-oxoG in vitiligo is significantly increased compared to controls indicating the high level of DNA damage caused by the mM level of H<sub>2</sub>O<sub>2</sub>, as correlated to low catalase levels in the same patients **(B&D)** (\*\*\*) (\*\*\* p<0.001, \*\* p<0.01, mean ± SD).



#### 4.10.2 Increased *in vitro* expression of 8-oxoG in vitiliginous MCs

The *in situ* results showed that 8-oxoG levels were significantly increased throughout the human epidermis. In order to further explore under *in vitro* conditions, we used cultured vitiliginous MCs originated from lesional skin of acute vitiligo. The staining showed that 8-oxoG levels were even increased vitiliginous MCs in the nucleus and cytoplasm compared to healthy MCs (**Figure 35; A,B,C&D**). This result is in agreement with the *in situ* observations, suggesting a control cellular problem and not just a regular response.



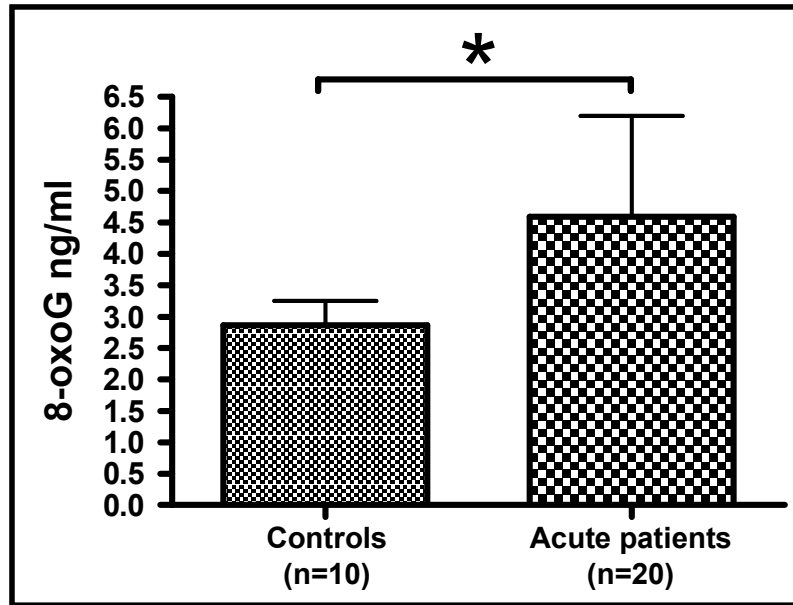
**Figure 35**

**Increased *in vitro* expression of 8-oxoG in MCs originated from lesional vitiligo**

(A): FITC staining of 8-oxoG in healthy MCs; (B): overlay of DAPI and FITC; (C): overexpression of 8-oxoG in cultured vitiliginous MCs in the cytoplasm and in the nucleus (inset); (D): overlay of DAPI and FITC, shows the co-localisation of 8-oxoG with DAPI in the nucleus (inset). The result shows that 8-oxoG levels are increased even under *in vitro* conditions compared to control.

#### 4.10.3 Increased plasma levels of 8-oxoG in vitiligo

Once 8-oxoG is formed, it is excised from the damaged DNA (Chung, Kasai et al. 1991; Chung, Kim et al. 1991; Tchou, Kasai et al. 1991; Boiteux 1993; Boiteux and Radicella 1999) and it is finally excreted in the urine without being further metabolised (Wu, Chiou et al. 2004). Therefore determination of 8-oxoG levels in different tissues, blood and also urine allows to monitor the extent of oxidative DNA damage (Nakano, Kawanishi et al. 2003; Wu, Chiou et al. 2004; Dincer, Erzin et al. 2007). Inspired by our *in situ* and *in vitro* findings, we wanted to know whether plasma levels of this compound would be increased in vitiligo patients. Using ELISA technique, our results revealed that plasma levels of 8-oxoG were significantly increased in all 20 patients examined in our experiment compared to the controls (**Figure 36**). Taken together, our results indicate that this patient group deals with significant DNA damage due to H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress.



**Figure 36**

**Increased plasma levels of 8-oxoG in vitiligo**

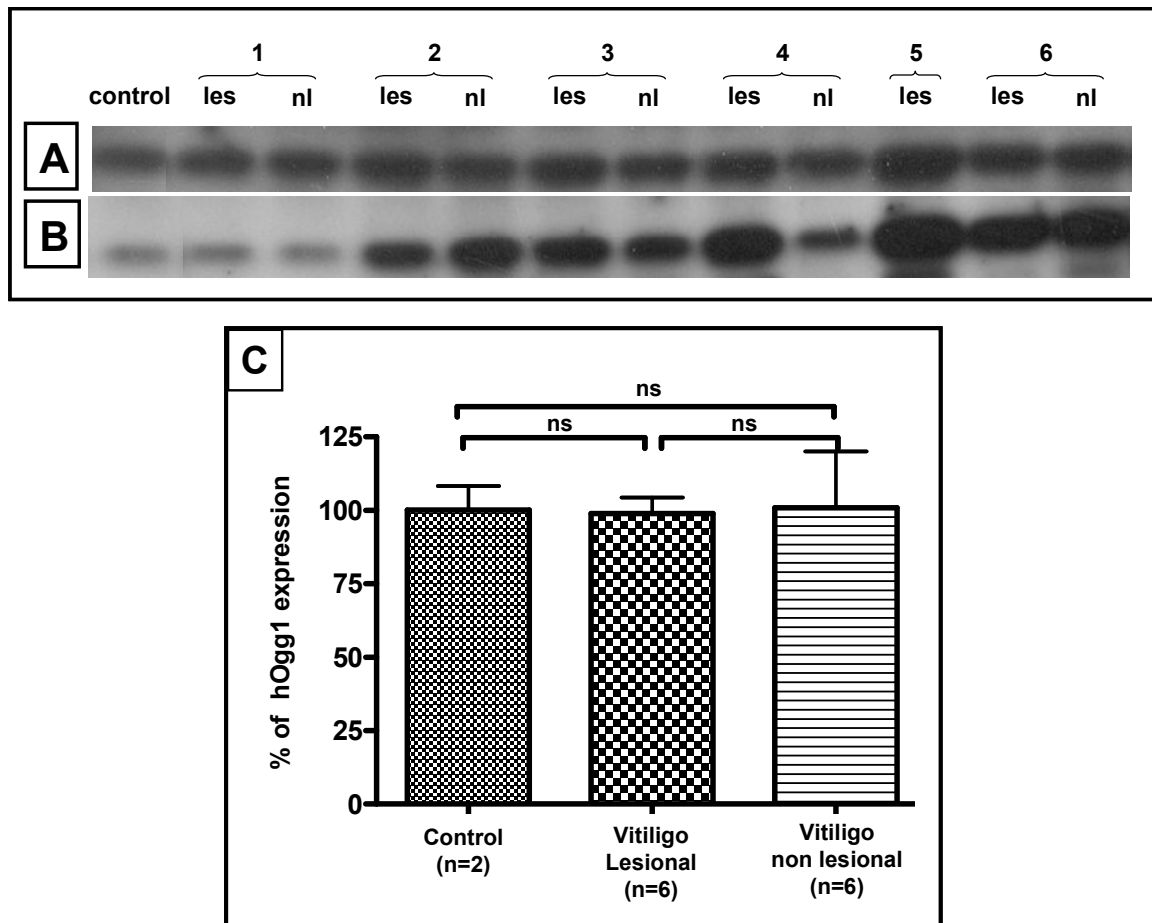
The figure shows that 8-oxoG levels is significantly increased in the plasma of acute vitiligo (n=20) compared to the controls (n=10) (\* p<0.05, mean  $\pm$  SD).

## 4.11 DNA repair in vitiligo

Genomic stability is highly controlled amongst living organisms; hence most organisms have evolved DNA repair mechanisms. Oxidative damage to DNA is repaired in living cells mainly via base-excision repair (BER) mechanisms (Boiteux and Radicella 1999).

### 4.11.1 hOgg1 expression in vitiligo

Once 8-oxoG is formed on DNA strands under oxidative stress, it has to be removed. This base lesion is recognised and excised from the damaged DNA by a glycosylase called hOgg1 initiating BER pathways (Boiteux 1993; van der Kemp, Thomas et al. 1996; Boiteux and Radicella 1999; Boiteux and Radicella 2000; Fortini, Pascucci et al. 2003; David, O'Shea et al. 2007). Consequently it was decided to investigate the expression of hOgg1 in epidermal cells in vitiligo. Western blot analysis proved the presence of this important enzyme in vitiligo. Notably, the results revealed that hOgg1 expression was not increased in vitiligo compared to controls (**Figure 37; A,B&C**). This result suggests that the expression of hOgg1 protein is independent of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in vitiligo. Our results are in agreement with previously published data by Saitoh et al. in 2001 (Saitoh, Shinmura et al. 2001; Fortini, Pascucci et al. 2003). These data emphasise the absolute importance of hOgg1 / BER for cell survival.



**Figure 37**

**Protein expression of hOgg1 in human epidermal suction blister tissue cell extract**

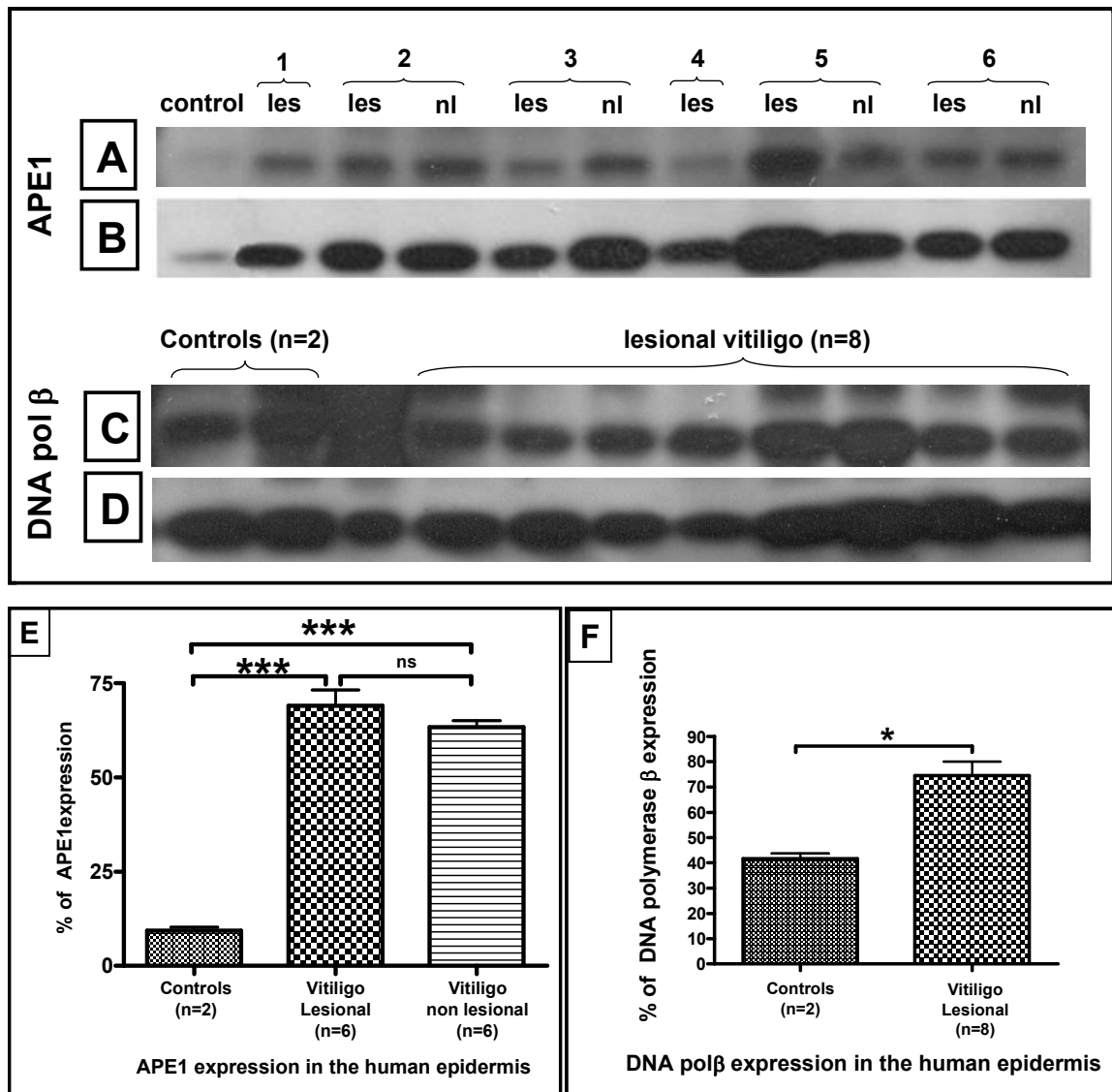
(A): hOgg1 protein is expressed in vitiliginous epidermal cell extracts (n=6) as well as in control (n=2). hOgg1 expression is not increased in vitiligo compared to controls ( $p>0.05$ ).

The numbers reflect the number of patients. (B): GAPDH staining of the same membrane to show protein loading. (c): Densitometry of the bands was performed to determine the percentage of hOgg1 expression related to GAPDH expression for each band (Plots are mean  $\pm$  SD).

From these results we can conclude that despite increased 8-oxoG levels (oxidative DNA damage) in the epidermis as well as in the plasma of the patients with vitiligo, the efficient BER mechanisms seems to be intact.

#### **4.11.2 Increased epidermal expression of APE1 and DNA polymerase $\beta$ in vitiligo**

In order to substantiate the presence of an effective BER mechanism as shown by the presence of an unaltered hOgg1, we wanted to explore DNA polymerase $\beta$  as well as APE1. It has been reported that a core of four enzymes are required to complete the repair of 8-oxoG. This includes besides hOgg1, APE1, DNA polymerase $\beta$  and DNA ligase I (Parikh, Mol et al. 1997; Fortini, Parlanti et al. 1999; Fortini, Pascucci et al. 2003; Chaudhry 2007). To address this scenario in more detail, again we utilised Western blot analysis of cell extracts from epidermal suction blister tissues from both controls and vitiligo patients. Our results revealed increased protein expression of these two enzymes.



**Figure 38**

**Increased APE1 and DNA polymerase $\beta$  expression in vitiligo**

(A): APE1 protein expression in vitiligo cell extracts and in controls. (C): DNA polymerase $\beta$  protein expression in vitiligo and in controls. The numbers reflect the number of patients (B&D): GAPDH staining of the membranes refers to protein loading. (E&F): Evaluation of the bands was correlated to loaded protein (GAPDH staining). The results show that both enzymes are over expressed in vitiligo compared to controls (\*\* p<0.01, mean  $\pm$  SD).



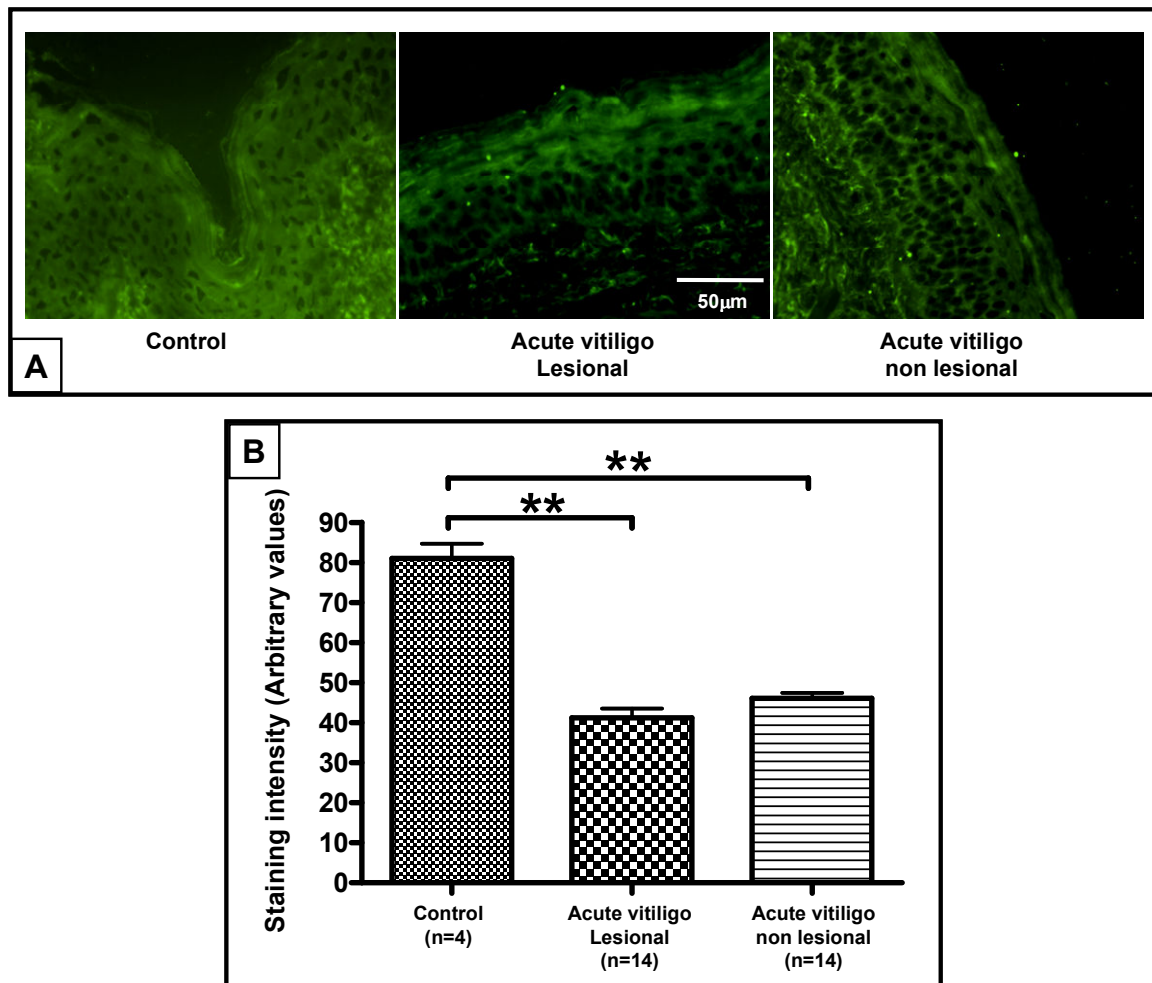
Taken together we have shown that patients with vitiligo have considerable DNA damage evidenced by increased 8-oxoG. Moreover, this patient group has the capacity to repair this DNA damage via hOgg1, APE1 and DNA polymerase $\beta$ . These results indicate the presence of efficient DNA repair machinery in patients with vitiligo.

#### **4.12 More support for absence of increased apoptosis in vitiligo**

DNA-damage responses are very complex involving numerous signals, proteins and specific cellular pathways including DNA repair mechanisms, cell cycle check points, cellular senescence and apoptosis (Schmitt, Paquet et al. 2007). Since epidermal cells have to cope with mM levels of H<sub>2</sub>O<sub>2</sub> in patients with vitiligo (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008) and the results from this thesis showed that epidermal DNA damage is increased in these patients, the question of interest was how do these patients handle this DNA damage besides DNA repair as shown before. In this context it was reported that epidermal functioning wild-type p53 is increased in vitiligo (Schallreuter, Behrens-Williams et al. 2003). Considering the major role of this protein in DNA repair and its regulatory role in apoptosis, it was decided to investigate a number of the apoptotic/non apoptotic epidermal cell responses in these patients despite several authors showed no evidence for increased apoptosis in vitiligo (van den Wijngaard, Aten et al. 2000). However, since the presence of increased apoptosis has also been shown by some other authors (Boissy and Nordlund 1997; Kemp, Waterman et al. 2001; Huang, Nordlund et al. 2002; Boissy and Manga 2004; Park, Kim et al. 2007; Ruiz-Arguelles, Brito et al. 2007; Song, Xu et al. 2008), it was decided to re-address this issue.

#### **4.12.1 Decreased *in situ* epidermal cytochrome c and caspase 3 expression in vitiligo**

To shed some more light on our findings, we turned our interest to epidermal cytochrome c and caspase 3 expression. Cytochrome c is an important intermediate in apoptosis. Following exposure to apoptotic stimuli, cytochrome c is rapidly released from the mitochondria into the cytosol, an event which is required for completion of apoptosis (Liu, Kim et al. 1996; Cai, Yang et al. 1998; Skulachev 1998). In turn, cytosolic cytochrome c functions in the activation of caspase 3, where it is a key effector of apoptosis (Boldin, Varfolomeev et al. 1995; Tewari, Quan et al. 1995; Kuida, Zheng et al. 1996; Abu-Qare and Abou-Donia 2001). Within this study, we evaluated cytochrome c and caspase 3 levels in the skin of patients with vitiligo as well as controls using immunofluorescence reactivity. Our *in situ* results showed that both epidermal cytochrome c and caspase 3 levels in vitiliginous epidermis of patients with acute vitiligo are significantly lower than controls.



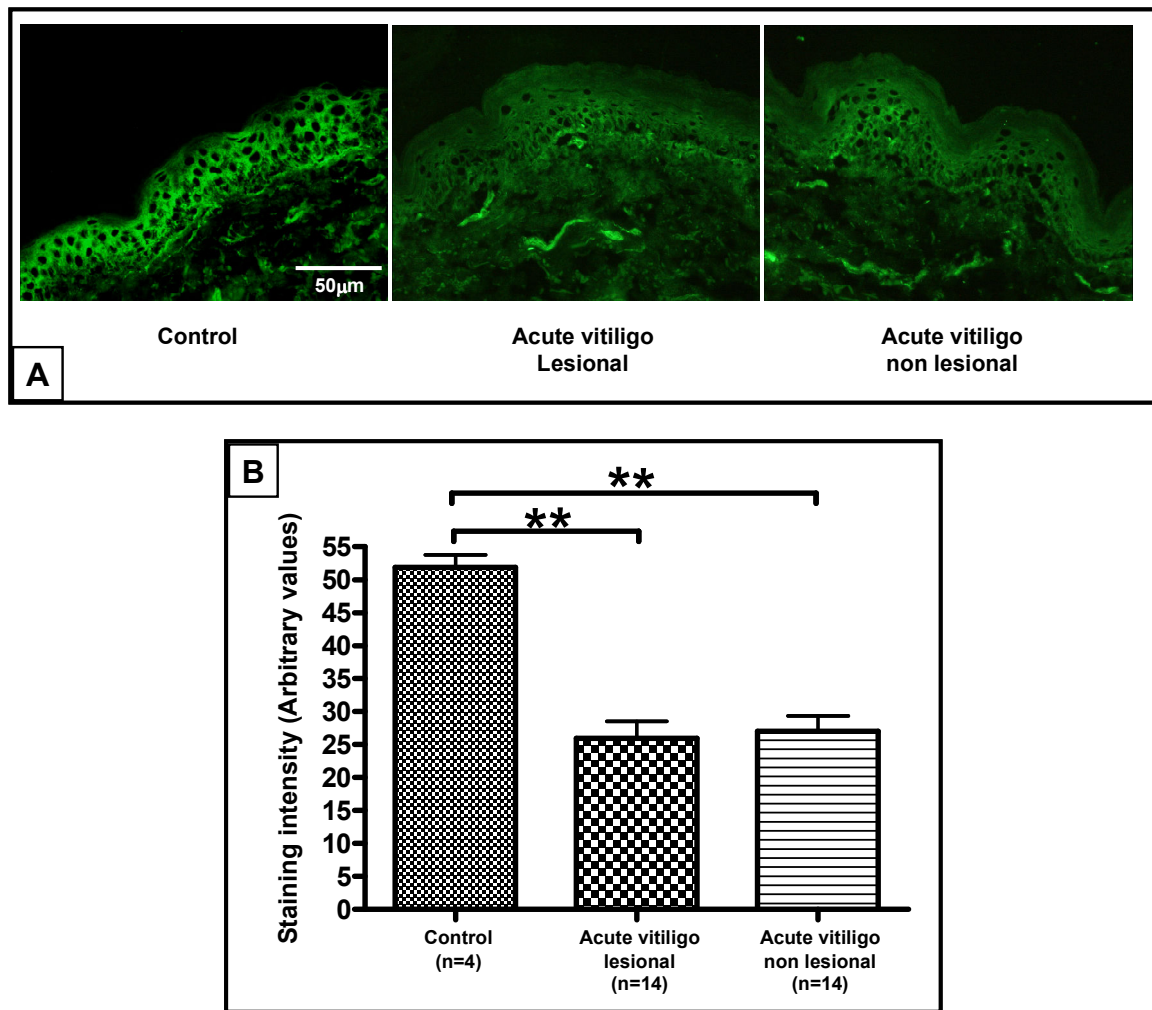
**Figure 39**

***In situ* expression of cytochrome c in both healthy skin and patients with vitiligo (skin phototype III, Fitzpatrick classification)**

**(A):** Decreased *in situ* expression of cytochrome c in vitiligo compared to control (skin phototype III, Fitzpatrick classification). The figure shows that cytochrome c levels are lower in both lesional and non lesional skin of patients with acute vitiligo (+H<sub>2</sub>O<sub>2</sub>) than controls.

The presence of H<sub>2</sub>O<sub>2</sub> was verified by low catalase level in these patients (Figure 33; C&D).

**(B):** Image analysis reveals significantly lower levels of cytochrome c in acute vitiligo compared to the controls ((\*\* p<0.05, mean ± SD).



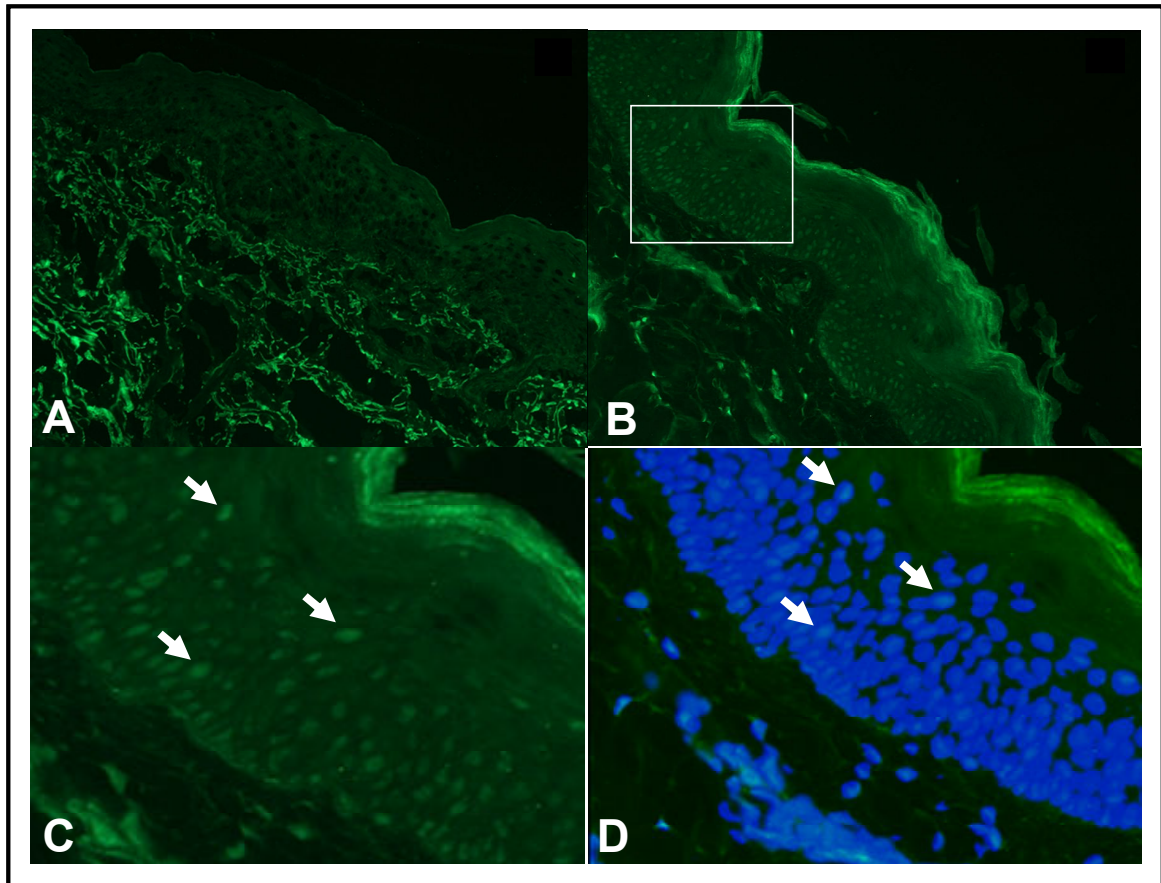
**Figure 40**

**Decreased *in situ* expression of caspase 3 in patients with vitiligo (skin phototype III, Fitzpatrick classification)**

**(A):** Caspase 3 levels are lower in the epidermis of acute vitiligo compared to controls. **(B):** Image analysis shows significantly decreased caspase 3 levels in acute vitiligo compared to controls (\*\*  $p < 0.05$ , mean  $\pm$  SD). The presence of  $H_2O_2$  was monitored by low catalase expression (Figure 33; C&D).

#### 4.12.2 Increased expression of Gadd45 $\alpha$ in vitiligo

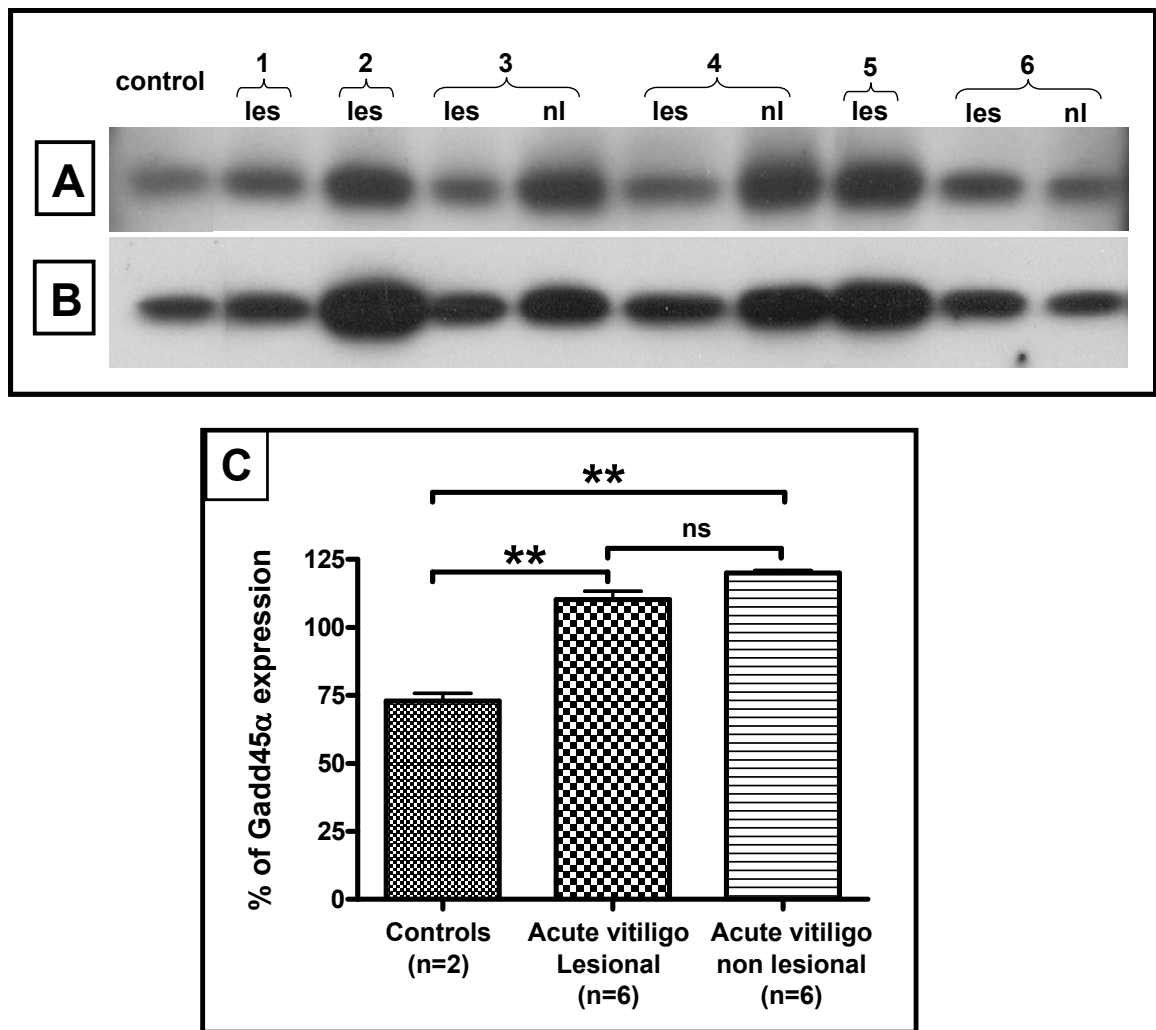
p53 plays a critical role in the normal cell's response to a variety of stress stimuli (Harris 1996). In response to genomic stress including DNA damage, this multifunctional protein is activated and in turn transactivates a series of its downstream effector genes involved in cell cycle arrest and DNA repair (including p21 and Gadd45 $\alpha$ ) and also apoptosis (including Bcl-2 and Bax) (Harris 1996). Gadd45 is a nuclear protein and it functions during cell cycle checkpoint in G2/M, DNA repair and in the apoptotic pathway (Fornace, Jackman et al. 1992; Wang, Zhan et al. 1999; Sheikh, Hollander et al. 2000; Maeda, Hanna et al. 2002; Zhan 2005). Earlier it has been shown that Gadd45 $\alpha$  is involved in the induction of G2/M cell cycle checkpoint following UVB irradiation in epidermal KCs, MCs and also in melanoma cells (Maeda, Hanna et al. 2002; Pedeux, Lefort et al. 2002). Based on these results, it has been suggested that the activation of Gadd45 $\alpha$  in MCs and melanoma cells may play a crucial role for their survival due to their ability to efficiently repair UVB-induced lesions (Pedeux, Lefort et al. 2002; Fayolle, Pourchet et al. 2006; Fayolle, Pourchet et al. 2008). In order to find out whether epidermal cells in vitiligo express Gadd45 $\alpha$  in response to oxidative stress, we investigated the presence of this protein in patients' skin. Our results show for the first time that Gadd45 $\alpha$  is indeed expressed *in situ* using immunoreactivity and Western blotting.



**Figure 41**

**Gadd45 $\alpha$  is present throughout the epidermis and in the nucleus**

(A): FITC-labelled Gadd45 $\alpha$  in healthy controls in which no Gadd45 $\alpha$  co-localisation is observed (n=3) (B): positive Gadd45 $\alpha$  staining (FITC) in the human epidermis, and inset, shows the expression of this protein in the nucleus (C&D): Enlargement of the marked area, shows Gadd45 $\alpha$ 's localisation and its co-localisation with DAPI in the nucleus (n=5) (magnification x 200).



**Figure 42**

**Western blot analysis of Gadd45α expression in vitiligo**

**(A):** The expression of Gadd45α in cell extracts from epidermal suction blister tissue of both healthy controls and vitiligo patients. The numbers reflect the number of patients. **(B):** GAPDH staining of the same membrane and **(C):** Densitometry of the bands was performed in correlation with Gadd45α levels and the loaded protein (GAPDH staining). The graph shows that Gadd45α is significantly overexpressed in lesional vitiligo compared to controls and non lesional epidermis (Plots are mean ± SD).



## 5.0 DISCUSSION

### 5.1 The presence of XO in the human epidermis and its regulation by H<sub>2</sub>O<sub>2</sub>

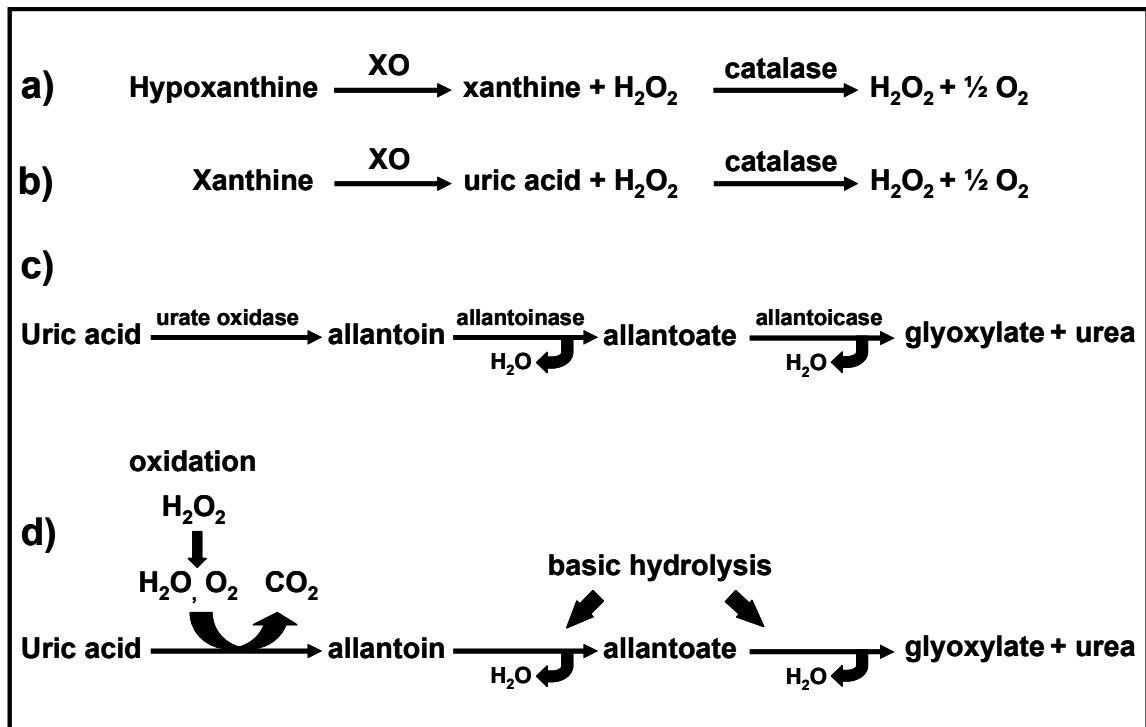
The role of ROS in the pathogenesis of various diseases has been the subject of intensive research during the past decades. In recent years vitiligo has been emerged as a biological model disease for epidermal oxidative stress (Schallreuter, Wood et al. 1991; Maresca, Roccella et al. 1997; Schallreuter 1999; Schallreuter, Moore et al. 1999; Dell'Anna, Maresca et al. 2001; Dell'Anna, Urbanelli et al. 2003; Hasse, Gibbons et al. 2004; Dell'Anna, Ottaviani et al. 2006; Dell'anna and Picardo 2006; Dell'Anna, Ottaviani et al. 2007; Maresca, Flori et al. 2008; Schallreuter, Bahadoran et al. 2008). The presence of mM H<sub>2</sub>O<sub>2</sub> concentrations in the epidermis of these patients, low catalase levels and impaired back-up systems provide ample evidence for this hypothesis contributing in turn to the pathogenesis of this disease (Schallreuter, Pittelkow et al. 1986; Schallreuter, Hordinsky et al. 1987; Schallreuter, Wood et al. 1991; Beazley, Gaze et al. 1999).

XO has been implicated in free radical-induced damage in a multitude of human diseases because this enzyme plays a major role in generating ROS (H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>•-</sup>). O<sub>2</sub><sup>•-</sup> is generated during the enzyme reaction and thereafter it is converted to H<sub>2</sub>O<sub>2</sub> via the action of superoxide dismutase to via spontaneous disproportionation. OH<sup>•</sup> is generated via the Fenton- or Haber-Weiss reaction (Manson, Anthenelli et al. 1983; Im, Shen et al. 1984; Chambers, Parks et al. 1985; Parks and Granger 1986; Picard-Ami, MacKay et al. 1991; Glantzounis, Tsimoyiannis et al. 2005). Therefore the

presence and activity of this enzyme in epidermal cells was of two-fold interest, in general and in the context of vitiligo. In order to assess XO activity in human MC and KC cell extracts, we developed and utilised a very sensitive TLC method using [<sup>14</sup>C]xanthine. The separation of xanthine and uric acid on the chromatogram is shown in **Figure 22**. One example of the standard assay is shown in **Figure 23**. The result shows that in the absence of catalase [<sup>14</sup>C]uric acid formation increases up to 5 min, followed by a decrease, implying oxidation of uric acid to allantoin by H<sub>2</sub>O<sub>2</sub> produced during the reaction. This assumption is supported by the increase in uric acid formation in the presence of catalase.

To the best of our knowledge, we here demonstrated for the first time that mRNA expression (**Figure 7**), protein expression (**Figures 8, 9, 10, 11&12**) and enzyme activity (**Figure 29**) of XO are present in both epidermal MCs and KCs. In melanocytes XO is predominantly distributed around the nucleus in a granular pattern (**Figure 9b**) where it co-localises with tyrosinase suggesting that XO is present in melanosomes (**Figure 9; c,d&e**). **Figure 11** demonstrates *in situ* that XO protein expression in vitiligo is not different compared to control. This result indicates that XO protein expression is not altered in vitiligo supporting that XO expression is not affected by H<sub>2</sub>O<sub>2</sub>. Also, our kinetic results presented in this thesis showed that XO functionality was only partially affected under severe oxidative condition since there is only one Trp residue in the flavin ring of FADH<sub>2</sub>, one Met residue in the moco centre and no Met/Trp residue in the active site of XO as shown in Figures 26, 27 & 28. Met and Trp are the most sensitive amino acids to H<sub>2</sub>O<sub>2</sub>-mediated oxidation. Therefore the presence of allantoin in the epidermis of patients with vitiligo was expected. Our results supported this assumption, as we identified significantly higher levels of allantoin in the lesional and non-lesional epidermis of all patients examined,

whereas this metabolite was absent in healthy controls (**Figure 30**). Since humans lack the enzyme uricase converting uric acid to allantoin, thus the formation of allantoin from uric acid can only be attributed to H<sub>2</sub>O<sub>2</sub> (**Figure 43**). Taken together, to our knowledge, this is the first time that the presence and activity of XO has been demonstrated in human epidermal MC and KC cell extracts (**Figure 29A**). It appears that XO activity is higher in KCs compared to MCs (**Figure 29B**).



**Figure 43**

**The final two steps in purine catabolism pathway**

XO catalyses the oxidation of hypoxanthine to xanthine and further to uric acid. These reactions are accompanied by the formation of 2 molecules H<sub>2</sub>O<sub>2</sub> as by-product. Under physiological conditions catalase removes H<sub>2</sub>O<sub>2</sub> and uric acid is excreted in the urine **(a&b)**.

Further metabolism of uric acid in mammals, turtles and molluscs proceeds from uric acid to allantoin by urate oxidase. Allantoin is hydrolysed to allantoate by allantoinase followed by further hydrolysis to glyoxylate and urea by allantoicase (Newsholm and Leech 1984) **(c)**.

In humans, under oxidative conditions, as observed in vitiligo, uric acid is oxidised by H<sub>2</sub>O<sub>2</sub> to allantoin (Grootveld and Halliwell 1987; Kaur and Halliwell 1990; Benzie, Chung et al. 1999; Yardim-Akaydin, Sepici et al. 2004; Yardim-Akaydin, Sepici et al. 2006). Allantoin is hydrolysed to allantoate and the latter to glyoxylate and urea (Newsholm and Leech 1984) **(d)**.

## 5.2 The presence of allantoin underlines oxidative stress in vitiligo

This is the first study investigating the levels of allantoin in vitiligo. Our data add to the list of oxidative stress in this disease (Shalhaf, Gibbons et al. 2008). The presence of epidermal allantoin in acute vitiligo mirrors and emphasises the involvement of H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress in this disease and also highlights the role of uric acid as an important antioxidant in humans because it can directly react with free radicals to produce relatively stable products (Ames, Cathcart et al. 1981; Kaur and Halliwell 1990; Becker 1993; Glantzounis, Tsimoyiannis et al. 2005). Therefore XO itself can contribute to ROS scavenging via the production of uric acid. It is also possible that uric acid is involved in scavenging of peroxynitrite (ONOO<sup>-</sup>) which has been extensively shown (Hooper, Spitsin et al. 1998; Kean, Spitsin et al. 2000). Since the epidermis of patients with vitiligo generates NO in 10<sup>-6</sup>M range via NOS, this could be a relevant mechanism (Schallreuter, Bahadoran et al. 2008). However, since oxidative stress via H<sub>2</sub>O<sub>2</sub> and ONOO<sup>-</sup> is presenting lesional and non-lesional skin, it still needs to be shown, why some patches are white, while others remain pigmented. Our other observation i.e. melanocyte detachment in vitiligo cannot be explained by our data (Gauthier, Cario Andre et al. 2003). Whether this detachment is specific for vitiligo is not yet clear. We have data that detachment of melanocytes can also be observed in healthy individuals (Schallreuter, unpublished data).

**Figure 24** demonstrates the separation of allantoin by TLC. The oxidation of uric acid to allantoin by H<sub>2</sub>O<sub>2</sub> in the absence of catalase is shown in **Figure 25**. After an initial increase in allantoin formation, the production of this metabolite decreases over time. Allantoin is further converted to allantoate and allantoate to glyoxylate and urea via

basic hydrolysis (**Figure 43**). However, unfortunately allantoate and glyoxylate do not have any UV-absorption, hence they are not detectable by UV light. Therefore it is not possible to measure these products using this separation system.

### 5.3 XO is inhibited by its own substrate

Spectrophotometric studies of XO activity with different substrate concentrations in the absence of catalase revealed that increase in the concentration of substrate decreases the formation of reaction product (**Figure 14**). As discussed earlier,  $H_2O_2$  is formed during the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid (**Figure 43**). Our result suggests that the decrease in the reaction product could be based on its oxidation to allantoin by  $H_2O_2$  (Newsholm and Leech 1984), or inhibition of the enzyme or a combination of both. The cause of the decrease in uric acid formation in the above experiment was confirmed after addition of catalase to remove  $H_2O_2$  produced in the reaction (**Figure 15**). The result revealed that XO activity was inhibited in a concentration dependent manner by the substrate, proving substrate inhibition of XO activity. However, XO inhibition by its substrate is observed in the presence of very high and non-physiological xanthine concentrations. This is a novel finding on the regulation of XO activity.

## 5.4 FADH<sub>2</sub> is directly affected by H<sub>2</sub>O<sub>2</sub> in XO

As mentioned earlier, unique information about the structure of both native and oxidised XO can be obtained from UV/visible spectroscopy (Hille and Nishino 1995; Stockert 2004 ). The UV/visible spectra of purified H<sub>2</sub>O<sub>2</sub>-oxidised XO (1.5M H<sub>2</sub>O<sub>2</sub>) showed that FADH<sub>2</sub> is oxidised to FAD under those conditions. In the presence of lower H<sub>2</sub>O<sub>2</sub> concentrations we could not appreciate the signal. Hence, we can conclude that only high non-physiological concentrations of H<sub>2</sub>O<sub>2</sub> can affect the FADH<sub>2</sub> cofactor of XO (**Figures 19&20**). These results are in agreement with previous work from Hille and Nishino (Hille and Nishino 1995). The UV/visible spectroscopic study of XO revealed that the maximum absorbance at 450nm is due both to the oxidised Fe-S and the FAD centres. However, the molybdopterin domain's absorbance (500-600nm) is too small (Hille and Nishino 1995; Stockert 2004 ). As mentioned earlier, Fe-S centres in the structure of the enzyme are coordinated to four cysteine residues, close to the FAD centre and these play a role in electron transfer derived from the substrate (Olson, Ballou et al. 1974; Kisker, Schindelin et al. 1997; Enroth, Eger et al. 2000). The oxidation of the cysteine residues coordinated to Fe-S centres by H<sub>2</sub>O<sub>2</sub> as well as the oxidation of FADH<sub>2</sub> to FAD can cause of the changes in the UV/visible spectra of the oxidised XO. Subsequently, the oxidation of the Fe-S centres and FADH<sub>2</sub> co-factor by H<sub>2</sub>O<sub>2</sub> could lead to malfunction in the intramolecular electron transfer of XO as it was shown in Figure 18. Since constantly H<sub>2</sub>O<sub>2</sub> levels are in the 10<sup>-3</sup>M range in acute vitiligo, this mechanism could indeed take place.



## 5.5 H<sub>2</sub>O<sub>2</sub> directly affects XO structure

In order to further investigate whether H<sub>2</sub>O<sub>2</sub> alters the structure of XO, we utilised computer molecular modelling. Using this tool, all the Met and Trp amino acid residues around the FADH<sub>2</sub> co-factor, moco and active site of XO were oxidised to methionine sulfoxide (MetSO) and 5-OH-Trp, respectively. Then the oxidised binding domains were compared to the native states and the changes were analysed. The analysis revealed that oxidation of the most noticeable Trp around the FADH<sub>2</sub> binding site (Trp<sup>336</sup>) disrupts  $\pi$ - $\pi$  stacking of Phe<sup>337</sup> and the H-bonding network to a small extent (**Figure 26; A&B**). It seems that this little change does not significantly affect the affinity of XO for FADH<sub>2</sub>, but it may affect the vulnerability of FADH<sub>2</sub> to oxidation. Moreover, this analysis showed that the oxidation of Met and Trp residues does not adversely influence the moco-cofactor binding site, whereas oxidation of Met and Trp residues around the active site causes shifts in the positions of several amino acid residues, affecting reaction rate and catalysis (**Figures 27&28**).

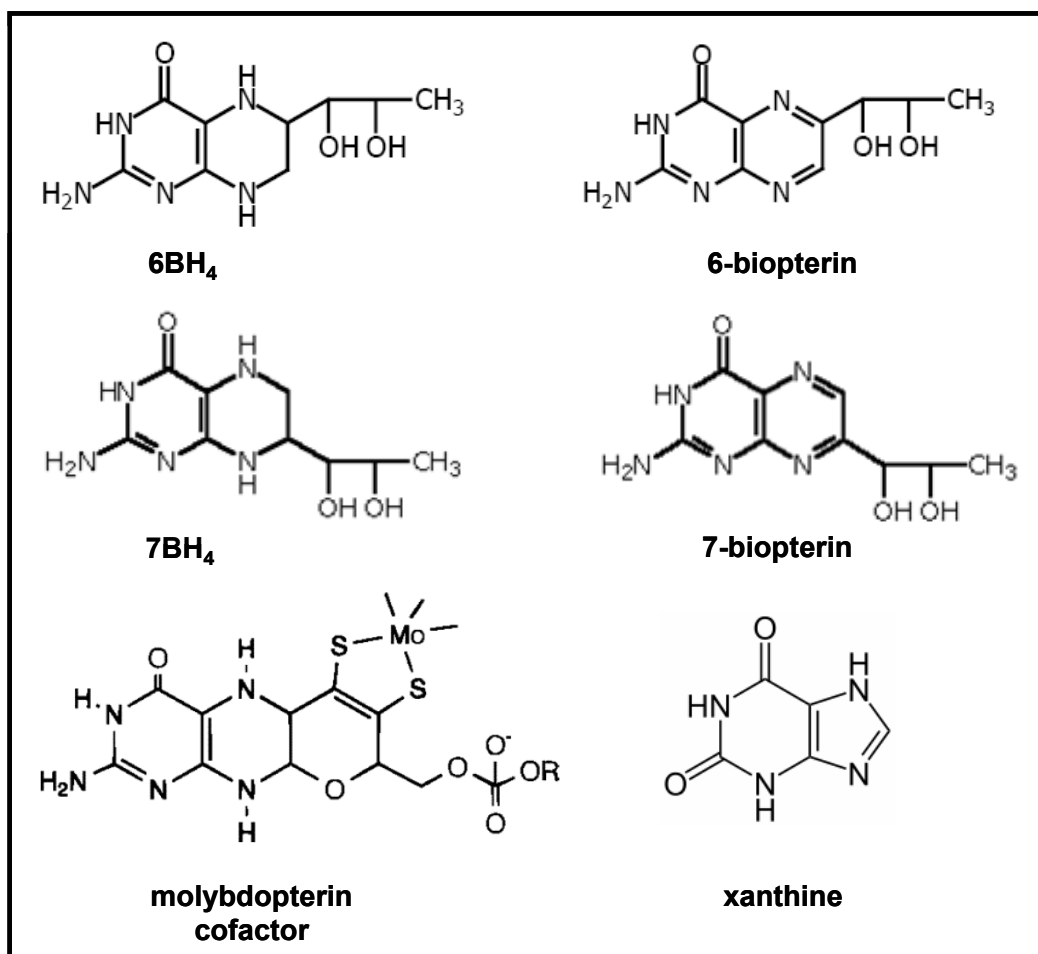
Taken together, these data strongly support our former results.

## 5.6 Inhibition of XO activity by 6/7BH<sub>4</sub> and 6/7-biopterin and binding of 6/7BH<sub>4</sub> to XO: A novel mechanism in regulation of melanogenesis?

Earlier studies have already shown that pteridines are able to inhibit XO activity in a concentration-dependent manner (Kalckar, Kjeldgaard et al. 1948; Kalckar and Klenow 1948; Hofstee 1949; Lowry, Bessey et al. 1949; Kalckar, Kjeldgaard et al. 1950; Isaka 1952; Wede, Altindag et al. 1998; Oettl and Reibnegger 1999). Amongst all pterins, 6-biopterin, the oxidation product of the ubiquitous cofactor (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH<sub>4</sub>) is the most potent inhibitor of XO (Wede, Altindag et al. 1998).

However, to date, there has been no report on inhibition of XO by 7BH<sub>4</sub> and/or 7-biopterin. Within this study, we showed for the first time that both the reduced as well as the oxidised 7-isomer of 6BH<sub>4</sub> are able to inhibit XO activity in a concentration dependent manner (**Figure 31**). The results confirmed that 6-biopterin is the strongest inhibitor of XO followed by 7-biopterin, 6BH<sub>4</sub> and 7BH<sub>4</sub>. Here it is noteworthy that 6BH<sub>4</sub> is composed of a pyrimidine and pyrazine ring with a side-chain attached to the 6<sup>th</sup> position (**Figure 44**). 7BH<sub>4</sub> has the same structure as 6BH<sub>4</sub> but the side-chain is on the 7<sup>th</sup> position (**Figure 44**). The difference between the structure of 6&7-biopterin is based on the move of the side chain on the pyrazine ring (**Figure 44**). Moreover, it has been proposed that the inhibitory effect of the pterins on XO activity may be due to the structural similarities between the pterins, xanthine and the molybdopterin cofactor (Wede, Altindag et al. 1998). Based on these similarities, the pterins may occupy the active site of XO, or they may engage moco binding domain without being a substrate for the enzyme (**Figure 44**) (Wede, Altindag et al. 1998).

Moreover, it has also been suggested that the possession of a side chain with several –OH groups in the 6<sup>th</sup> position could play an important role in pterins' interactions with XO (Wede, Altindag et al. 1998). However, our results with 7BH<sub>4</sub> and 7-biopterin suggest that the side chain in position 7 of those pterins could be an explanation for inhibition of XO activity by the 7-isomer as shown in this thesis. The stronger inhibitory effect of the oxidised pterins (6&7-biopterin) compared to their reduced state may be attributed to the presence of two double bonds in the pyrazine ring. This oxidation dependent interaction with molybdopterin cofactor could influence more effectively the electronic properties of the molybdenum cofactor (Schindelin, Kisker et al. 1996).



**Figure 44**

**Structure of four pterins compared to molybdopterin cofactor of XO and xanthine**

6-biopterin is the most potent inhibitor of XO activity followed by 7-biopterin, 6BH<sub>4</sub> and 7BH<sub>4</sub>, respectively. All these pterins have a hydroxylated side chain attached to the either 6<sup>th</sup> or 7<sup>th</sup> position of the pyrazine ring. The strong inhibitory effect of these pterins has been attributed to presence of this side chain, whereas due to the absence of the side chain in leucopterin, the inhibitory effect of this pterin has been shown to be drastically weaker compared to pterins with a side chain (Wede, Altindag et al. 1998; Oetl and Reibnegger 1999).

In the context presented herein it is interesting that the presence of both 6BH<sub>4</sub> and 7BH<sub>4</sub> has been documented in melanosomes (Schallreuter, Moore et al. 1999; Spencer, Chavan et al. 2005). In this thesis we show for the first time that XO is also present in this organelle (**Figure 9**).

What could be the function of this enzyme in this pigment forming organelle?

Considering that XO generates O<sub>2</sub><sup>•-</sup> which in turn is an effective activator of tyrosinase (Wood and Schallreuter 1991), and that both 6BH<sub>4</sub> and 7BH<sub>4</sub> are potent inhibitors of tyrosinase and XO, it was tempting to invoke XO in a concerted action with tyrosinase and 6BH<sub>4</sub>/7BH<sub>4</sub> in regulation of melanogenesis.

We therefore wanted to know whether XO have the capacity to act as a possible activator of the tyrosinase-6BH<sub>4</sub> inhibitor complex as well as the tyrosinase-7BH<sub>4</sub> inhibitor complex. Our results showed that the tyrosinase-inhibitor complex can indeed be reactivated upon addition of XO (**Figure 32; A&B**). We demonstrated that the 6BH<sub>4</sub>-tyrosinase inhibitor complex is very fast reactivated by XO (**Figure 32A**). To further substantiate these observations, we used [<sup>3</sup>H]6BH<sub>4</sub> to follow its binding to XO. The result confirmed that XO can bind 6BH<sub>4</sub> (**Figure 33**).

We then showed that XO can also reactivate the 7BH<sub>4</sub>-tyrosinase inhibitor complex. However, reactivation needs more XO (**Figure 32**). This result suggests that the pterin binding site on XO is different for both pterins.

To our knowledge, we here show for the first time that XO strongly binds both 6 and 7BH<sub>4</sub> which in turn enables XO to be an activator of the tyrosinase inhibitor complex. Hence, the presence of XO in the melanosomes might be important for this reason. However, as said above, on one hand XO generates O<sub>2</sub><sup>•-</sup> which is an activator of tyrosinase; on the other hand both pterins inhibit XO (**Figure 31**). In this context it is noteworthy that α-MSH and β-MSH can also bind the pterins with 1: 1 stoichiometry

(Moore, Wood et al. 1999; Schallreuter, Moore et al. 1999; Spencer, Chavan et al. 2005). So far we have not compared the affinity of XO with both  $\alpha$ -MSH and  $\beta$ -MSH. However, the presence of XO, 6BH<sub>4</sub> and 7BH<sub>4</sub> in the melanosome could provide an effective machinery in control of the tyrosinase inhibitor complex. This could be another mechanism within the melanocyte, whereby tyrosinase can be re-activated in a receptor independent manner, as shown earlier by  $\alpha$ -MSH and recently by  $\beta$ -MSH (Moore, Wood et al. 1999; Spencer, Chavan et al. 2005).

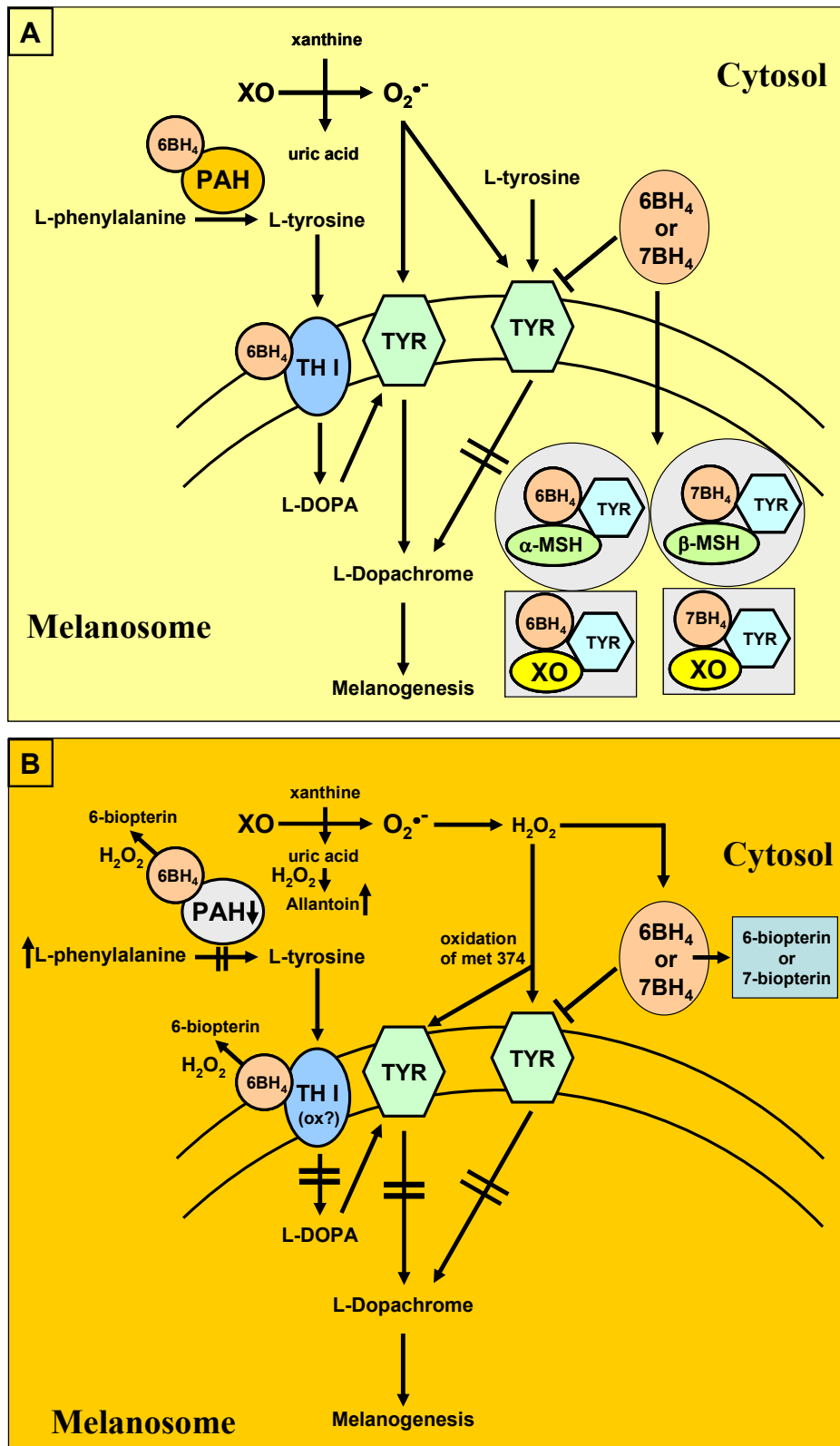


Figure 45

**Inhibition/activation of tyrosinase, the key enzyme in melanogenesis**

**A: Normal skin.** Conversion of the amino acid L-phenylalanine to L-tyrosine via PAH initiates melanin synthesis (Schallreuter and Wood 1999). TH I which is situated side by side

with TYR on the melanosomal membrane, converts L-tyrosine to L-DOPA (Marles, Peters et al. 2003). L-DOPA activates TYR which in turn forms L-Dopachrome yielding finally to melanin formation. Both 6 and 7BH<sub>4</sub> inhibit TYR (Wood, Schallreuter-Wood et al. 1995). This inhibition is reversible by the binding of  $\alpha$ -MSH to 6BH<sub>4</sub> and  $\beta$ -MSH to 7BH<sub>4</sub>, thus reactivating TYR (Moore, Wood et al. 1999; Spencer, Chavan et al. 2005). In this thesis we identified the presence of XO in melanosomes (**Figure 9**) (Shalhaf, Gibbons et al. 2008). Moreover, we showed that both 6 and 7BH<sub>4</sub> can bind to XO, inhibiting its activity (**Figure 31**). We also showed that the binding of 6 and 7BH<sub>4</sub> to XO can reactivate pterin inhibited TYR (**Figure 32**). XO converts xanthine to uric acid. In this reaction O<sub>2</sub><sup>•-</sup> is produced (Newsholm and Leech 1984). TYR's activity has been shown to be 40-fold higher in the presence of O<sub>2</sub><sup>•-</sup> (Wood and Schallreuter 1991).

Taken together, interactions between XO, 6BH<sub>4</sub>, 7BH<sub>4</sub>,  $\alpha$ -MSH,  $\beta$ -MSH and tyrosinase provide potential alternative back up mechanisms within the melanocyte/melanosome in the regulation of tyrosinase in a receptor-independent manner.

**B: Vitiligo.** The presence of 10<sup>-3</sup>M H<sub>2</sub>O<sub>2</sub> within melanocytes, causes the oxidation of 6/7BH<sub>4</sub> to 6-/7-biopterin respectively. H<sub>2</sub>O<sub>2</sub>-mediated oxidation of the key enzymes involved in melanogenesis tyrosinase (Tyr) and phenylalanine hydroxylase (PAH) alters their structure and functionality. Recently it was shown that H<sub>2</sub>O<sub>2</sub>-mediated oxidation affects met 374 in the active site of tyrosinase leading to deactivation of the enzyme (Wood, Decker et al. 2009) which in turn affects melanin biosynthesis. As a result of the deactivation of PAH, L-phenylalanine levels are increased within the melanocytes. Uric acid, the end product of purine degradation pathway is oxidised to allantoin by H<sub>2</sub>O<sub>2</sub>. Increased epidermal allantoin levels in vitiligo have been shown in this thesis.



## **5.7 Patients with vitiligo combat with high levels of oxidative DNA damage in their epidermis**

DNA is continuously damaged by ROS generated as a result of normal biochemical reactions in metabolic pathways and also generated by various environmental agents, leading in turn to the release of purine bases from the damaged DNA. The purine bases need to be degraded (Malins 1993; Hattori-Nakakuki, Nishigori et al. 1994; Hattori, Nishigori et al. 1996; Marnett 2000; Ichihashi, Ueda et al. 2003; Smith, Marks et al. 2004). 8-oxoG has been regarded as the major oxidative DNA-base lesions and they have been used to evaluate the extent of oxidative stress in individuals (Kasai and Nishimura 1984; Kasai and Nishimura 1984; Kasai, Crain et al. 1986; Adelman, Saul et al. 1988; Loft, Fischer-Nielsen et al. 1993; Kasai 1997; Steenken and Jovanovic 1997; Helbock, Beckman et al. 1999; Bruner, Norman et al. 2000; Kasai 2002; Ichihashi, Ueda et al. 2003; Neeley and Essigmann 2006). Elevated levels of this oxidised DNA base have been identified in various diseases caused by oxidative stress (Fraga, Shigenaga et al. 1990; Loft, Vistisen et al. 1992; Hattori-Nakakuki, Nishigori et al. 1994; Hattori, Nishigori et al. 1996; Tsuboi, Kouda et al. 1998; Ahmed, Ueda et al. 1999; Lovell, Gabbita et al. 1999; Nunomura, Perry et al. 1999; Nunomura, Perry et al. 1999; Honda, Yamada et al. 2000; Ishizakai, Yoshida et al. 2004; Wu, Chiou et al. 2004; Wang, Schmeichel et al. 2005; Perra, Maxia et al. 2006; Shiihara, Kato et al. 2006; Bahar, Feinmesser et al. 2007; Chen, Wu et al. 2007; Chen, Liou et al. 2007; Dincer, Erzin et al. 2007; Kinoshita, Wanibuchi et al. 2007; Dong, Cui et al. 2008; Fukuda, Yamauchi et al. 2008; Ku, Jin et al. 2008).

Nowadays, there is no doubt that the entire epidermis of patients with vitiligo is under oxidative stress due to accumulation of mM range of H<sub>2</sub>O<sub>2</sub> (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008). Hence, we postulated high levels of oxidative DNA damage resulting in high levels of 8-oxoG in the epidermis of patients with vitiligo.

To the best of our knowledge, so far there has been no report of increased 8-oxoG levels in the epidermis of patients with vitiligo. Within this thesis we demonstrate for the first time significantly elevated levels of this oxidised DNA base both *in situ* and *in vitro* in lesional and non-lesional epidermis. Moreover, we also show *in vitro* higher levels in vitiliginous epidermal MCs of patients with acute vitiligo compared to healthy controls (**Figure 34; A&B and Figure 35; A, B, C&D**). Notably, 8-oxoG is not degradable, it is finally excreted from plasma in the urine (Wu, Chiou et al. 2004). Our immuno reactivity results were backed up by demonstrating significantly elevated plasma levels of 8-oxoG in all 20 patients compared to healthy controls (**Figure 36**). These results imply that patients with vitiligo are dealing with high levels of oxidative DNA damage due to H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress in their epidermis.

In this context it has been well documented that 8-oxoG lesions induce potentially mutagenic G:C to T:A transversion unless repaired prior to DNA replication. Thus it is considered to be a pro-mutagenic DNA lesion produced by oxygen radicals (Floyd 1990; Wood, Dizdaroglu et al. 1990; Shibutani, Takeshita et al. 1991; Cheng, Cahill et al. 1992; Maki and Sekiguchi 1992; Grollman and Moriya 1993; Moriya 1993; Le Page, Margot et al. 1995; Johnson, Yu et al. 1996; Hussain and Harris 1998; Sunaga, Kohno et al. 2001; Bjelland and Seeberg 2003; Hussain, Hofseth et al. 2003). This oxidised base is expected to be involved not only in carcinogenesis, but also in tumour biology (Hattori, Nishigori et al. 1996). High percentage of G:C to T:A

transversion in human skin tumours has been attributed to 8-oxoG formation by UVA-generated ROS (Daya-Grosjean, Dumaz et al. 1995). Moreover, this mutagenic transversion has been observed in UVB-induced skin cancers in mice (Nishigori, Wang et al. 1994) and also in human non-melanoma skin cancer (van der Schroeff, Evers et al. 1990). Therefore, oxidative damage to DNA has been assigned to be one of the major causes of mutations, apoptosis and cancer (Hattori-Nakakuki, Nishigori et al. 1994; Johnson, Yu et al. 1996; Marnett 2000; Cooke, Evans et al. 2003; Hussain, Hofseth et al. 2003).

Taken together, based on all the facts present at this time a high incidence of skin cancer would be expected in patients with vitiligo.

Along this line it was reported that there was no difference in apoptotic regulatory molecules in vitiligo compared to healthy controls (Tobin, Swanson et al. 2000; van den Wijngaard, Aten et al. 2000), while significantly increased epidermal functioning wild-type p53 expression was documented (Schallreuter, Behrens-Williams et al. 2003). Taking into consideration that the outcome of p53 activation is not only apoptosis but also DNA repair (“the two face theory of p53”) (Smith and Fornace 1997), the presence of increased epidermal oxidative DNA damage, in association with increased functioning wild-type p53 along with unchanged apoptotic regulatory molecules in vitiligo suggested that only one face of the two face theory needed to be proven i.e. “DNA REPAIR”.

## **5.8 Enhanced epidermal DNA repair in vitiligo**

### **5.8.1 Epidermal cells induce Gadd45 $\alpha$ expression in vitiligo**

In order to prevent the deleterious effects of DNA oxidation such as mutagenesis and carcinogenesis, living organisms have developed protective strategies. The most effective strategy in combating oxidative DNA damage is thought to be exclusively via base-excision repair (BER) (Dempfle and Harrison 1994; Boiteux and Radicella 1999; Dianov, Souza-Pinto et al. 2001). The repair of the damaged DNA involves the activation of multiple genes inducing inhibition of cell division and prolongation of cell-cycle arrests and checkpoints. Two major kinases are responsible to induce cellular checkpoints in response to DNA damage i.e. ATM and ATR (Schmitt, Paquet et al. 2007). The induction of these proteins leads to the activation of the “guardian of the genome p53” (Kastan, Onyekwere et al. 1991; Schmitt, Paquet et al. 2007) which in turn transactivates its downstream effector genes and proteins including p21 and Gadd45 $\alpha$  leading in turn to the induction and prolongation of G1/S and G2/M phases respectively (el-Deiry, Harper et al. 1994; Kearsley, Coates et al. 1995; Wang, Zhan et al. 1999). These proteins can be regulated in a p53-dependent pathway as observed after ionizing radiation (Kastan, Zhan et al. 1992; Carrier, Smith et al. 1994; Zhan, Fan et al. 1996; el-Deiry 1998; Kachnic, Wu et al. 1999; Pedeux, Lefort et al. 2002; Smith and Seo 2002; Zhan 2005; Fayolle, Pourchet et al. 2006). These proteins can also be regulated in a p53-independent pathway in response to DNA damage caused by UV radiation as shown in the case of Gadd45 $\alpha$  (Russo, Zambrano et al. 1995; Zhan, Fan et al. 1996; Jin, Fan et al. 2001; Zhan 2005). Gadd45 $\alpha$  induces G2/M cell cycle checkpoint in KCs, MCs and melanoma cells following UVB irradiation (Maeda, Hanna et al. 2002; Pedeux, Lefort et al. 2002). Furthermore, melanoma cells

can efficiently repair UVB-induced lesions and therefore their survival may be based on the activation of Gadd45 $\alpha$  (Pedeux, Lefort et al. 2002; Fayolle, Pourchet et al. 2006; Fayolle, Pourchet et al. 2008).

As mentioned above, epidermal functioning wild-type p53 is increased in the epidermis of patients with vitiligo (Schallreuter, Behrens-Williams et al. 2003). Moreover, it has been shown that H<sub>2</sub>O<sub>2</sub> activates p53 and induces Gadd45 $\alpha$  expression in KCs and HeLa cells (Guyton, Xu et al. 1996; Wan, Wang et al. 2000). The data presented within this thesis demonstrate for the first time that Gadd45 $\alpha$  is significantly over-expressed in the lesional and non-lesional epidermis of patients with vitiligo compared to healthy controls as shown by *in situ* immuno-reactivity and Western blotting (**Figures 41&42**). Since these patients accumulate mM levels of H<sub>2</sub>O<sub>2</sub> in their epidermis (Schallreuter, Moore et al. 1999; Schallreuter, Bahadoran et al. 2008), the induction of epidermal Gadd45 $\alpha$  could be ascribed to H<sub>2</sub>O<sub>2</sub>. However, the precise mode of action needs yet to be shown.

Gadd45 $\alpha$  directly interacts with both p21 and PCNA and this activity is thought to be vital for the modulation of the cell cycles and for the inhibition of DNA replication (Smith, Chen et al. 1994; Chen, Smith et al. 1995; Hall, Kearsley et al. 1995; Kearsley, Coates et al. 1995; Vairapandi, Balliet et al. 1996; Vairapandi, Azam et al. 2000).

Salem showed that both epidermal p21 and PCNA expression are also upregulated in vitiligo (Salem 2009).

Taken together our data further highlight a role for Gadd45 $\alpha$  in the cellular response to increased oxidative DNA damage in vitiligo.

### 5.8.2 Vitiliginous epidermal cells express hOgg1 to excise 8-oxoG from 8-oxoG/C pairs in DNA

The repair of oxidative DNA damage is exclusively accomplished via BER pathways (Sancar 1994; Parikh, Mol et al. 1997; Fortini, Parlanti et al. 1999; Dianov, Souza-Pinto et al. 2001; Dogliotti, Fortini et al. 2001; Lindahl 2001; Lu, Li et al. 2001; Fromme and Verdine 2004). This mechanism is initiated by DNA glycosylases. In human, the key enzyme hOgg1 detects and removes 8-oxoG, leaving an AP site (Boiteux and Radicella 1999; Kinoshita, Wanibuchi et al. 2002). Using Western blot analysis, our results revealed that hOgg1 is indeed expressed in epidermal cells in vitiligo. Interestingly, the expression of this enzyme showed no difference between vitiligo and controls (**Figure 37; A, B&C**). These results suggest that H<sub>2</sub>O<sub>2</sub> does not alter the protein expression of this key enzyme in vitiligo. These data are in agreement with previously published work by Saitoh and colleagues (Saitoh, Shinmura et al. 2001; Fortini, Pascucci et al. 2003). After the removal of the damaged base by hOgg1 other steps are needed to complete the repair process. The repair mode of oxidative DNA damage is predominantly via short-patch BER where the enzyme DNA polymerase $\beta$  plays a critical role (Dianov, Prasad et al. 1999; Prasad, Dianov et al. 2000). In this pathway, APE1 and DNA polymerase $\beta$  excise the remaining sugar moiety and reinstall an undamaged nucleotide respectively. This step is followed by DNA ligase I activity sealing the nick thereby repairing the damaged base site (Parikh, Mol et al. 1997; Gros, Sapparbaev et al. 2002; Fortini, Pascucci et al. 2003; Chaudhry 2007). As expected, protein expression of these two enzymes is significantly increased in the epidermis of patients with vitiligo (**Figure 38**).

To sum up, these results imply that patients with vitiligo develop effective DNA repair machinery via hOgg1, APE1 and DNA polymerase $\beta$  to combat oxidative DNA damage as evidenced by increased 8-oxoG levels.

Earlier it has been suggested that Ogg1 gene is directly involved in tumour prevention (Xie, Yang et al. 2004). These authors reported that *Myh* and *Ogg1* knockout mice showed 65.7% increase in tumour predisposition, predominantly lung and ovarian tumours in addition to lymphomas (Xie, Yang et al. 2004). Given the high levels of H<sub>2</sub>O<sub>2</sub> in vitiligo skin, oxidative DNA damage was expected. As said above oxidative DNA damage is regarded as one of the major contributors to tumorigenesis (Ames 1989).

However, there are only scattered reports of non-melanoma skin cancers (NMCS, squamous cell carcinoma and basal cell carcinoma) in patients with vitiligo (Calanchini-Postizzi and Frenk 1987; Westerhof and Schallreuter 1997; Schallreuter, Tobin et al. 2002). Skin cancer was never diagnosed in 2500 patients with vitiligo (Westerhof and Schallreuter 1997), even lower risk of NMCS has been reported in this disorder by other authors (Lisi 1972; Ortonne, Pelletier et al. 1978; Schallreuter, Tobin et al. 2002).

Taking into consideration that the patients lack increased development of skin cancers, our data suggest that BER is a major player in the hierarchy to combat oxidative stress and to prevent ROS-caused tumorigenesis in the epidermis of patients with vitiligo.

## **5.9 In vitiligo epidermal cells do not undergo apoptosis in response to oxidative DNA damage**

Within this thesis we showed that DNA repair is significantly overriding increased oxidative DNA damage in epidermal cells of patients with vitiligo compared to healthy controls. Moreover, epidermal functioning wild-type p53 is increased (Schallreuter, Behrens-Williams et al. 2003). Taking into consideration that the activation of p53 is also leading to transactivation of its downstream genes involved in apoptosis, we here investigated protein expression of two major apoptotic regulators i.e. cytochrome c and caspase 3. The results revealed that in vitiligo, epidermal cells express even lower cytochrome c and caspase 3 proteins compared to controls (**Figures 39&40**). Since apoptotic cells show induction of cytochrome c and caspase 3 as crucial components in the apoptotic cascade (Liu, Kim et al. 1996; Nicholson and Thornberry 1997; Yang, Liu et al. 1997; Finucane, Bossy-Wetzel et al. 1999; Li, Kolluri et al. 2000; Abu-Qare and Abou-Donia 2001), then decreased levels of these two key regulators of apoptosis fuels the hypothesis that epidermal cells in vitiligo do not undergo programmed cell death. Based on our data discussed earlier, we show that vitiliginous cells seem to combat H<sub>2</sub>O<sub>2</sub>-induced oxidative DNA damage by induction of the powerful BER machinery.

However, it is noteworthy that the results presented in this thesis demonstrate protein expression of DNA repair enzymes or apoptotic regulators in all epidermal cells and not specifically in melanocytes. Considering that almost 80% of the epidermal cell population are KCs, the results of this thesis mainly focus on KC responses to oxidative DNA damage. The reports in the literature are conflicting, ranging from no increased to increased apoptosis in vitiliginous MCs compared to normal controls



(Boissy and Nordlund 1997; Tobin, Swanson et al. 2000; van den Wijngaard, Aten et al. 2000; Kemp, Waterman et al. 2001; Huang, Nordlund et al. 2002; Boissy and Manga 2004; Park, Kim et al. 2007; Ruiz-Arguelles, Brito et al. 2007; Song, Xu et al. 2008). Our additional data from this thesis provide more support for the absence of increased apoptosis in vitiligo.

## 6.0 CONCLUSION

### **The puzzle comes together**

Given that despite loss of the protecting pigment and the overwhelming evidence for H<sub>2</sub>O<sub>2</sub> induced oxidative stress in the epidermis of these patients, there is no increased risk or a higher prevalence for sun induced skin cancer (Calanchini-Postizzi and Frenk 1987; Schallreuter, Tobin et al. 2002).

However, vitiligo is associated with DNA damage as evidenced by the presence of high 8-oxoG levels in the epidermis and in the plasma of patients. The results of this study imply that patients with vitiligo must develop effective DNA repair machinery.

One way to combat DNA damage is BER repair via the key enzyme hOgg1 followed by activities of APE1 and DNA polymerase $\beta$ . Both enzymes are up-regulated in vitiligo. Since it has been shown that hOgg1 expression is not different in these patients from healthy controls, it can be concluded that 8-oxoG is glycosylated and the repair would be completed by APE1 and DNA polymerase $\beta$ . Hence our results support enhanced DNA repair in vitiligo by this cascade.

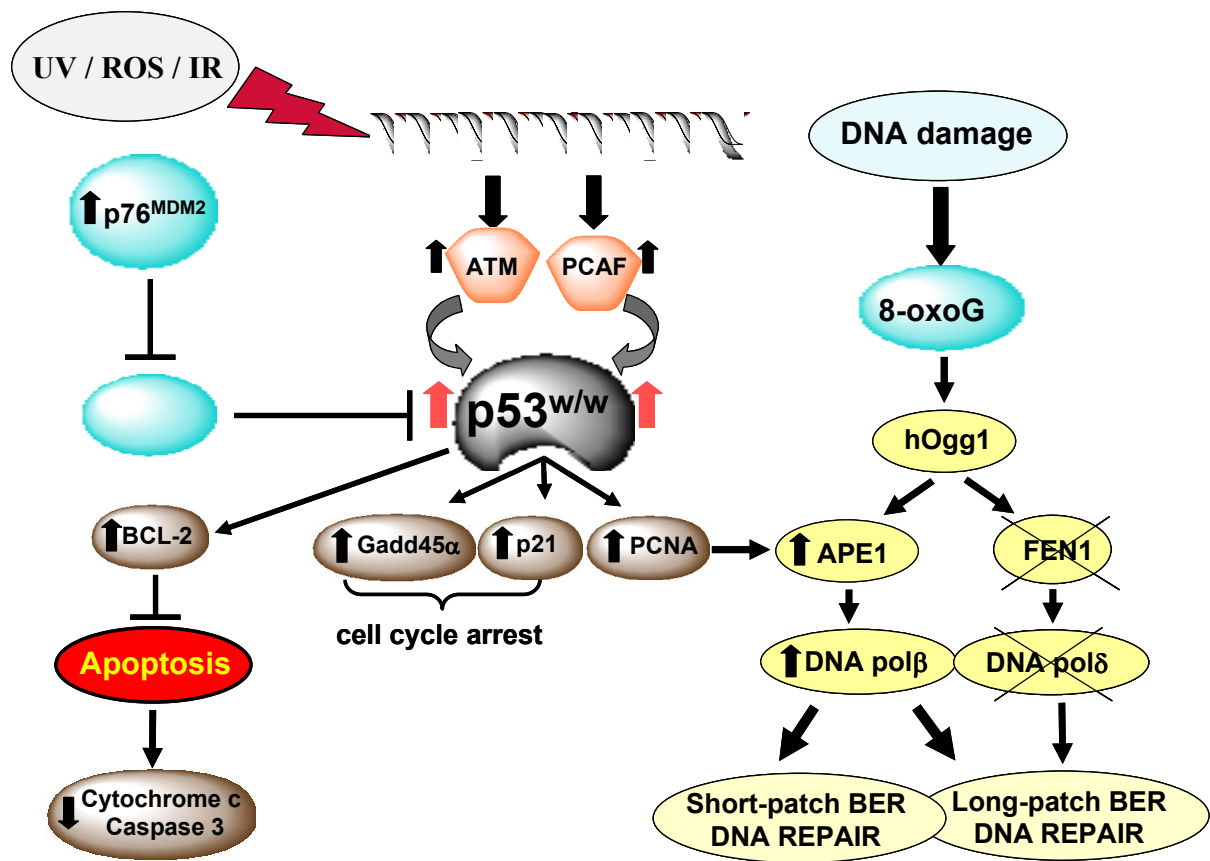
The presence of increased Gadd45 $\alpha$  expression favours cell cycle arrest. However, Gadd45 $\alpha$  directly interacts with both p21 and PCNA and this activity is thought to be vital for the modulation of the cell cycles and for the inhibition of DNA replication (Smith, Chen et al. 1994; Chen, Smith et al. 1995; Hall, Kearsley et al. 1995; Kearsley, Coates et al. 1995; Vairapandi, Balliet et al. 1996; Vairapandi, Azam et al. 2000). In this context it was shown that both PCNA as well as p21 are up-regulated in vitiligo (Salem 2009). There is no evidence for apoptosis in this disease (Tobin, Swanson et al. 2000; van den Wijngaard, Aten et al. 2000). These results are further substantiated

by the absence of caspase 3 and cytochrome c as shown in this thesis. More support stems from the up-regulation of the anti-apoptotic protein Bcl-2 (Salem 2009).

This conclusion is even more supported by earlier report that acetylcholine esterase protein expression and activity is very low in epidermal cells in vitiligo compared to healthy controls (Schallreuter, Elwary et al. 2004). This enzyme has been shown to be an apoptotic regulator since its overexpression inhibits cell proliferation and promotes apoptosis, whereas the inhibition of acetylcholine esterase gene expression enhanced cell proliferation and suppressed apoptosis-associated DNA fragmentation (Soreq, Patinkin et al. 1994; Soreq and Seidman 2001; Park, Kim et al. 2004; Jiang and Zhang 2008). To sum up, all data available at the present time argue against increased apoptosis in epidermal cells in vitiligo.

The crucial question to be answered is why wild-type p53 is up-regulated in vitiligo. In this context it was shown that epidermal p76<sup>MDM2</sup> in patients with vitiligo is up-regulated (Salem 2009). This isoform counteracts p90<sup>MDM2</sup> which in turn would degrade p53 (Perry, Mendrysa et al. 2000).

Consequently the question arose, whether p53 could be the master regulator in DNA repair. In this context it was shown by Salem that p53 in the presence of H<sub>2</sub>O<sub>2</sub> together with peroxynitrite enhances the capacity of DNA binding (Salem 2009). It was also shown that p53 levels are not affected by the removal of epidermal H<sub>2</sub>O<sub>2</sub> by a pseudocatalase PC-KUS (Salem 2009). Since p53 stimulates BER through binding of APE1, hOgg1 and DNA polymerase $\beta$  (Sengupta and Harris 2005), our findings would strongly support that p53 is of overriding importance in DNA repair in vitiligo thus explaining the lack of increased skin cancer. The strength of all results resides in the evaluation of the same patient and control group throughout the entire investigation on this subject. Our current understanding is summarised in **Scheme 1**.



**Scheme 1**

**Up-regulated wild type p53 as the main conductor of ROS-mediated DNA damage / repair in vitiligo**

ROS – mediated DNA damage in vitiligo leads to increased 8-oxoG levels in the epidermis and in plasma of patients with vitiligo. Phosphorylation is a crucial step for p53 stabilization to perform its function as a transcription factor (Giaccia and Kastan 1998; Appella and Anderson 2001; Ito, Shinkai et al. 2001). In this context it has been shown that phosphorylation of p53 protein is initiated by DNA damage. Up regulated wild type p53 is phosphorylated on ser9 and ser15, proving functionality of up-regulated ATM in this disease (Salem 2009). Acetylation is one step of posttranslational modifications (Polevoda and Sherman 2000). Up regulated epidermal PCAF in vitiligo is functioning as shown by the presence of acetylated lysine residues at position 373 and 382 (Salem 2009). Both steps support functioning stabilised p53 as shown earlier (Schallreuter, Behrens-Williams et al. 2003). Under normal conditions p53 is degraded by p90<sup>MDM2</sup> (Oliner, Kinzler et al. 1992).

The isomer p76<sup>MDM2</sup> binds to p90<sup>MDM2</sup>, preventing binding of p90<sup>MDM2</sup> to p53, stopping in turn degradation of p53 (Perry, Mendrysa et al. 2000). Our results revealed for the first time high epidermal p76<sup>MDM2</sup> levels in vitiligo, whereas p90<sup>MDM2</sup> expression is not affected in this compartment (Salem 2009). Functioning stabilised p53 leads to transcription of p21, Gadd45 $\alpha$  and PCNA (Smith and Seo 2002; Rozan and El-Deiry 2007; Shu, Li et al. 2007). Both p21 and Gadd45 $\alpha$  induce cell cycle arrest and both signals are up regulated in vitiligo (Salem 2009). Oxidative DNA damage is repaired via BER, which is initiated by hOgg1 excising 8-oxoG (Michaels, Tchou et al. 1992; Boiteux 1993; Boiteux and Radicella 1999; Kinoshita, Wanibuchi et al. 2002). Depending on the type of DNA damage, the repair pathways would involve either short- or long-patch BER, although these pathways can switch between each other (Wilson and Thompson 1997; Dogliotti, Fortini et al. 2001; Fromme and Verdine 2004). PCNA binds to DNA polymerase $\delta$  and works as a processivity factor for this enzyme, which together with FEN1 is involved in long-patch BER (Matsumoto, Kim et al. 1994; Frosina, Fortini et al. 1996; Klungland and Lindahl 1997; Fromme and Verdine 2004). Notably, we were unable to detect epidermal DNA polymerase $\delta$  and FEN1 in our patients, but we found upregulated epidermal APE1 and DNA polymerase $\beta$  levels. This result favours short patch BER in vitiligo. However, DNA polymerase $\beta$  can function in both repair pathways (Dianov, Prasad et al. 1999; Prasad, Dianov et al. 2000). In this context it is of interest that PCNA can interact directly with APE1 (Dianova, Bohr et al. 2001). Moreover, hOgg1, APE1 and DNA polymerase $\beta$  are directly regulated by p53 (Sengupta and Harris 2005). Therefore it can be concluded that oxidative DNA damage in vitiligo can be repaired through short-patch BER in cooperation with a specified long-patch BER in the absence of FEN1 and DNA polymerase $\delta$ . Epidermal programmed cell death is ruled out because up regulated Bcl-2 favours anti-apoptotic activity which is further supported by decreased levels of cytochrome c and caspase 3. These new findings are further supported by unremarkable levels of the pro-apoptotic stimulus BAX and low acetylcholine esterase levels/activities in vitiligo as shown earlier (van den Wijngaard, Aten et al. 2000; Schallreuter, Elwary et al.

2004). Taken together, patients with vitiligo can efficiently combat epidermal  $\text{H}_2\text{O}_2/\text{ONOO}^-$  induced DNA damage with up regulated wildtype p53 as main conductor in the scenario (Salem 2009).

## 7.0 FUTURE WORK

The data presented in this thesis has opened a new window in to the repair of oxidative DNA damage. Future work could focus on:

1. Investigation of PCNA / DNA polymerase $\beta$  regulation in long-patch DNA repair in vitiligo.
2. Since xanthine oxidase appears to be in the nucleus of both epidermal melanocytes and keratinocytes, future work could address these questions.
  - What is the purpose of XO in the nucleus?
  - What is its role?
  - Why is the presence of XO in the nucleus of keratinocytes stronger than in melanocytes?
3. Our results showed for the first time that 7BH<sub>4</sub> and 7-biopterin are inhibitors of XO. Since 6 and 7BH<sub>4</sub> have been shown to be present in melanosomes together with XO, the possible role for XO in pigmentation needs further investigation.
4. One major question needs to be answered in future. Why does H<sub>2</sub>O<sub>2</sub>/ONOO<sup>-</sup> mediated oxidative stress affect both lesional and non-lesional skin in vitiligo despite only the lesional skin is depigmented?

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## 9.0 APPENDIX

### Working solutions for SDS-PAGE

All chemicals were from Sigma, Dorset, UK unless otherwise stated.

#### Solution B (4x separating buffer or resolving gel):

1.5M Tris base, 0.4% SDS, pH ???, ??ml final volume made using dH<sub>2</sub>O.

#### Solution C (4x stacking buffer):

0.5M Tris base, 0.4% SDS, pH ???, ??ml final volume made using dH<sub>2</sub>O.

	5% resolving gel	10% resolving gel	13% resolving gel	3% stacking gel
dH <sub>2</sub> O	13.6 ml	9.6 ml	7.2 ml	4.87 ml
Solution B/C	6 ml sol. B	6 ml sol. B	6 ml sol. B	1.87 ml sol. C
Acrylamide	4 ml	8 ml	10.4 ml	750 µl
TEMED	8 µl	8 µl	8 µl	10 µl
APS (10%)	90 µl	90 µl	90 µl	75 µl

**Table 3:** The quantities of each solution required for preparation of polyacrylamine gels for SDS-PAGE.



**Sample buffer (x5):** 10% SDS 4ml, mercaptoethanol 1ml, glycerol 2ml, 0.5M Tris/HCl 1ml, distilled water 2ml, and a trace of brilliant blue.

**Running buffer (x4):** 192mM glycine, 0.025M Tris base, 0.1% SDS, made to a final volume of 1L with dH<sub>2</sub>O.

**Transfer buffer (x4):** 0.2M glycine, 25mM Tris base, 20% methanol, made to a final volume of 1L with dH<sub>2</sub>O.

**TBS/Tween (NATT) buffer (x4):** 150mM NaCl, 20mM Tris base, 0.047% Tween 20, pH 7.4, made to a final volume of 1L with dH<sub>2</sub>O.

**ECL1:**

250mM luminol in DMSO	1ml
90mM p-coumaric acid in DMSO	0.44ml
1M Tris base (pH 8.5)	10ml
dH <sub>2</sub> O to a final volume of	100 ml

**ECL2:**

30% H <sub>2</sub> O <sub>2</sub>	64μl
1M Tris base (pH 8.5)	10ml
dH <sub>2</sub> O to a final volume of	100 ml