IN VITRO STUDIES ON LIPID METABOLISM IN KERATINOCYTES OF DIFFERENT RACIAL ORIGIN

A THESIS SUBMITTED TOWARDS PARTIAL FULFILLMENT OF

BS-MS DUAL DEGREE PROGRAMME

To the

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

By

RITESH RAGHAVAN

Written under the guidance of

DR. ANITA DAMODARAN

HINDUSTAN UNILEVER RESEARCH CENTER

WHITEFIELD, BANGALORE

CERTIFICATE

This is to certify that this thesis entitled "*In vitro* studies on Lipid metabolism in Keratinocytes of different Racial origin" submitted towards partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research Pune represents original research carried out by "Ritesh Raghavan" at "Hindustan Unilever Research Center, Bangalore", under the supervision of "Dr. Anita Damodaran" during the academic year 2012-2013.

Date: 12th April 2013
Place: Bangalore

Dr. Anita Damodaran
Supervisor

DECLARATION

I hereby declare that the matter embodied in the thesis entitled "*In vitro* studies on Lipid metabolism in Keratinocytes of different Racial origin" are the results of the investigations carried out by me at Hindustan Unilever Research Center, Whitefield, Bangalore under the supervision of Dr. Anita Damodaran and the same has not been submitted elsewhere for any other degree.

Ritesh Raghavan
Student

ABSTRACT

Lipids play a predominant role in the barrier function of the skin, with different lipid classes playing unique parts in the maintenance of a competent barrier. Comparison of clinical studies conducted on Indian and Caucasian volunteers reveal differences in their skin lipid profile. Our study aimed to examine the possible reasons behind this observation, by evaluating lipid profiles of epidermal keratinocytes from Caucasian and Indian origin cultured under various growth conditions, and expression profiles of genes involved in lipid metabolism.

We have not found much difference in the constitutive lipid profiles between cultured keratinocytes having different ethnic origin, both in the proliferating as well as differentiated states, except in case of cholesterol sulphate and phospholipids, which seem to be markedly increased in the Caucasian cells. When the cells were given high-glucose treatment for 48h, Indian cells show increased levels glycerolipids and cholesterol esters as opposed to Caucasians, with differentiation also influencing the lipid profiles. LDL treatments also show an increase in levels of glycerolipids and cholesteryl esters in Indians, which might be due to uptake.
Further, qRT-PCR studies showed that there were no stark differences in expression patterns of genes involved in ceramide or cholesterol metabolism between the two groups, whereas those for glycerolipid metabolism showed some characteristic differentiation associated trends, and also higher expression in Indian keratinocytes, notably in *DGAT2*, which catalyzes the final step in biosynthesis of triglycerides.

Physiological significance of our results may be predicted only after further confirmation with multiple sets of cultures and *in vivo* studies. Trends are indicative and give possible directions which may be explored to give conclusive answers.

ACKNOWLEDGEMENTS

I would take this opportunity to express my deep sense of gratitude to my respected guide and mentor, Dr. Anita Damodaran for her invaluable guidance, unwavering encouragement, careful nurturing and commitment to go beyond the path of necessity; that have contributed greatly to my development, and without which I could never have successfully culminated the project. I am thoroughly indebted to my guide for her contributions and dedicate my thesis to her.

I am also ever grateful to my very special “buddies”, Mr Sreenivas K. T. and Mr Rezwan Shariff, who have very capably taught me the nuances of the scientific methods and modes of inquiry required for my studies, stuck with me selflessly through a number of hurdles which could not have been crossed if it weren’t for their expertise, provided useful scientific inputs and supported me constantly in every aspect of my stay in Unilever.

I would like to give a special note of thanks to Ms BL Maria Juliet for her great help with PCR studies, informed discussions and words of encouragement without which part of the project would have been difficult if not impossible to complete; Mr Rajkumar RS for unmatched lateral support with tissue culture materials and methods, and advice regarding the same; Mr. Aryasekhar Sanyal, who has been like a big brother, a source of immense support both inside and outside the lab and a faithful friend in times of trouble, for which I am greatly indebted; Dr Jyoti Tiwari, for inspiring discussions and sound advice that is sure to hold true wherever I may go; Mr Rakesh Kumar Bandi for great support at a personal level and friendly words of encouragement; and not the least, to all the members of the Skin Appearance lab: Dr Ganesh C, Ms Nirmala Nair, Ms Aparna Damle, Ms Nivedita Patil, Ms Vaidehi Kale and Ms Maitreyee Dutta for the stimulating discussions, great support and vibrant atmosphere which has made my time in the lab a fun-filled learning experience. It was lovely to be a part of the team!
I must also make a special mention of Dr Shilpa Vora, who despite the pre-occupations

of her position, has been in constant contact and always radiated a sense of inclusion and warmth.

I would also like to thank members of the Organic Chemistry lab; Mr Praful Lahorkar, Ms Ramitha K, Mr Ravikant Shukla, Mr Balu K and others for their great support with troubleshooting of instruments, problems in analytic techniques, conceptual queries and for making me feel at home during the large part of my time I spent in their lab.

I am also grateful to my dear friends Ms Ankita Jha, Mr Sharad Kamble and Mr Ashish Yekhe for all the great times, which relieved the boredom and frustration of the mundane, and also for great support at a personal level.

I would like to acknowledge all the employees of Unilever I interacted with and HR person Roopa R for making my stint a memorable and enjoyable one.

Most importantly, I would like to express my gratitude to the authorities of both IISER-Pune and Unilever Ltd who allowed me to conduct my 5^{th} year project at HURC, Bangalore. I would like to specially thank Prof Shouvik Dutta of IISER-Pune and Dr Dileep Rout of Unilever, who have been notable in their support, and without whom this project would not have even started; Dr M Jayakannan, project co-ordinator, IISER-Pune and Dr Krishna N. Ganesh, Director, IISER-Pune for their role as able administrators and Dr Vilas P. Sinkar, site-head of HURC, Bangalore for his kind hospitality.

Lastly I would like to give a big hug of thanks out to my parents, whose unwavering faith, love and patience have been like pillars of strength; and who made my life a whole lot easier by inevitably joining me in Bangalore during the final months of my project.

Ritesh Raghavan

CONTENTS

List of Figures

1. INTRODUCTION

The skin forms the exterior covering of our body. Skin tissues along with appendages like nails and hair comprise the integumentary system. Being located at the interface of the body and its immediate physical environment, the skin plays a pivotal role in maintaining homeostasis and protection against environmental stress factors.

1.1. SKIN PHYSIOLOGY

The skin is divided into two primary layers, the dermis and the epidermis, which are subdivided into layers as illustrated in Figure 1.1. The skin tissue is composed of a number of different cell types, which confer the functional diversity required for its unique role in maintaining homeostasis and interacting with the immediate environment. They include dermal fibroblasts, which form the dermal matrix onto which the sweat glands, mechanosensory neurons, hair follicles and pilosebaceous glands are embedded; and epidermal keratinocytes, which construct a protective barrier against external stress factors, melanocytes, which produce melanin pigment which bestows protection against harmful UV radiation and immune response cells which act as a first line of defense against invading pathogens. For further reading, Fitzpatrick's *Dermatology in General Medicine* is recommended.

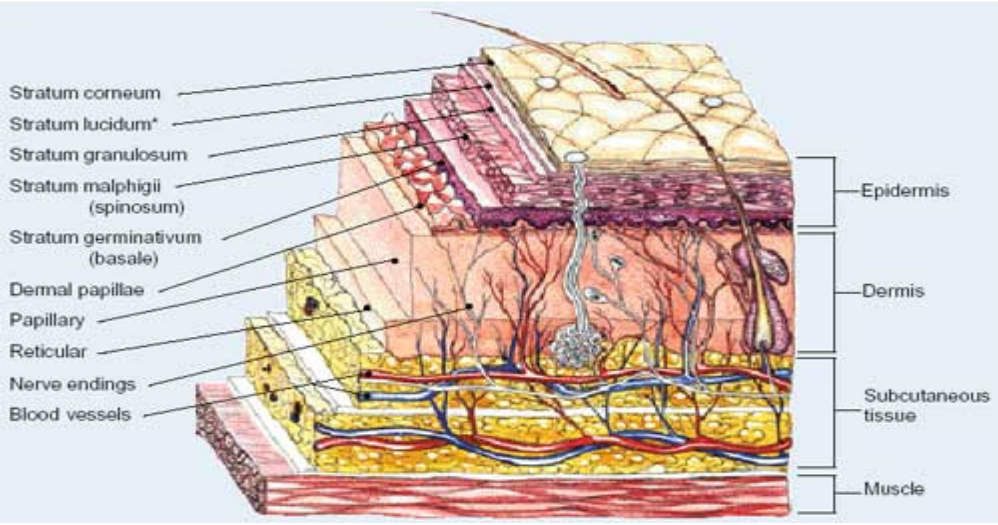

Figure 1.1 Different layers and cells of the skin
(Image courtesy Espace Beauty, Romania)

1.2. THE EPIDERMAL BARRIER

Arguably, one of the most important functions of the skin is the construction and maintenance of the protective barrier (Feingold, 1991), which fulfills the important functions of waterproofing, protection from a vast number of stress factors encountered frequently and prevention of desiccation of internal tissues. The epidermis has evolved as a specialized skin tissue whose primary function is to construct and maintain the barrier (Madison, 2003; Spearman, 1964), without which life in a terrestrial environment would be impossible. The epidermis is classically described as a "stratified squamous epithelium" and consists of five layers, as illustrated in Figure 1.2.

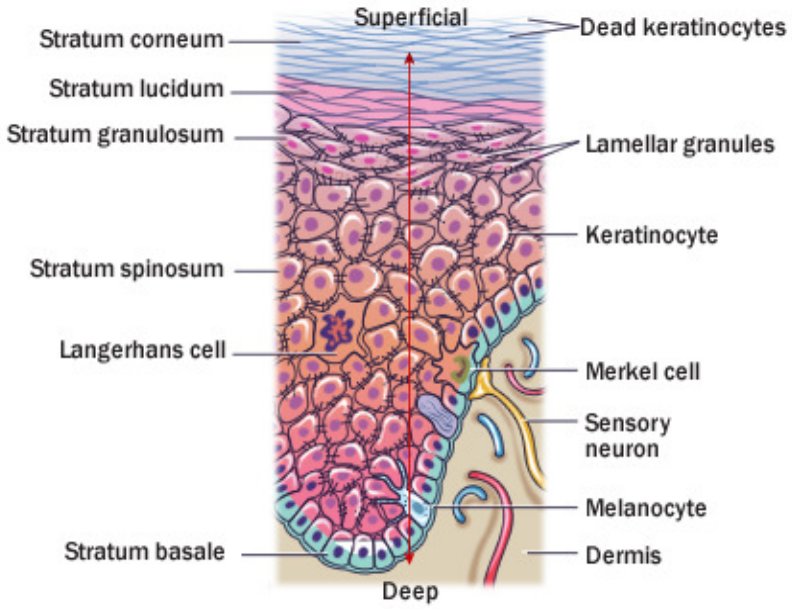

Figure 1.2 The Epidermis

Keratinocytes form the major cell type of the epidermis. Keratinocytes are derived from epidermal stem cells, which are attached to the basement membrane at the junction of the epidermis and dermis. Nascent or "basal" keratinocytes undergo proliferation in the stratum basale, after which they embark on a terminal differentiation pathway and simultaneously begin to move suprabasally, undergoing morphologic and biochemical changes along the way (Koster, 2009). The layers of the epidermis are characterized by various stages of keratinocyte differentiation. At the final stage of differentiation, in the stratum corneum (SC), the cells become enucleated, undergo apoptosis and become a

structural unit of the barrier; and are rightly rechristened as corneocytes. At the most superficial layer of the SC, corneocytes are continuously shed due to loss of the inter-cellular associations, in a process called desquamation.

STRUCTURE

The stratum corneum is the site of the skin permeability barrier. The corneocytes are hardened due to accumulation of keratins, which are fibrous proteins, within the cell; and a cornified envelope made of structural proteins viz. involucrin, filagrin, loricrin etc. They are embedded in a matrix of lipids viz. ceramides, fatty acids and cholesterol. The important structural components of the skin are described in Figure 1.3. This structure resembles a brick wall, an analogy that is drawn from one of the generally accepted models of barrier structure, the “brick -and-mortar” model (Elias, 1988).

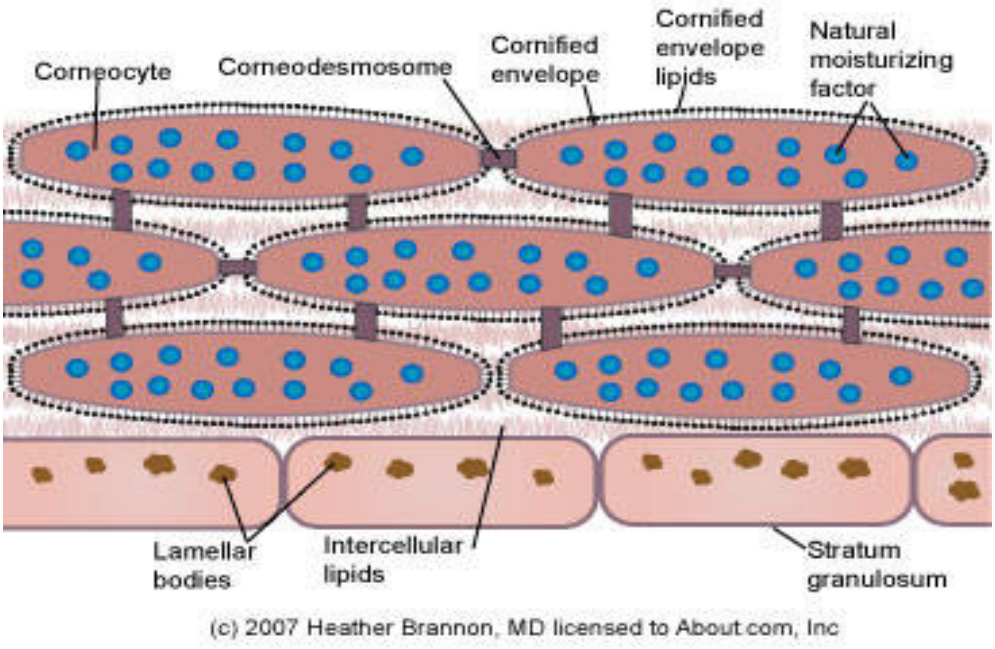

Figure 1.3 The stratum corneum barrier
(Image courtesy About.com, reproducible for educational purposes)

The stratum corneum has a unique lipid composition, consisting roughly by weight 45-50% ceramides, 25%cholesterol, 10-15% free fatty acids and less than 5% several other lipids such as cholesteryl sulphate, triglycerides etc. (Madison, 2003). Lipids are in the human SC are known to be arranged as orthorhombic lateral stacks(Bouwstra and Gooris, 2010), with a class of ceramides covalently bonded with the corneal envelope.

The lipid lamellae form the barrier against percutaneous water permeability (Feingold, 2007). The process by which this structure is generated is discussed next.

1.3. THE FORMATION OF THE BARRIER

Barrier lipids are generally synthesised and stored in lamellar bodies as precursor lipids (glucocylceramides, cholesterol, glycerophospholipid) along with enzymes involved in their processing. This step occurs in the stratum spinosum; and is accompanied by keratinisation- the intracellular accumulation of keratin fibers and keratohyalin, and formation of a hard protein envelope around the cells. The SC barrier is formed when the keratinocytes of the stratum granulosum (SG) extrude the lamellar bodies into the extracellular space at the SG-SC interface, leading to enzymatic conversion of the precursors and assembly of the lipids into domains. Figure 1.4 gives a pictorial account of the events involved in barrier formation. The whole process is referred to as cornification.

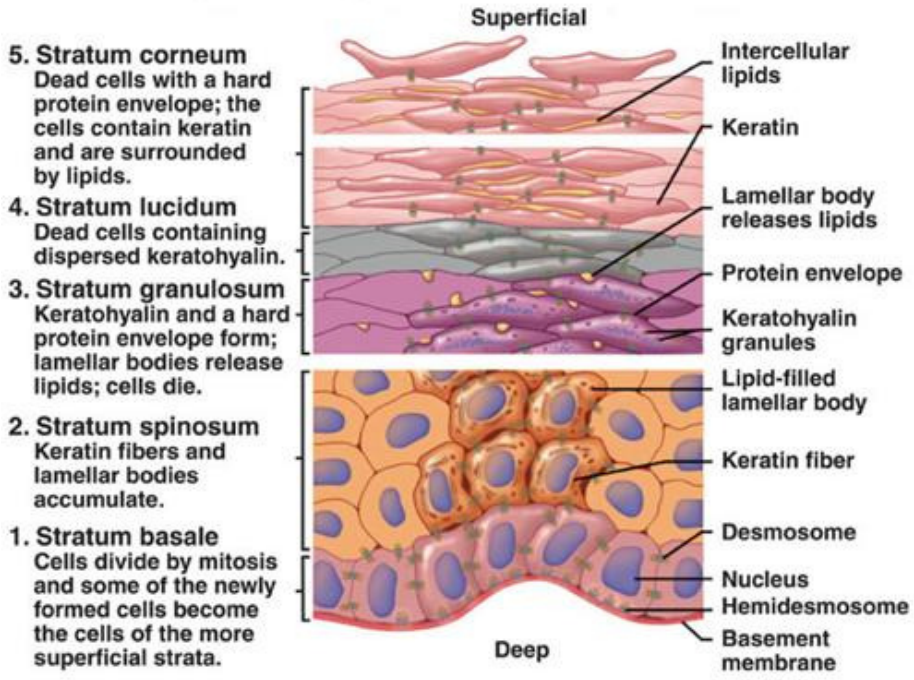

Figure 1.4 Formation of the SC barrier
(Image courtesy Tata-McGrawhil Pvt Ltd)

The importance of lipids with regard to barrier function has been stressed upon previously. Their metabolism is therefore a primary function of keratinocytes, with the epidermis being one of the most active sites of lipid synthesis in the body (Feingold, 2011), specific aspects of which are described in the next section.

1.4. Biosynthesis of Epidermal Lipids

1.4.1 Ceramides

Ceramides constitute approximately 45-50% by weight of the stratum corneum. Structurally ceramides are composed of a sphingoid moiety linked to a fatty acid residue by an amide bond. There are 11 different types of ceramides found in the human stratum corneum (Mizutani et al., 2009), as shown in Figure 1.5 with variations in the length of fatty acid chains and addition of hydroxyl groups at different positions on the sphingoid moiety. The ω-O-acylCERs, EOS, EOH, and EOP are unique to the epidermis and critical for barrier function.

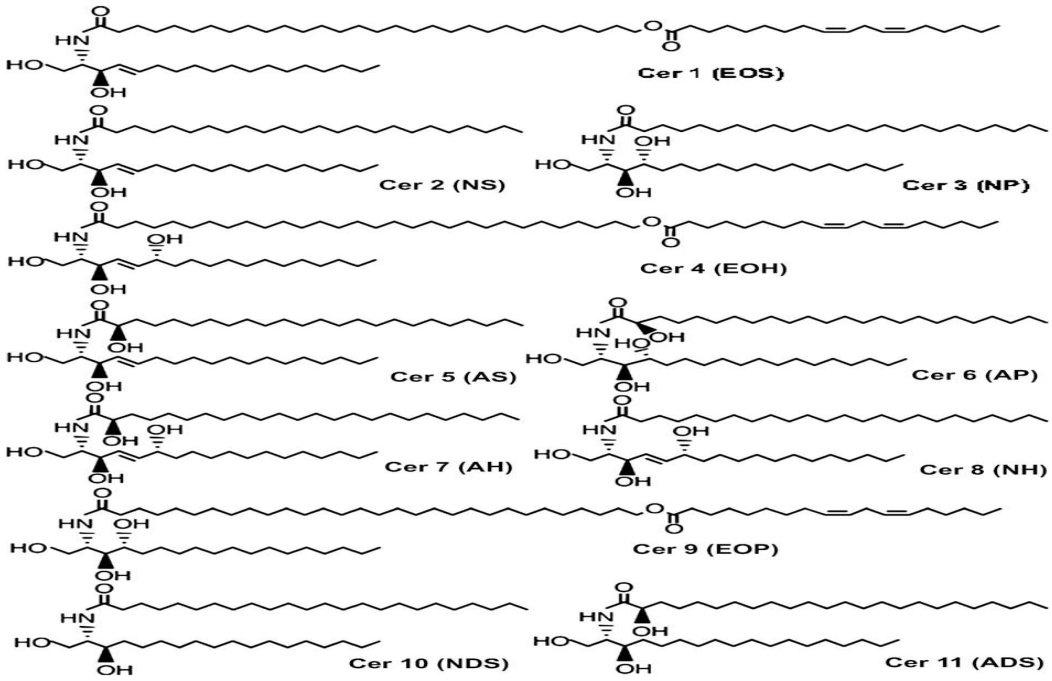

Figure 1.5 Structures of different CERs in the SC of humans. Nomenclature after Masukawa et al. N, A, and EO indicate amide-linked FA species: N, non-hydroxy FA; A, a-hydroxy FA; and EO, u-O-esterified FA. S, DS, P, and H indicate sphingoid bases: S, Sph; DS, dihydro-Sph; P, phyto-Sph; and H, 6-hydroxy-Sph.

Ceramides are synthesized *de novo* by keratinocytes via the pathway outlined in Figure 1.6. The first step is the condensation of palmitate and serine to form 3-keto-dihydrosphingosine. This reaction is catalyzed by *serine palmitoyltransferase* with long chain substrate affinity (SPTLC) and is the rate-limiting step of the pathway, with S*PTLC2* being predominantly expressed in keratinocytes (Farrell et al., 1998),. This is followed by the reduction of 3-keto-dihydrosphingosine to dihydrosphingosine, which is then acylated by *(dihydro)ceramide synthase (CerS)* to produce dihydroceramide. Finally, ceramide is produced by the action of *dihydroceramide desaturase* (DES).

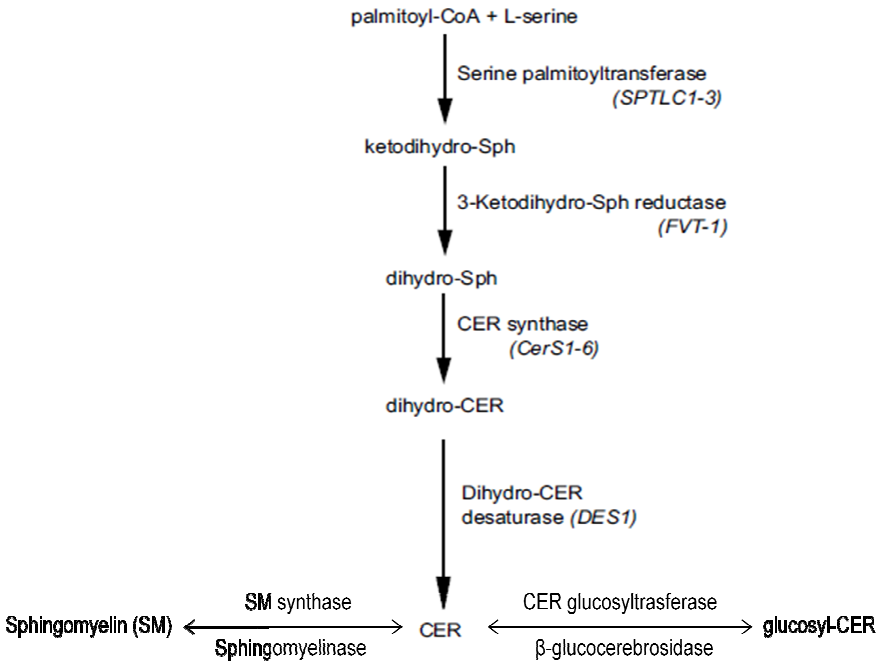

Figure 1.6 Pathway of ceramide synthesis
(Adopted from Mizutani et al)

1.4.2 CHOLESTEROL

Cholesterol is synthesized by a complex sequence of reactions called the mevalonate pathway. This pathway begins with the condensation of two acetyl-CoA units to form acetocetyl-CoA, which is condensed with another acetyl-CoA unit to make 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), a reaction catalysed by *HMG-CoA synthase*. Subsequently, HMG-CoA is reduced irreversibly to mavelonic acid, by *HMG-CoA*

reductase (HMGCR). This is the rate limiting, regulated step of the pathway. From here, there are 34 more steps which lead to the synthesis of cholesterol, with important intermediaries such as lanosterol and lathosterol, which are diverging points for the synthesis of other biologically important steroids.
Cholesterol can also be taken up by basal keratinocytes by means of low-*density lipoprotein (LDL)* receptors, which mediate the endocytosis of LDL particles that are the vehicles for the transport of cholesterol in circulation (Ponec et al., 1992)

1.4.3 FATTY ACIDS

Biosynthesis of fatty acids (FAs) is initiated by the carboxylation of acetyl-CoA to malonyl CoA by *acetyl Co-A carboxylase beta (ACCβ or ACACB)*, a reaction that is also the rate limiting step for the entire process. Malonyl CoA, in turn, forms the substrate for *fatty acid synthase-1 (FAS1),* a large enzyme complex that sequentially adds carbons to malonyl CoA, leading to the synthesis of palmitate. There are mechanisms to further modify the synthesized saturated fatty acid, such as by elongation of chain length and adding unsaturation or a side group, to make a diverse range of biologically active fatty acids.
Additionally, FA uptake by the action of *fatty acid transport protein-4 (FATP4)* plays an important role in keratinocytes (Khnykin et al., 2011; Schmuth et al., 2005), because certain FAs, which play a vital role in barrier function, cannot be synthesized de novo in the epidermis. These essential fatty acids (EFAs), a notable example of which is linoleic acid, need to be taken up from circulation and their deficiency leads to dermatitis and impaired permeability barrier function (Schürer et al., 1999).

1.4.4 TRIGLYCERIDES

In keratinocytes, triglyceride synthesis is majorly through the glycerophosphate or Kennedy pathway, whose starting substrate is glycerol-3-phosphate (G-3-P). An outline of the pathway is given in Figure 1.7. One fatty acyl chain is added to the 1’ position of G-3-P, mediated by *G-3-P acyltransferase (GPAT)* to yield lysophosphatidic acid (LPA), which is acted upon by *AGPAT*, also called *LPAAT*, giving phosphatidic acid. PA is dephosphorylated by *lipin,* also called PA phosphatase, to give diacylglycerol (DAG). PA and DAG are branch points from which synthesis of phospholipids may be initiated.

The final step is the addition of one more fatty acid chain to DAG by the action of *DGAT* enzyme to yield triacylglycerol. The isoforms of the key enzymes in this pathway found to be expressed in the epidermis are GPAT 1,3; *Lipin 1,2*; and *DGAT2* (Jiang and Feingold, 2011a).

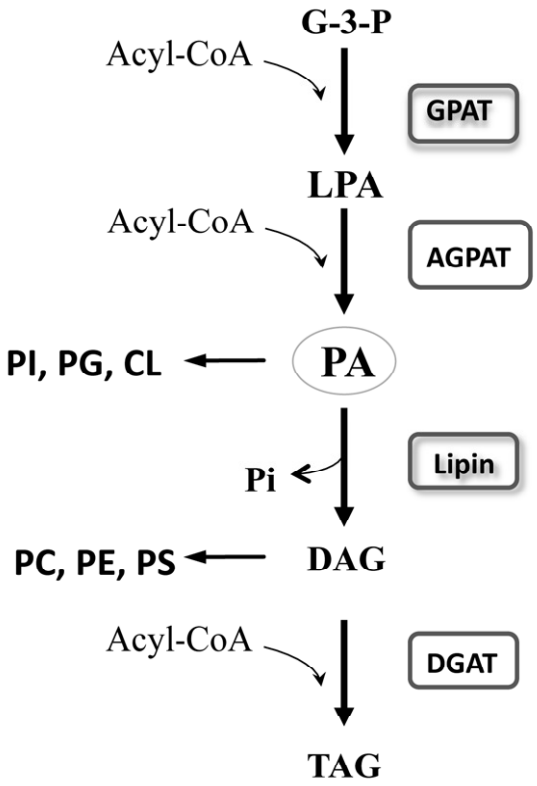

Figure 1.7; The glycerophosphate pathway for triglyceride/phospholipid synthesis (Adopted from Feingold and Jiang, 2009)

1.5. LIPIDS AND PATHOLOGICAL CONDITIONS

The importance of lipid in the formation of efficient skin barrier is evident from the various diseases, pathological conditions and animal model studies. **Dorfman-Chanarin syndrome (DCS)** is a neutral lipid storage disease caused by mutations in the CGI-58 gene (Ujihara et al., 2010), which is an activator of adipocyte triglyceride lipase (ATGL). This leads to accumulation of triglycerides in a number of tissues including the epidermis, where it results in ichthyosis, defined by dry, flaky or scaly skin. This gives evidence that at least a part of the total barrier lipids are derived from products of TG catabolism. **DGAT2 knockdown mice** are known to be born with

significant permeability barrier defects and die shortly after birth due to excessive water loss (Jiang and Feingold, 2011a). As expected triglyceride content of *Dgat2*$^{-/-}$ mice epidermis is 96% lower, but levels of other lipids are not significantly different. These models also show lower amounts of epidermal linoleic acid, which might one of the reasons behind loss of functional barrier. **Atopic dermatitis** is characterized by impaired barrier function and associated high trans-epidermal water loss (TEWL) with patients showing higher levels of cholesterol and lower ceramides in the skin (Di Nardo et al., 1998). **Xerosis**, or parching of skin, which can be a seasonal disorder or a symptom, is also associated with higher TEWL (Saint-Léger et al., 1989), with depletion in cholesterol levels. In **lamellar ichthyosis**, covalent linking of ω-acylceramides to the corneocyte envelope is impaired leading to dramatic changes in SC phenotype and loss of barrier function (Huber et al., 1995). Whereas in psoriatic skin, the corneal envelope bound ceramides containing linoleate are seen to increase, while levels of Cer-1 decrease, giving rise to formation of local plaques and barrier impediment (Motta et al., 1994; Proksch et al., 2008). **Recessive X-linked ichthyosis** is caused by mutations in the gene *sterol sulphatase (STS)*, whose substrate in the epidermis is cholesterol sulphate (CSO4). CSO4 is extruded from the lamellar bodies at the SC-SG interface, where it can be acted upon by intercellular STS, thus supplementing a fraction of SC cholesterol, while the progressive decline in CSO4, which is a serine protease inhibitor, permits corneodesmosome degradation leading to normal desquamation (Zettersten et al., 1998). **UV-linked photoaging**: Fatty acid and triglyceride levels are found to decrease in UV-exposed skin taken from human volunteers. *Matrix mettaloproteinase-1*, a collegenase known to cause UV-induced skin loosening, has been shown to be downregulated upon treatment with triolein, both in human cultured keratinocytes as well as murine epidermis (Kim et al., 2010).

It is thus very well established that defects of lipid metabolism in keratinocytes can potentially lead to altered skin barrier functions and various pathological conditions.

1.6. AIM AND OBJECTIVE OF THE STUDY

Inter-racial differences in skin phenotype beyond pigmentation level or “colour” have been reported (Rawlings, 2006). Racial susceptibility to transcutaneous penetration to nicotinates showed that black skin resists the penetration of some chemicals better than whites which could be due to differences in surface lipid (Berardesca and Maibach, 1996; RICHARDS et al., 2003). There were significant differences in TEWL among the ethnic groups with TEWL at baseline being higher in blacks than white (Wesley and Maibach, 2003; WILSON et al., 1988) along with the desquamation rate which was also 2.5 times higher in blacks than whites. This was suggested to be the reason for increased skin xerosis seen in blacks. It has also been shown that levels of SC ceramides are significantly higher among Asians, followed by Caucasians and Negroes, which are inversely co-related with barrier function as measured by trans-epidermal water loss or TEWL (Sugino et al., 1993).

So far very no studies have been published on skin functions or skin lipids in Indian Asian population. A Unilever study carried out on solvent-extracted skin surface lipids from Indian volunteers showed a large variation in their lipid profile from that published for Caucasian population using similar methodology as well as in general.

These studies suggest that there may be physiological difference in the keratinocyte lipid metabolism in different ethnic groups and this could be attributed to possible difference in their skin barrier functions. It is possible that the observed difference in skin lipid profile is genetic or may be induced due to the diet or environment. Therefore in the present study we aimed to evaluate differences in vitro, if any, in the lipid profile of primary keratinocytes derived from Caucasian and Indian origin. Further we also evaluated the lipid profile of these cells in a modified environment in the presence of higher levels of glucose and LDL. Possible differences in the expression of the genes involved in the cellular lipid metabolism were also evaluated in the keratinocytes of different racial origin. This will give an indication if there are inter-racial differences in lipid metabolism at the phenotypic and at the molecular level.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

Human primary keratinocytes from neonatal foreskin, and of Caucasian (sourced from Lonza, India) as well as Indian origin (derived from discarded clinical samples) were cultured in flasks (Greiner Bio-One, Germany) with Cascade Biologics® Epilife™ medium (Invitrogen, India). At confluence, cells were split 1:3, and expanded up to the sixth passage. Seeding for experiments was done between the 3^{rd} and 6^{th} passages in 12-well culture plates (BD-Falcon) at a density of $5X10^4$ cells/well. Cells were harvested, for lipids by trypsinizing and the pellets were stored at -70^oC until used; and for RNA analysis by lysing with RLT buffer (Qiagen), and storing the lysate at -70^oC until used.

Treatments

Indian and Caucasian primary keratinocytes were seeded in 12 well plates as mentioned before. After 48hrs of plating the cells were treated with following.

i. Ca^{2+}: CaCl2 (Merck Millipore, India), which dissociates to give Ca2+ ions- a known inducer of differentiation (Watt and Green, 1982), was added to the culture media to give a final concentration of 2mM

After 48 hours of Ca^{2+} treatment, both differentiated as well as control cells were treated with the following for 48 hours

ii. LDL: Cells were treated with media enriched with 20 ppm LDL (Sigma-Aldrich, India)

iii. Glucose: Media was supplemented with additional 1.5 mg/mL dextrose (Sigma-Aldrich, India).

The timeline for seeding, treatment and harvesting is illustrated in Figure 2.1.

Samples have been given abbreviated labels, which will also be used in subsequent discussions.

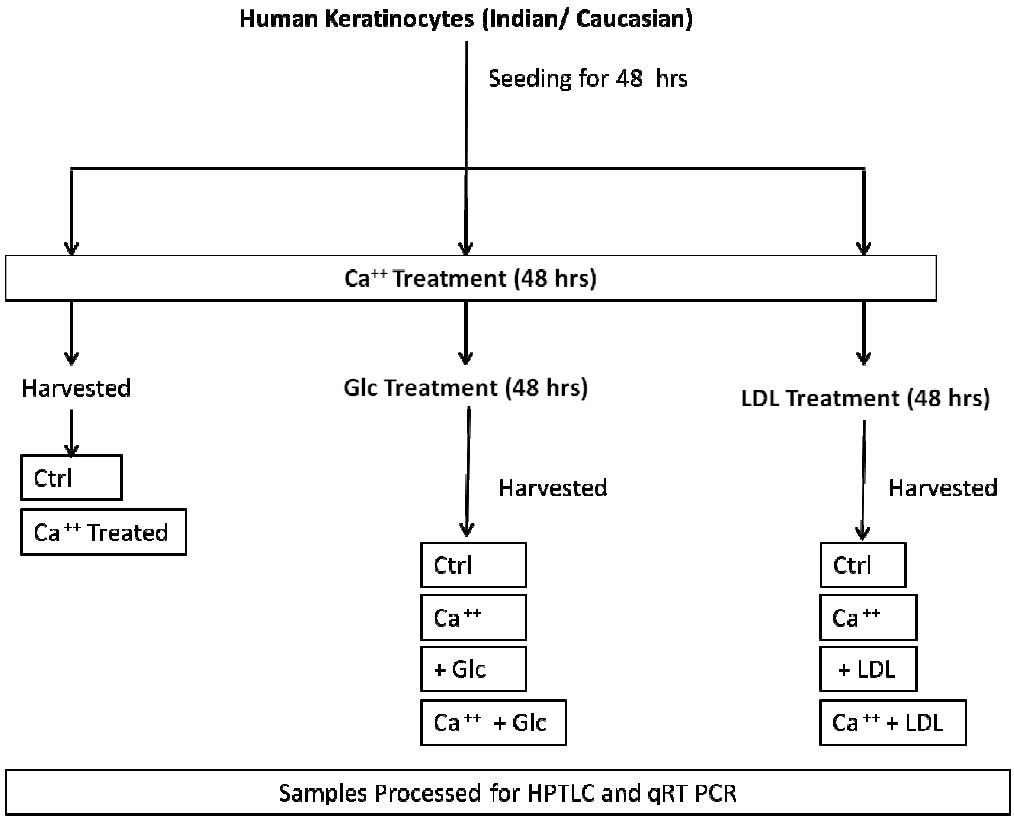

Figure 2.1 Cell culture methodology with timelines and sample labels

2.2. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Cell pellets were homogenized in 1 mL water by sonication and lipid extraction was conducted as per the Bligh and Dyer method (Bligh and Dyer, 1959). Briefly, 1.25 mL chloroform ($CHCl_3$) and 2.5 mL methanol (MeOH) were added to the homogenized sample in a 15 mL centrifuge tube and the mixture vortexed for 1 minute, following which 1.25 mL of water and 1.25 mL of $CHCl_3$ were added and again vortexed for 1 minute. The tube was stored overnight at 4°C in a standing position, to facilitate separation of the mixture into $CHCl_3$ and MeOH/H_2O layer. The $CHCl_3$ layer, which should contain the majority of extractable lipids, was dried under vaccum (Rotavac, Heidolph, Germany) and the lipid mixture was reconstituted in 200-300µL of 1:1 $CHCl_3$ + MeOH. Standard mixtures were prepared which contained 100 ppm each of ceramides (Cer-2), fatty acids (oleic acid), triglycerides (triolein), cholesterol, cholesterol-3-sulphate, cholesterol esters (cholesterol oleate), phospholipids(PC), sphingosine and squalene (all standards from Sigma, India).

High performance thin layer chromatography (HPTLC) is a high throughput separation technique involving automated sample application onto a TLC plate, chromatographic separation, and post-separation analysis. HPTLC is a standard method for the routine analysis of biological lipids (Shantha and Napolitano, 1998). HPTLC apparatus, consisiting of sample applicator, plate documenter and densitometric scanner were from CAMAG, Switzerland. Samples and standards were applied onto a 20X20 Silica-60 gel TLC plate (Merck, Germany) using the CAMAG Linomat 5 HPTLC sample applicator. Chromatographic separation was done using three solvent systems developed up to different heights to clearly resolve all lipids: $CHCl_3$+MeOH+H_2O (95:20:1), till 6 cm (performed twice); n-hexane + diethyl ether + acetic acid (80:20:1) till 8.5 cm; and PET benzene, till 13 cm (all solvents were analysis grade from Merck, India). After separation, the plates were visualized under long-UV light (366 nm). Derivatization was done using a 3% solution of $CuSO_4$ in 8% o-phosphoric acid, which was sprayed onto the plate, followed by drying and charring at 120^oC for 15 minutes to visualize bands. Scanning of bands was done using the CAMAG TLC Scanner3; scanning was at 340nm, peak integration was performed using the standard algorithm built into CAMAG's "WinCATS" TLC management software.

2.3. PROTEIN ESTIMATION

Protein concentrations in samples were estimated by Bradford assay (Bradford, 1976), standardized for a 24-well microtitre plate (BD Falcon™, India) format. Briefly 0-5 ug of standard protein- bovine serum albumin (Sigma-Aldrich, India) was added to 6 different wells, and the volume was made up to 100uL using distilled water. Similarly, 10-20 uL test protein solution was dispensed in different wells, and volume made up to 100uL. Then, 400 uL of Bradford's solution (100mg Coomassie Brilliant Blue in 50 mL methanol + 100 mL conc phosphoric acid; q.s. to 1000mL with distilled water) was added per well, and the plate was covered with aluminum foil and incubated for 15 minutes at room temperature. Absorbance was measured at 540 nm using a TECAN M1000 plate reader (TECAN, Switzerland). A representative standard curve is shown in figure 2.1.

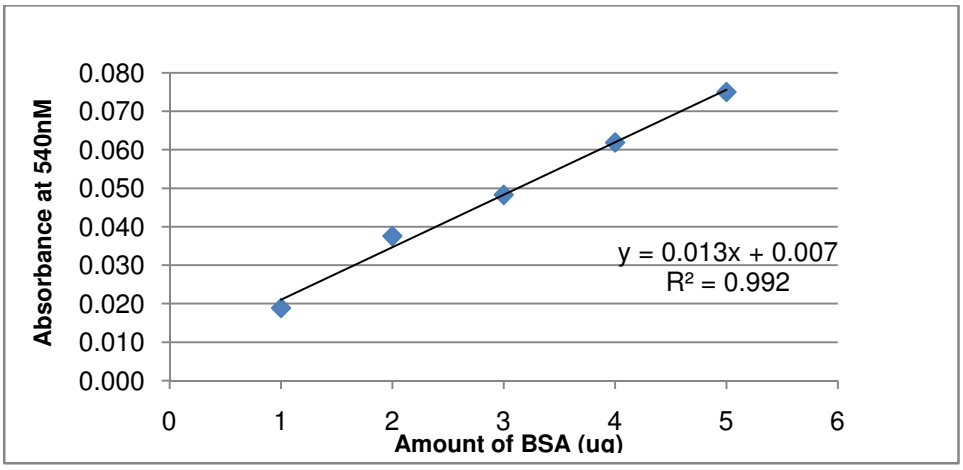

Figure 2.2 Standard curve for standard protein (BSA)

2.4. QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

2.4.1 RNA EXTRACTION

RNA extraction from the RLT lysed cell samples was carried out using RNeasy minikit (Qiagen, Germany) according to manufacturer's protocol. RNA was eluted with 20-30 uL of Ultrapure water. The purity and the concentration of extracted samples were checked spectro-photometrically using the Nanodrop 2000 (Thermo Scientific, USA). RNA samples were stored at -70℃ until used.

2.4.2 cDNA SYNTHESIS

cDNA synthesis for Real Time PCR was carried out by using iScript cDNA synthesis kit (Bio-Rad, USA). An aliquot of total RNA (250 to 500ng) was used to make cDNA by using the cDNA synthesis program. In brief, sample was pre denatured at 65℃ for 5min followed by 25℃ for 5min, and then cDNA synthesis was done at 42℃ for 30min. After cDNA synthesis sample was denatured at 85℃ 5min and then were left at 4℃ till removed.

2.4.3 REAL TIME PCR

Real time PCR was carried out using SYBR green method as per the manufacturer's (Bio-Rad, USA) instructions using thermal controlled Chromo4 machine (Bio-Rad, USA). 1-1.5pM of specific primers were used in each reaction by using a standardized PCR program i.e. "94℃ for 5min" followed by 50 cycles of "94℃ for 15sec, 60℃ for 30sec, 72℃ for 30sec" and results were analyzed by using **2–ΔΔCT** method (Pfaffl, 2001).

3. RESULTS AND DISCUSSION

Skin is the major barrier tissue protecting the body from unwanted influences from the environment. The organization of lipid domains on stratum corneum is considered to be very important for skin barrier function. The lipid matrix constitutes approximately 20% of SC volume (about 15% of dry weight). An accurate picture of epidermal lipid composition and SC lipid composition was established in 1980s (Melnik et al., 1989), wherein thin layer chromatographic separation of solvent extractable lipids from SC revealed an unusual lipid composition consisting of roughly equimolar mixture of ceramides (45-50%), cholesterol (25%), free fatty acids (10-15%) and 5% each of some other lipids, like cholesterol esters, cholesterol sulphate and glycosylceramides which seems to play a critical role in normal barrier function. SC lipids are generally synthesised and stored in lamellar bodies as precursors lipids (glucocylceramides, cholesterol, glycerophospholipid) along with enzymes involved in its processing, in keratinocytes. These precursor lipids are extruded by lamellar bodies at the stratum granulosum (SG)-SC interface, and lipids, along with hydrolytic enzymes are discharged into the intercellular spaces (Holleran et al., 1993; Mao-Qiang et al., 1995, 1996). These enzymes then convert precursor lipids into ceramide, cholesterol and fatty acids, the organization of which in proper ratio is essential to maintain the SC integrity.

The synthesis and metabolism of the major lipids is well studied in Keratinocytes (Feingold, 2011). However not much is known about triglyceride metabolism in keratinocytes except for few recent studies by Feingold et al. Ethnic differences in the skin barrier as well as skin parameters have been suggested (Rawlings, 2006), but so far, no studies have been carried out to determine the difference in skin lipid in various ethnic population. This would be the first attempt in this direction.

Our study therefore aimed to evaluate differences in keratinocytes from Indian and Caucasian origin cultured *in vitro*, under different growth conditions, by comparing cellular lipid profiles by HPTLC and understand the molecular basis behind these by studying the expression profiles of genes known to be involved in lipid metabolism.

3.1. LIPID PROFILES OF KERATINOCYTES

Indian and Caucasian keratinocytes at 3rd to 6th passage were cultured for 48hrs post incubation and then for another 48 hrs in presence or absence of 2mM $CaCl_2$; as Ca^{2+} is known to induce differentiation in cultured keratinocytes (Ponec et al., 1988).
As shown in Figure 3.1, keratinocytes in presence of Ca2+ undergo typical morphological changes associated with differentiation viz. loss of polygonal morphology, appearance of stratification etc. These cells were also tested for involucrin gene which is established marker of keratinocyte differentiation by qRT-PCR (data not shown).

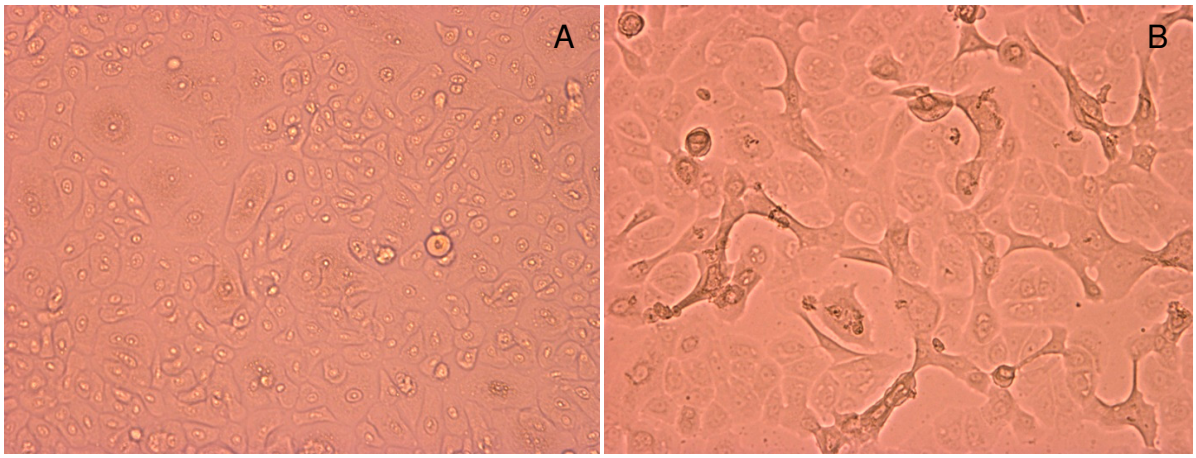

Figure 3.1 Cultured Keratinocytes(A)Incubated with normal media (B)Incubated with media supplemented with 2mM CaCl2

Both the treated and untreated cells were harvested and lipids were extracted as mentioned in Materials and methods. Cellular lipids were analyzed using various lipid standards as mentioned in Materials and methods. A test plate with different concentrations of standard lipids was run, and the chromatograph is shown in Fig. 3.2. Different doses of all lipids were analyzed to get a standard mass calibration curve as shown for triglycerides (triolein) and cholesterol in figure 3.3.
Once lipids were separated on HPTLC using the 3 solvent systems, each of the lanes were scanned using the densitometer and the values of areas under peaks associated with the different lipid classes were calculated by matching Rf values with standards and using the integration tool in the WinCATS software. A representative scan, of standards, is shown in figure 3.4.

A representative HPTLC plate of the cellular lipids extracted from the keratinocytes is shown in figure 3.5.

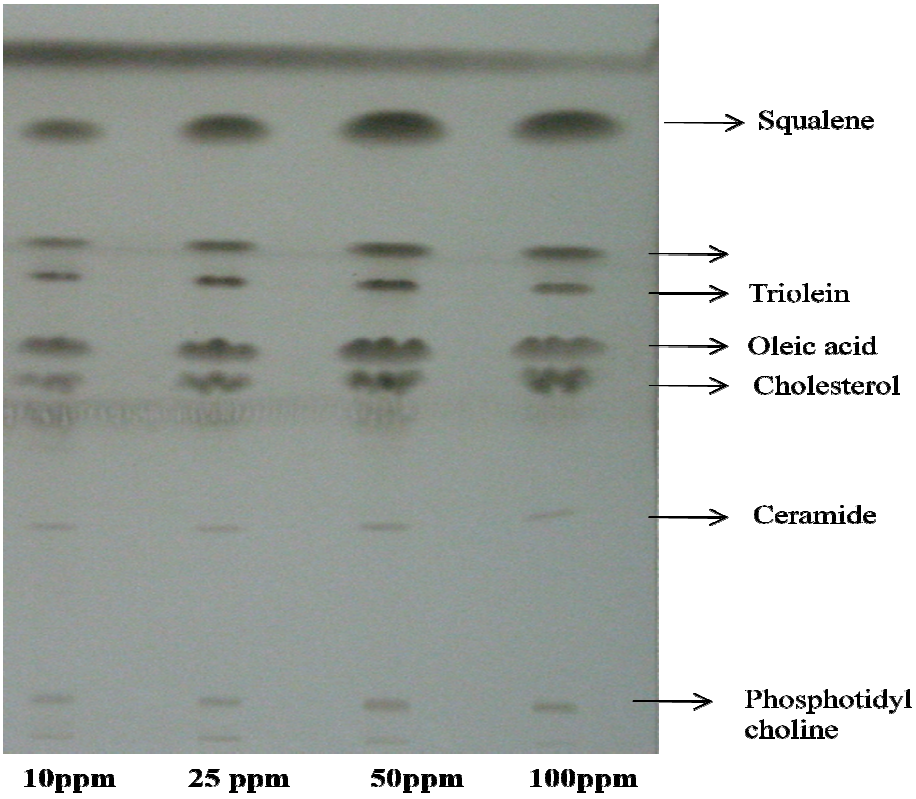

Figure 3.2 Lipid standard mixture of different concentrations run for calibration purpose.

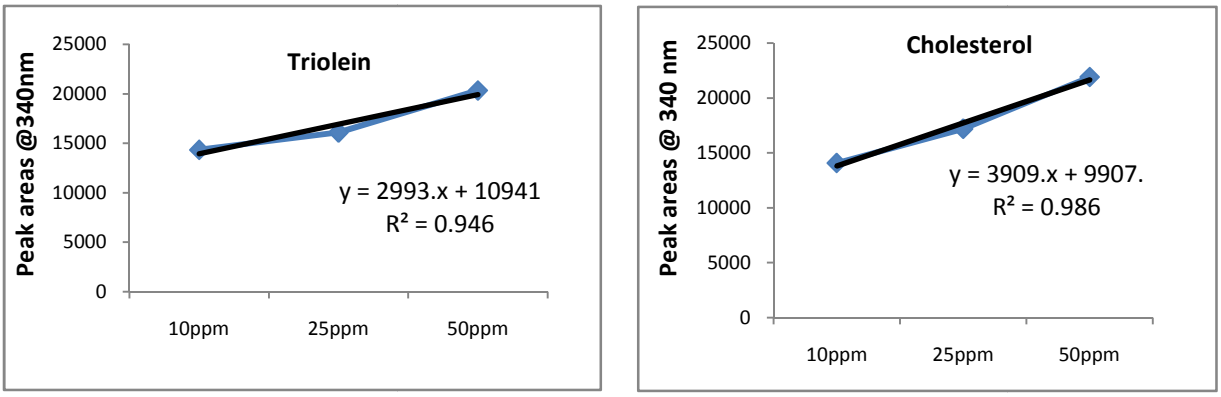

Figure 3.3 Standard curves for Triolein and cholesterol for mass calibration

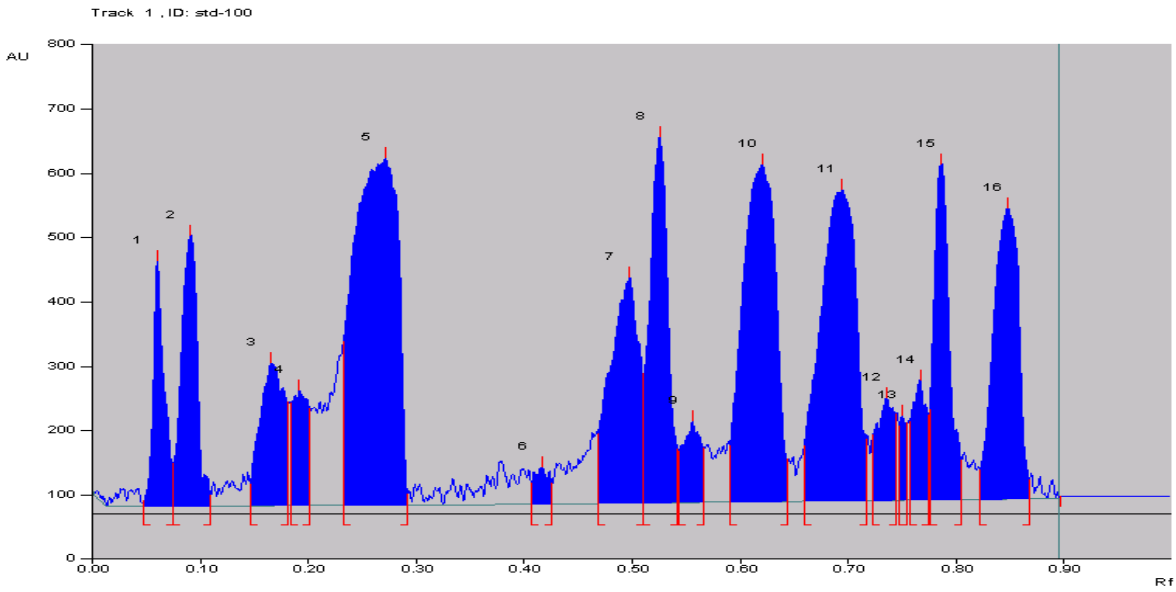

Figure 3.4 Scanning profile of a representative lipid pattern of standards. The peaks are labelled based on the Rf values of each lipid. A chart associated with the graph gives the corresponding peak area of each lipid for each concentration. A calibration curve is obtained for each lipid based on the peak area values and the corresponding concentration.

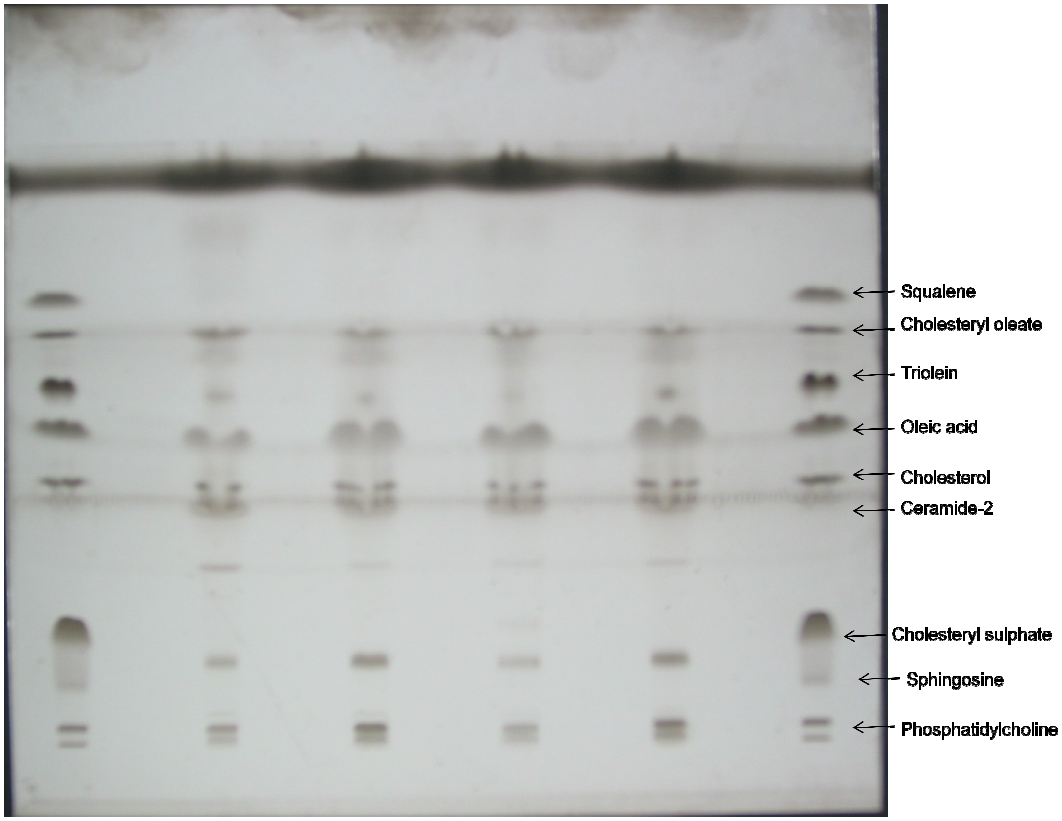

Figure 3.5 HPTLC chromatogram of lipids extracted from cells of Set-2. Lane (1) Standards' mixture (2) NHIEK-untreated (3) NHIEK-differentiated (4) NHCEK-untreated (5) NHCEK-differentiated (6) Standards' mixture

As shown in Figure 3.6 analysis and comparison of lipid profiles of Keratinocytes from both populations did not show much difference in the lipid profile either in proliferating or 48 hr differentiated cultures. However, Cholesterol sulphate (C-3-S) and phospholipids seem to be higher in Caucasian than Indian cells, both in differentiated and proliferating states. C-3-S and phospholipids are the precursors for skin barrier lipids. The data shows average values from analysis of 2 sets of samples (N=2).

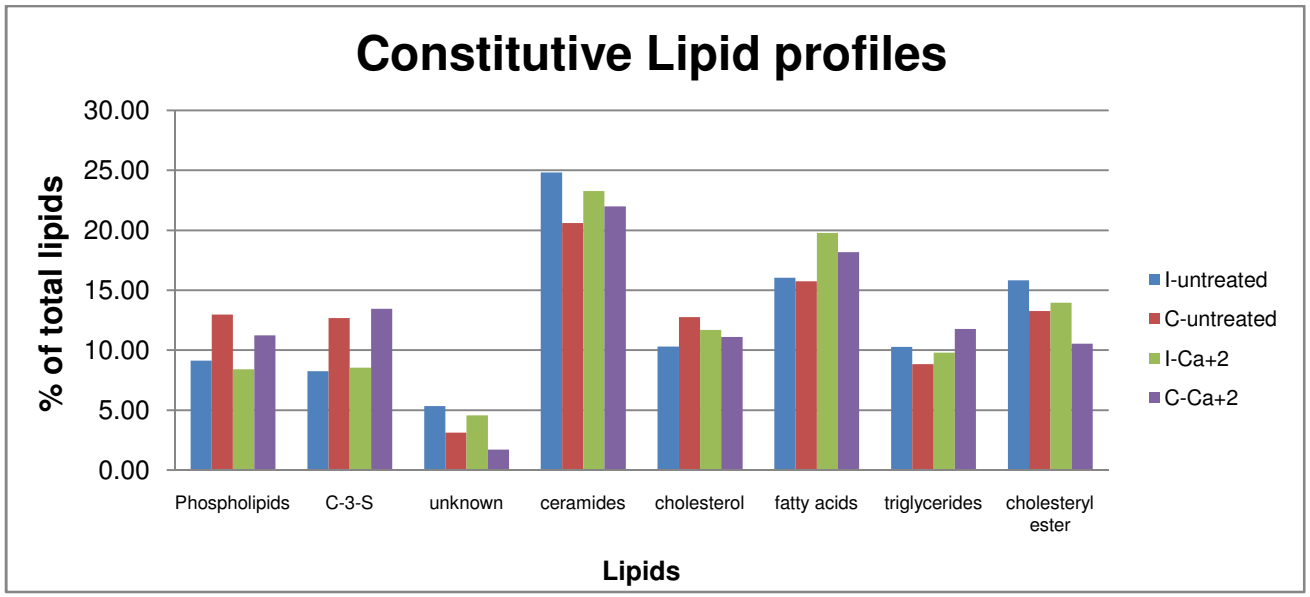

Figure 3.6 Comparison of lipid profile of cauciasian and Indian keratinocytes. The figures represent keratinocytes derived from four different individuals.

3.2. TREATMENT WITH GLUCOSE

To replicate the situation where changes in the physiological environment influences lipid metabolism in the cells; proliferating as well as differentiated keratinocytes were incubated with higher levels of glucose. Keratinocytes were cultured for 48hrs and then either treated or not treated with CaCl2 for inducing differentiation, for 48hrs and then both the sets were exposed to glucose for another 48 hrs.

Figures 3.7 A-G depicts the lipid profile of Indian and Caucasian keratinocytes treated with Ca++, glucose and both as well as respective controls. One set of samples was taken (N=1) for analysis.

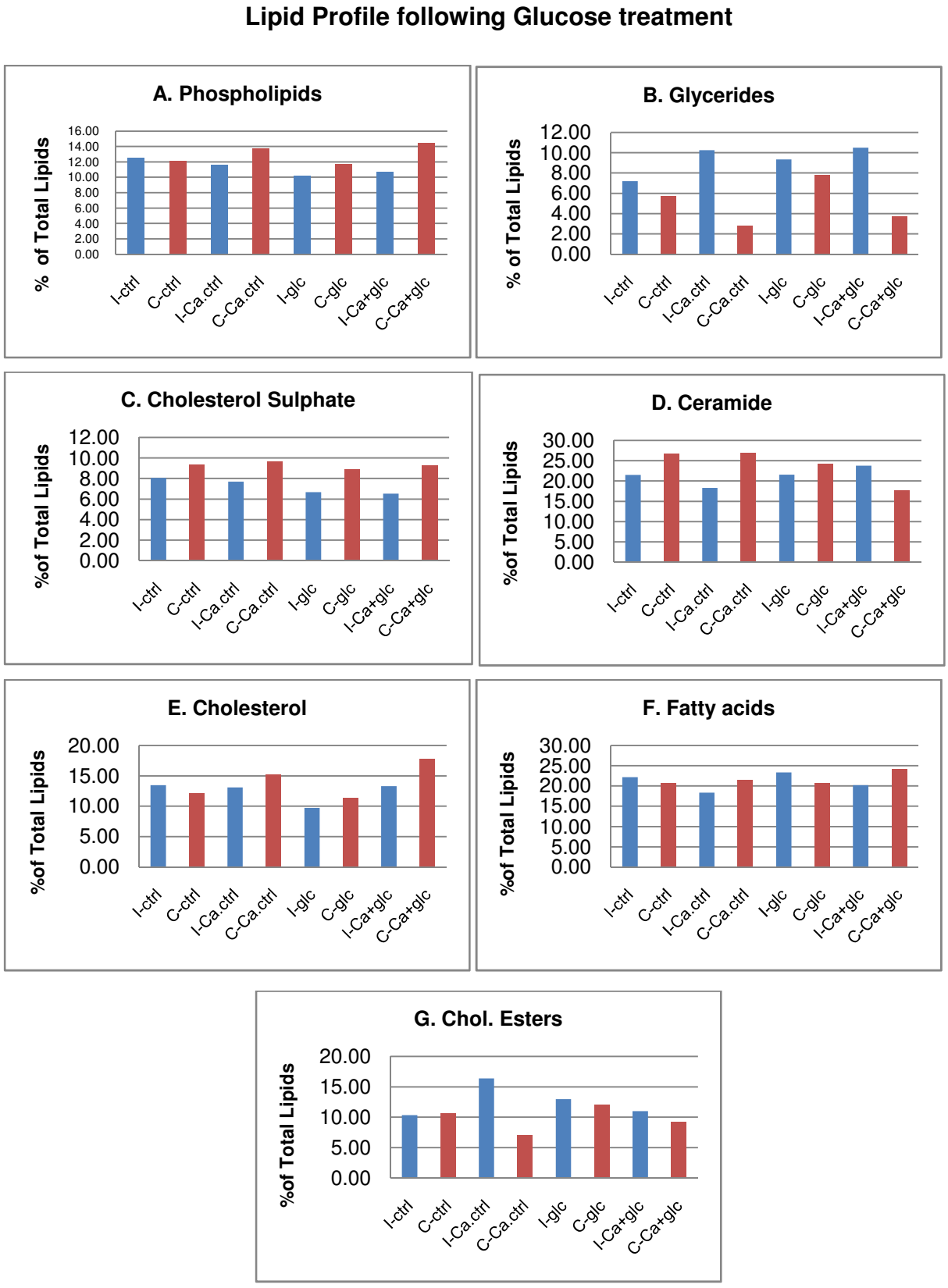

Figure 3.7 Graphs A-G give levels of different lipids in Indian and Caucasian keratinocytes under different growth conditions viz. Ca2+, glucose, Ca2+ and glucose treated, and respective controls.

As seen in Figure 3.7, addition of excess glucose caused increased production of glycerides in Indian keratinocytes as compared to Caucasian keratinocytes.

Exposure to calcium for 96 hrs in case of non glucose treated controls also increased the levels of glycerides as compared to just 48hrs calcium treated cells (figure 3.6). This was also observed in case of cholesterol ester build up in Indian keratinocytes. While addition of glucose to proliferating cells increase glycerides and cholesterol esters to some extent the same is not seen in differentiated keratinocytes.

In Caucasian keratinocytes on the other hand there is also increase in glycerides on exposure to glucose but this ability is lost when the glucose addition occurs after the cells have differentiated.

Further experimentation needs to be conducted with multiple sets of keratinocytes to confirm these observations.

3.3. TREATMENT WITH LDL

There are physiological differences in the plasma lipids and propensity of lipid associated diseases in Asians compared to Caucasians. A considerably high amount of triglyceride has been reported to be present in the blood of Asian Indians (Madhavan et al., 2005; Misra et al., 2004). There is a possibility that this difference in blood lipid profile in these populations may also reflect on their skin surface lipid. One can argue in favor of the said hypothesis as there are plenty of anecdotal evidences for the effect of diet and nutrition on skin health and for uptake of lipids from extracutaneous sites or from blood (Feingold, 2007).

Therefore in the present setup, we also attempted to examine the contribution of circulating lipids in determining the keratinocytes' lipid profile. This investigation would indicate the difference in the mechanism of lipids absorption from the environment between the two populations of keratinocytes. Here, the methodology followed was similar to that for glucose with cells incubated with media enriched with 20 ppm LDL for 48 hrs. Figure 3.8 shows the lipid profiles. One set of samples was taken (N=1) for analysis.

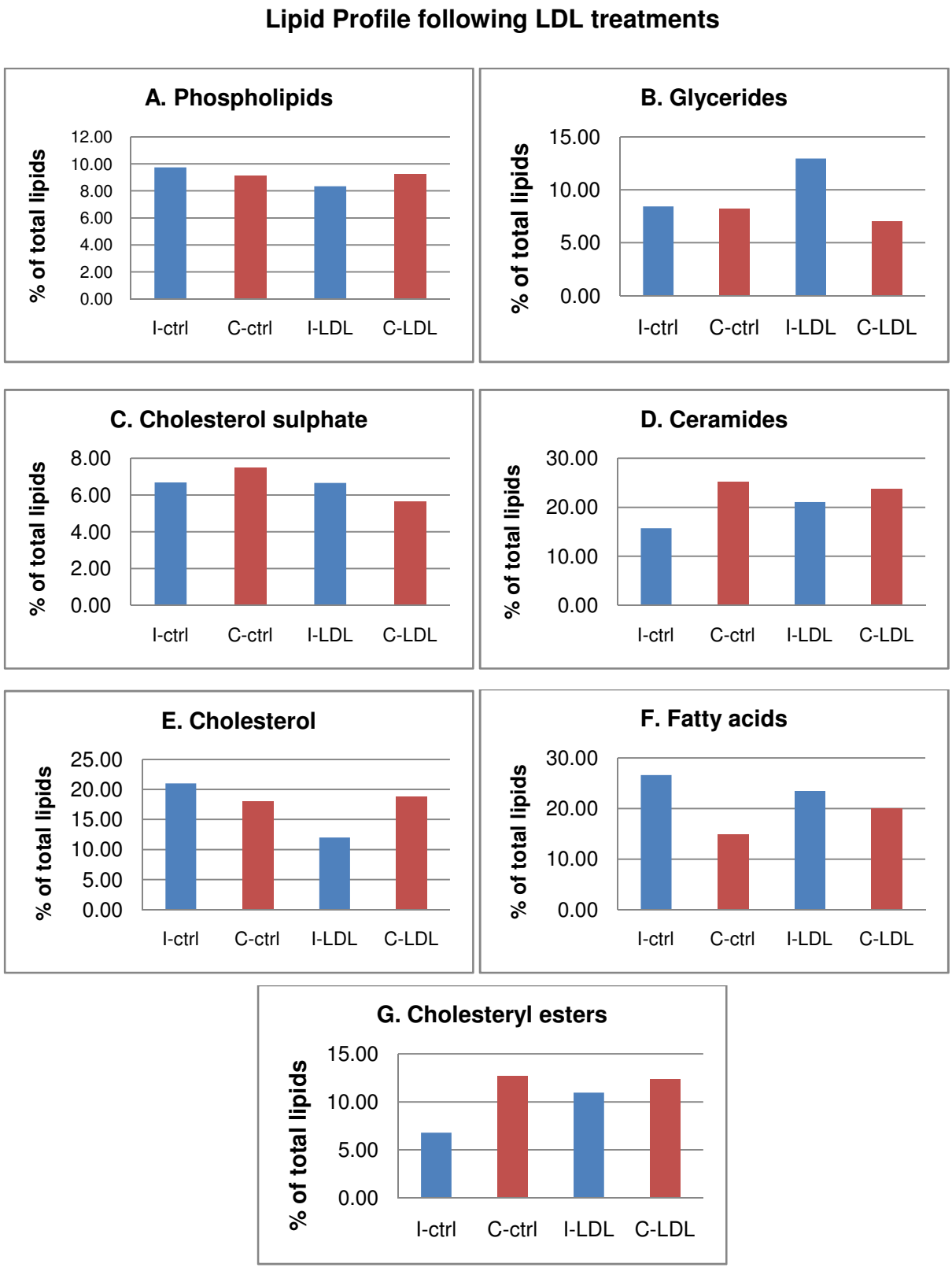

Figure 3.8 Graphs A-G give levels of different lipids in Indian and Caucasian keratinocytes under control and LDL-treated conditions

As shown in figure 3.8 the levels of glycerides and cholesterol ester seems to have increased on addition of LDL while the same is not observed with the Caucasian keratinocytes.

Thus, the current study suggests though the ethnic variations at the constitutive levels do not seem to be apparent with the methodologies used between the Indian and Caucasian keratinocytes. However state of differentiation, presence of glucose and circulating lipids may contribute to variation in the skin lipid profile in Indian Keratinocytes versus Caucasian. Higher sample size and additional human *in vivo* studies would need to be done to confirm the above observations.

3.4. QRT-PCR FOR GENES INVOLVED IN LIPID METABOLISM

Real time PCR was carried out the on the RNA isolated from the treated and untreated keratinocytes from both ethnic origin. A change was considered significant if it 0.5 or more fold different. One set of samples was taken (N=1) for analysis.

The genes studied were the ones involved in FAs and Glyceride synthesis (Figure 3.9), ceramide synthesis (Figure 3.10A) and cholesterol synthesis and absorption/uptake (Figure 3.10B).

As shown in Figure 3.9, ACAB gene expression was found to be higher in Indian keratinocytes compared to Caucasian. As reported earlier (Jiang and Feingold, 2011b), the CT values for GPAT and DGAT were higher than the other genes (~30 cycles) suggesting that the copy numbers of these genes are low in keratinocytes.

Further it was seen that the GPAT expression is differentiation associated in Indian keratinocytes as reported earlier (Feingold et al 2012). GPAT1 was expressed at higher level in proliferating cells (Figure 3.9) which reduced in differentiated cells while the reverse was observed in case of GPAT3. There was no difference in DGAT1 in both cell types but Caucasian keratinocytes had low DGAT2 compared to Indians and moreover on differentiation the transcripts of DGAT2 genes in Indian K was depressed.

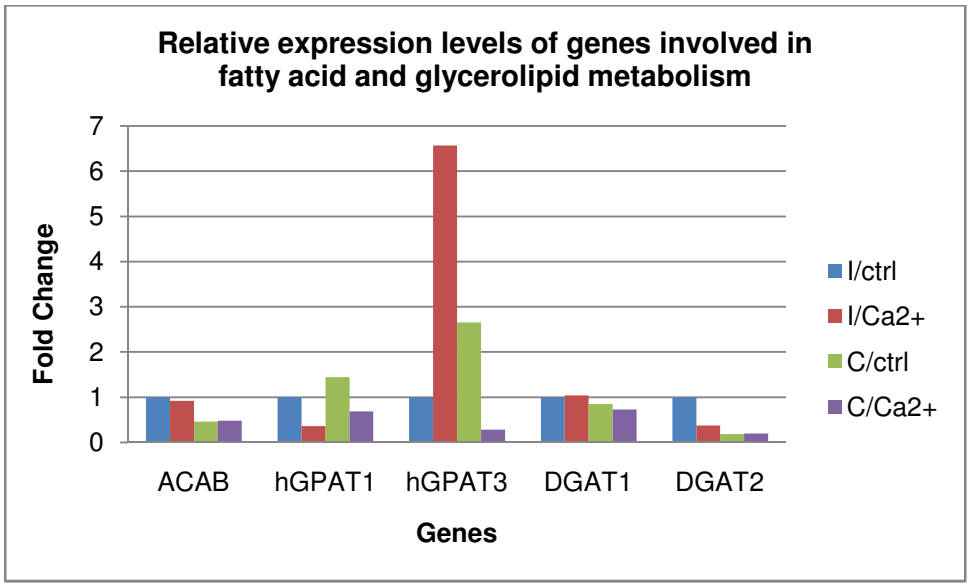

Figure 3.9 Relative expression levels of important genes involved in FA and glycerolipid metabolism in Indian and Caucasian cultured keratinocytes

There was no difference in either cholesterol or ceramide metabolism related genes at constitutive levels or on calcium treatment in both cell types.

The differences in the expression of the genes involved in lipid metabolism in proliferating and differentiating cells suggests that the metabolism of lipids are regulated differently at different stages of keratinocyte growth.

However the differences between keratinocytes of Indian and Caucasian origin would need to be confirmed with few more studies.

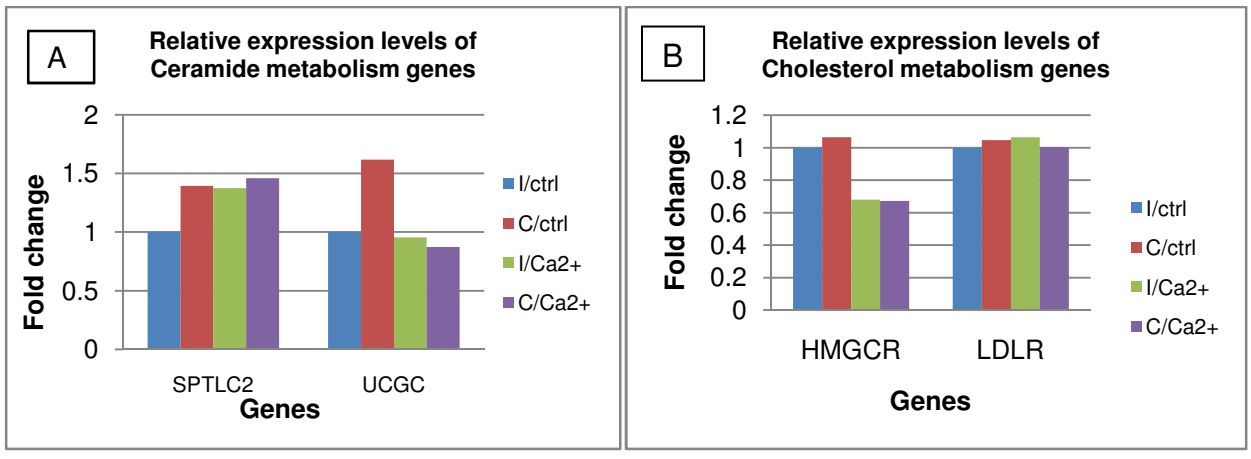

Figure 3.10 Relative expression levels of important genes involved in (A) Ceramide and (B) Cholesterol metabolism in Indian and Caucasian cultured keratinocytes

4. REFERENCES

Berardesca, E., and Maibach, H. (1996). Racial differences in skin pathophysiology. Journal of the American Academy of Dermatology *34*, 667–672.

Bligh, E., and Dyer, W. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and ….

Bouwstra, J.A., and Gooris, G.S. (2010). The Lipid Organisation in Human Stratum Corneum and Model Systems. 10–13.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry *72*, 248–254.

Elias, P.M. (1988). Structure and function of the stratum corneum permeability barrier. Drug Development Research *13*, 97–105.

Farrell, A.M., Uchida, Y., Nagiec, M.M., Harris, I.R., Dickson, R.C., Elias, P.M., and Holleran, W.M. (1998). UVB irradiation up-regulates serine palmitoyltransferase in cultured human keratinocytes. Journal of Lipid Research *39*, 2031–2038.

Feingold, K.R. (1991). The regulation and role of epidermal lipid synthesis. Advances in Lipid Research *24*, 57–82.

Feingold, K.R. (2007). Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. Journal of Lipid Research *48*, 2531–2546.

Feingold, K.R. (2011). Lipid metabolism in the epidermis. Dermato-endocrinology *3*, 52.

Holleran, W.M., Takagi, Y., Menon, G.K., Legler, G., Feingold, K.R., and Elias, P.M. (1993). Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. The Journal of Clinical Investigation *91*, 1656–1664.

Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavrijsen, S., Ponec, M., Bon, A., Lautenschlager, S., Schorderet, D., and Hohl, D. (1995). Mutations of keratinocyte transglutaminase in lamellar ichthyosis. Science *267*, 525–528.

Jiang, Y.J., and Feingold, K.R. (2011a). The expression and regulation of enzymes mediating the biosynthesis of triglycerides and phospholipids in keratinocytes/epidermis. Dermato-endocrinology *3*, 70–76.

Jiang, Y.J., and Feingold, K.R. (2011b). The expression and regulation of enzymes mediating the biosynthesis of triglycerides and phospholipids in keratinocytes/epidermis. Dermato-endocrinology *3*, 70–76.

Khnykin, D., Miner, J.H., and Jahnsen, F. (2011). Role of fatty acid transporters in epidermis: Implications for health and disease. Dermato-endocrinology *3*, 53–61.

Kim, E.J., Jin, X.-J., Kim, Y.K., Oh, I.K., Kim, J.E., Park, C.-H., and Chung, J.H. (2010). UV decreases the synthesis of free fatty acids and triglycerides in the epidermis of human skin in vivo, contributing to development of skin photoaging. Journal of Dermatological Science *57*, 19–26.

Koster, M.I. (2009). Making an epidermis. Annals of the New York Academy of Sciences *1170*, 7–10.

Madhavan, M., Pandey, R.M., Misra, A., Vikram, N.K., Dhingra, V., Luthra, K., and Wasir, J.S. (2005). Centile values for serum lipids and blood pressure for Asian Indian adolescents. Lipids in Health and Disease *4*, 20.

Madison, K.C. (2003). Barrier Function of the Skin: "'La Raison d'EŒ tre"'of the Epidermis. 231–241.

Mao-Qiang, M., Feingold, K., Jain, M., and Elias, P. (1995). Extracellular processing of phospholipids is required for permeability barrier homeostasis. J. Lipid Res. *36*, 1925–1935.

Mao-Qiang, M., Jain, M., Feingold, K.R., and Elias, P.M. (1996). Secretory Phospholipase A2 Activity Is Required for Permeability Barrier Homeostasis. Journal of Investigative Dermatology *106*, 57–63.

Masukawa, Y., Narita, H., Shimizu, E., Kondo, N., Sugai, Y., Oba, T., Homma, R., Ishikawa, J., Takagi, Y., Kitahara, T., et al. (2008). Characterization of overall ceramide species in human stratum corneum. Journal of Lipid Research *49*, 1466–1476.

Melnik, B.C., Hollmann, J., Erler, E., Verhoeven, B., and Plewig, G. (1989). Microanalytical Screening of All Major Stratum Corneum Lipids by Sequential High-performance Thin-layer Chromatography. Journal of Investigative Dermatology *92*, 231–234.

Misra, A., Luthra, K., and Vikram, N.K. (2004). Dyslipidemia in Asian Indians: determinants and significance. The Journal of the Association of Physicians of India *52*, 137–142.

Mizutani, Y., Mitsutake, S., Tsuji, K., Kihara, A., and Igarashi, Y. (2009). Ceramide biosynthesis in keratinocyte and its role in skin function. Biochimie *91*, 784–790.

Motta, S., Monti, M., Sesana, S., Mellesi, L., Ghidoni, R., and Caputo, R. (1994). Abnormality of water barrier function in psoriasis. Role of ceramide fractions. Archives of Dermatology *130*, 452–456.

Di Nardo, A., Wertz, P., Giannetti, A., and Seidenari, S. (1998). Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. Acta Dermato-venereologica *78*, 27–30.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research *29*, e45.

Ponec, M., Weerheim, a, Kempenaar, J., Mommaas, a M., and Nugteren, D.H. (1988). Lipid composition of cultured human keratinocytes in relation to their differentiation. Journal of Lipid Research *29*, 949–961.

Ponec, M., Te Pas, M.F.W., Havekes, L., Boonstra, J., Mommaas, A.M., and Vermeer, B.J. (1992). LDL Receptors in Keratinocytes. Journal of Investigative Dermatology *98*, 50S–56S.

Proksch, E., Brandner, J.M., and Jensen, J.-M. (2008). The skin: an indispensable barrier. Experimental Dermatology *17*, 1063–1072.

Rawlings, A. V (2006). Ethnic skin types: are there differences in skin structure and function? International Journal of Cosmetic Science *28*, 79–93.

RICHARDS, G.M., ORESAJO, C.O., and HALDER, R.M. (2003). Structure and function of ethnic skin and hair. Dermatologic Clinics *21*.

Saint-Léger, D., François, A.M., Lévêque, J.L., Stoudemayer, T.J., Kligman, A.M., and Grove, G. (1989). Stratum corneum lipids in skin xerosis. Dermatologica *178*, 151–155.

Schmuth, M., Ortegon, A.M., Mao-Qiang, M., Elias, P.M., Feingold, K.R., and Stahl, A. (2005). Differential expression of fatty acid transport proteins in epidermis and skin appendages. The Journal of Investigative Dermatology *125*, 1174–1181.

Schürer, N.Y., Rippke, F., Vogelsang, K., Schliep, V., and Ruzicka, T. (1999). Fatty acid uptake by cultured human keratinocytes grown in medium deficient in or supplemented with essential fatty acids. Archives of Dermatological Research *291*, 47–53.

Shantha, N., and Napolitano, G. (1998). Chapter 9. Analysis of Lipids by Thin-Layer Chromatography. In Advanced Chromatographic and Electromlgration Methods In Biosciences, pp. 371–402.

Spearman, R. (1964). The evolution of mammalian keratinized structures. Zoological Society of London. Symposia *12*, 67–81.

Sugino, K., Imokawa, G., and Maibach, H.I. (1993). Ethnic difference of stratum corneum lipid in relation to stratum corneum function. J Invest Dermatol *100*, 587.

Ujihara, M., Nakajima, K., Yamamoto, M., Teraishi, M., Uchida, Y., Akiyama, M., Shimizu, H., and Sano, S. (2010). Epidermal triglyceride levels are correlated with severity of ichthyosis in Dorfman-Chanarin syndrome. Journal of Dermatological Science *57*, 102–107.

Watt, F.M., and Green, H. (1982). Stratification and terminal differentiation of cultured epidermal cells. Nature *295*, 434–436.

Wesley, N.O., and Maibach, H.I. (2003). Racial (Ethnic) Differences in Skin Properties. American Journal of Clinical Dermatology *4*, 843–860.

WILSON, D., BERARDESCA, E., and MAIBACH, H.I. (1988). In vitro transepidermal water loss: differences between black and white human skin. British Journal of Dermatology *119*, 647–652.

Zettersten, E., Man, M.Q., Sato, J., Denda, M., Farrell, A., Ghadially, R., Williams, M.L., Feingold, K.R., and Elias, P.M. (1998). Recessive x-linked ichthyosis: role of cholesterol-sulfate accumulation in the barrier abnormality. The Journal of Investigative Dermatology *111*, 784–790.