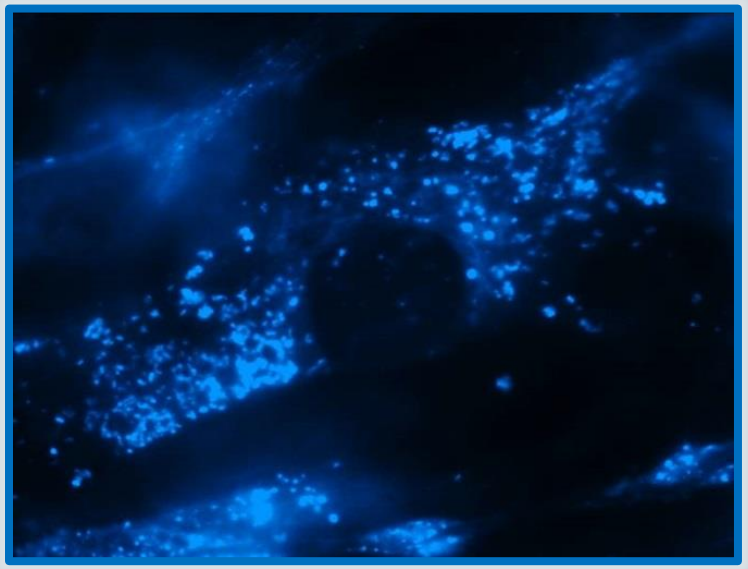
Autophagy as a main driver on Smith-Lemli-Opitz



Maria Luís Cardoso 25-05-2018

Nobel Prize in Physiology or Medicine 2016

The word ***autophagy*** originates from the Greek words *auto (*self) and *phagein*, (to eat). Thus, autophagy denotes "self eating". This concept emerged during the 1960's, when researchers first observed that the cell could destroy its own contents by enclosing it in membranes, forming sack-like vesicles that were transported to a recycling compartment, called the *lysosome,* for degradation.

Difficulties in studying the phenomenon meant that little was known until, in a series of brilliant experiments in the early 1990's, Yoshinori Ohsumi used baker's yeast to identify genes essential for autophagy.

He then went on to elucidate the underlying mechanisms for autophagy in yeast and showed that similar sophisticated machinery is used in our cells.

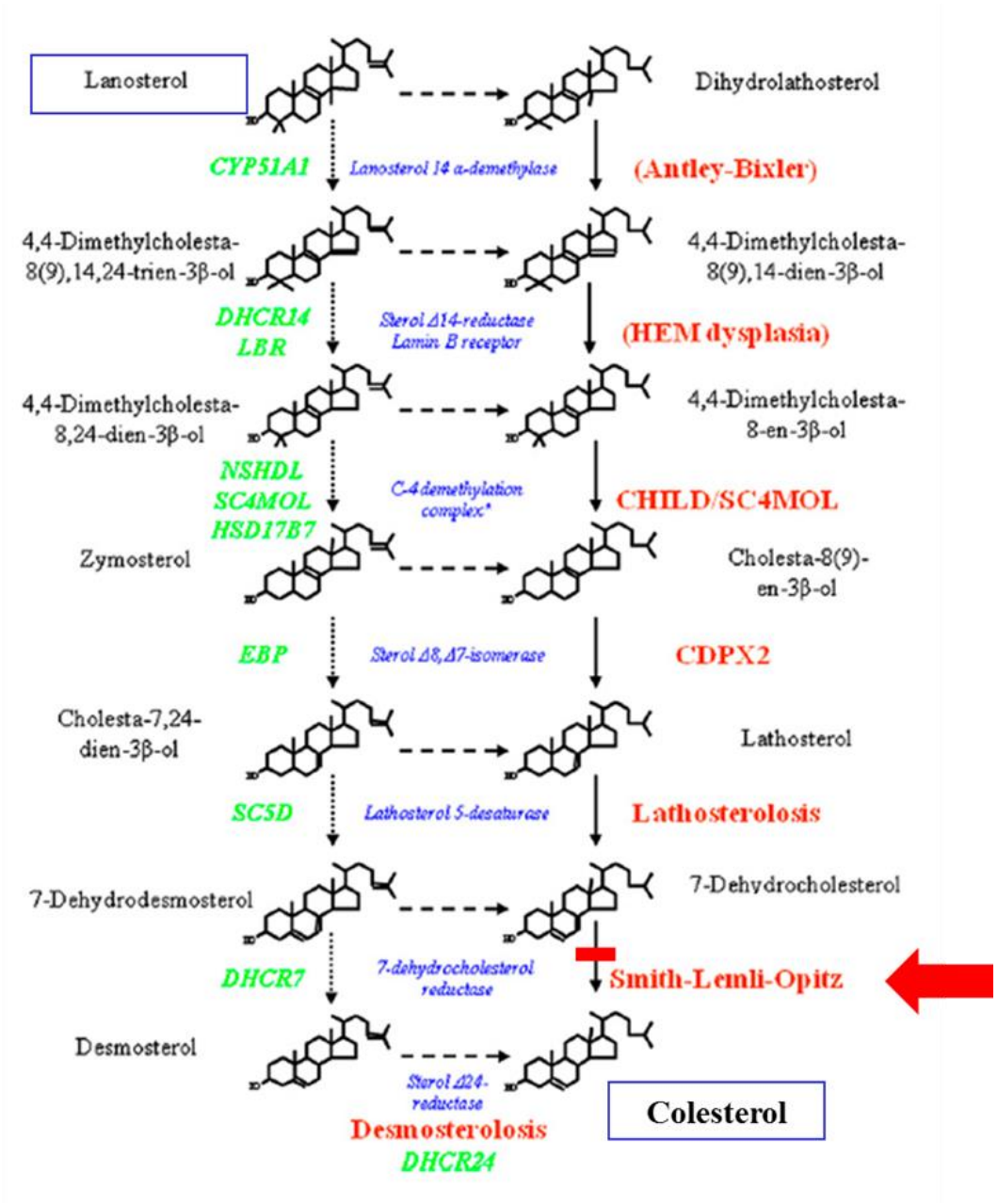
Ohsumi's discoveries led to a new paradigm in our understanding of how the cell recycles its content. His discoveries opened the path to understanding the fundamental importance of autophagy in many physiological processes, such as in the adaptation to starvation or response to infection. Mutations in autophagy genes can cause disease, and the autophagic process is involved in several conditions including cancer and neurological disease.

Yoshinori Ohsumi
Nobel Prize in Physiology or Medicine 2016.

INTRODUCTION

- **Cholesterol** is an important structural component of cellular membranes and myelin and it was found to play an essential role on **embryogenesis and development**.
- In the past most of the scientific investigations concerning this lipid were focused on the consequences of high levels of cholesterol and only a **few studies reported the dramatic consequences of low cholesterol levels for cells.**
- Furthermore such studies were performed using either **chemical inhibitors** of cholesterol biosynthesis either methyl-β-cyclodextrin, bringing to the experimental platform other factors than cholesterol deficiency itself.
- **Individuals with naturally occurring mutations** on the genes codifying for enzymes of cholesterol biosynthesis pathway can provide new and reliable tools for the **study of cellular consequences of low cholesterol availability**.
- In the present investigation we used **fibroblasts from Smith-Lemli-Opitz syndrome patients** (SLOS) - a metabolic genetic disease affecting the cholesterol biosynthesis pathway - to investigate the consequences of cholesterol deficiency on cell morphology and protein expression.

Défices da biossíntese do colesterol



MATERIALS AND METHODS

Morphological studies

- Optical microscopy
- MTT test
- Monodansylcadaverine coloration
- Acridine orange coloration
- Immunocytochemistry for LC3
- Electron microscopy

Proteomic analysis

Cellular homogenates treated with amine-reactive isobaric tagging reagents– iTRAQ were analyzed by LC-MALDI-TOF/TOF–MS.

were performed on fibroblasts from:

SLOS patients + Human controls

naturally deficient in a key enzyme of cholesterol biosynthesis pathway: 7-DHCR

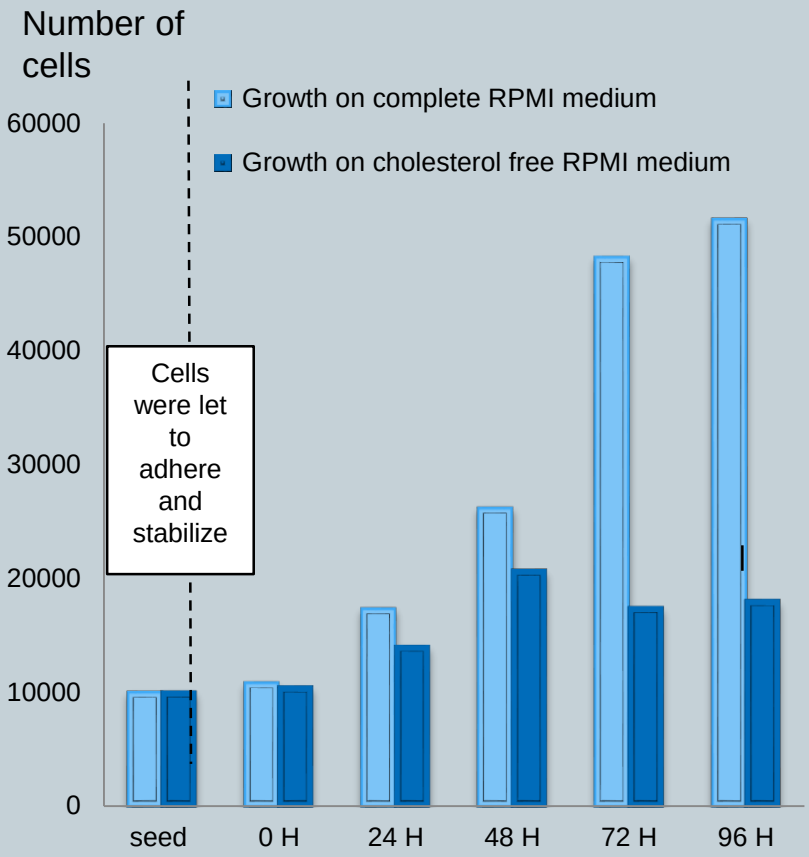
simultaneously cultivated on :

standard conditions

+

cholesterol depleted media

RESULTS
(Morphological studies)



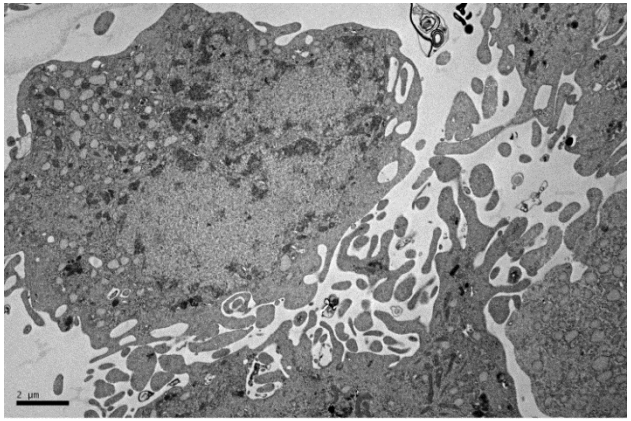
Different growth of 7-DHCR deficient fibroblast subcultures on standard conditions and cholesterol free medium

As expected, **control cells** showed morphology consistent with cells undergoing division during incubation in standard conditions, however when the culture medium was replaced by a cholesterol free one, such cells were forced to adapt their metabolism and to use their own cholesterol biosynthetic enzymatic machinery to produce this lipid, showing small adaptive changes, but maintaining a regular growth in such conditions.

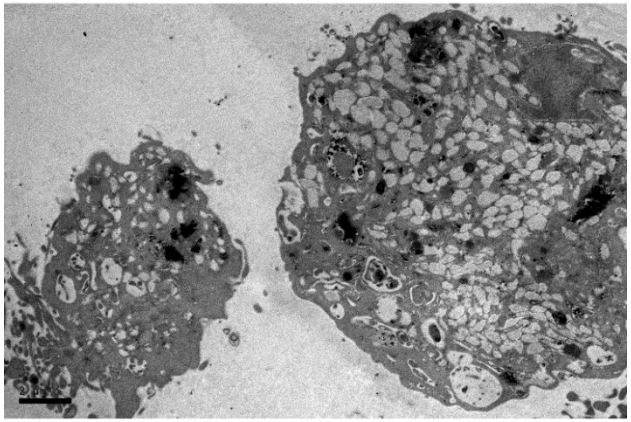
In the other hand, fibroblasts from SLOS patients, **deficient in 7-DHCR** exhibit a compromised cell proliferation which became dramatic by 96h of incubation although no increase cell death was observed.

This result confirm the data previously published (Fernandez et al, 2004) that reported a reduced rate of SLOS cell proliferation *in vitro*, thus reinforcing the idea that **cholesterol availability is critical for cells** whether it is supplied by exogenous lipoproteins or through endogenous synthesis.

RESULTS
(Morphological studies)



a	Normal control - 96h complete RPMI medium



b	SLOS - 96h cholesterol free medium

7-DHCR deficient fibroblasts also displayed morphological changes at ultrastructure.

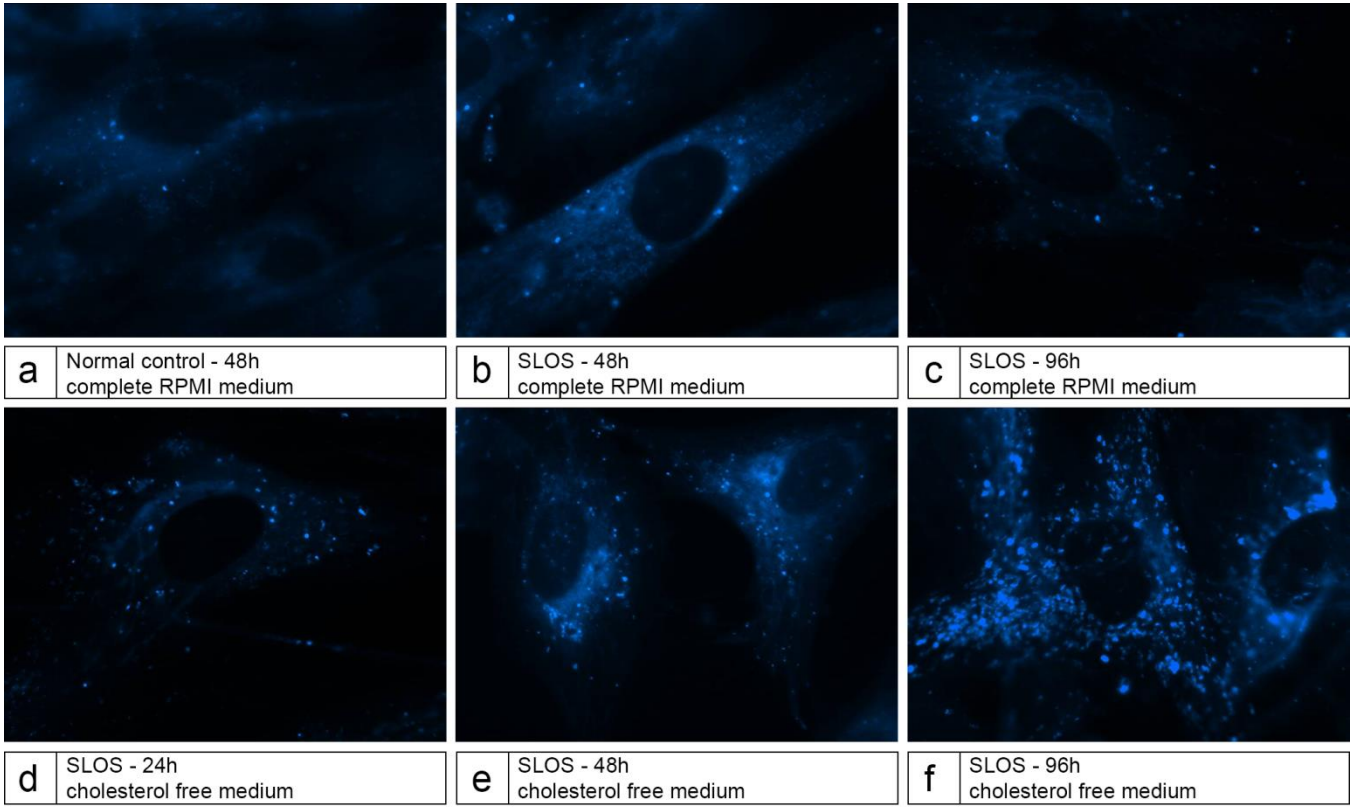
These changes became prominent when cells were cultivated in cholesterol depleted medium.

By 96h of incubation exhibited a **marked amount of vesicular organelles mostly at nucleus periphery .**

Ultrastructural features of normal and 7-DHCR deficient fibroblasts.
(a) Human fibroblasts, showing its characteristic elongated nucleus and organelles.
(b) 7-DHCR deficient fibroblasts by 96h of incubation, in cholesterol free medium, showing prominent structural changes namely a marked amount of vesicular organelles.

In order to characterize the nature of these cytoplasmatic vesicles, two vital autofluorescent dyes **Monodansylcadaverine** (MDC) and **Acridine Orange** (AO) were used. Both compounds differentially stain acid vesicular organelles commonly associated to autophagy.

MDC



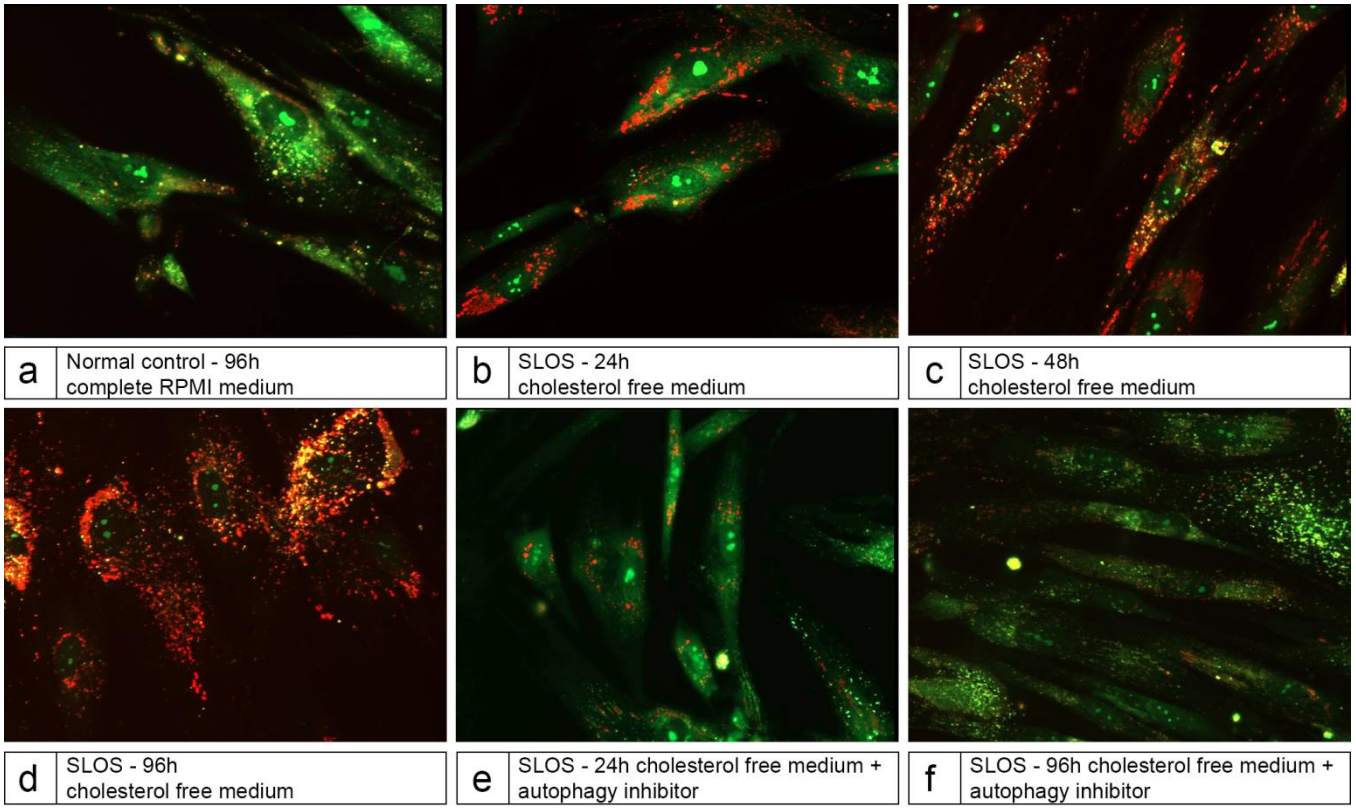
Complete RPMI

Cholesterol Free

Visualization of autophagic vacuoles (fluorescent dot-like structures dispersed in the cytoplasm or in the perinuclear regions) by MDC coloration.
There is **no apparently significant differences** between normal human control fibroblasts (a) and 7-DHCR deficient cells (b, c) when they are cultivated **in standard conditions**.
In the other hand **7-DHCR deficient cells seeded in cholesterol free medium** (d, e, f) show a **progressively increasing number of fluorescent autophagic vacuoles** (from 24 till 96h of incubation).

Supra vital cell staining with AO for autophagy detection (cytoplasm stains green whereas acid vesicular organelles are visualized as yellow/orange/red fluorescent structures)**.**

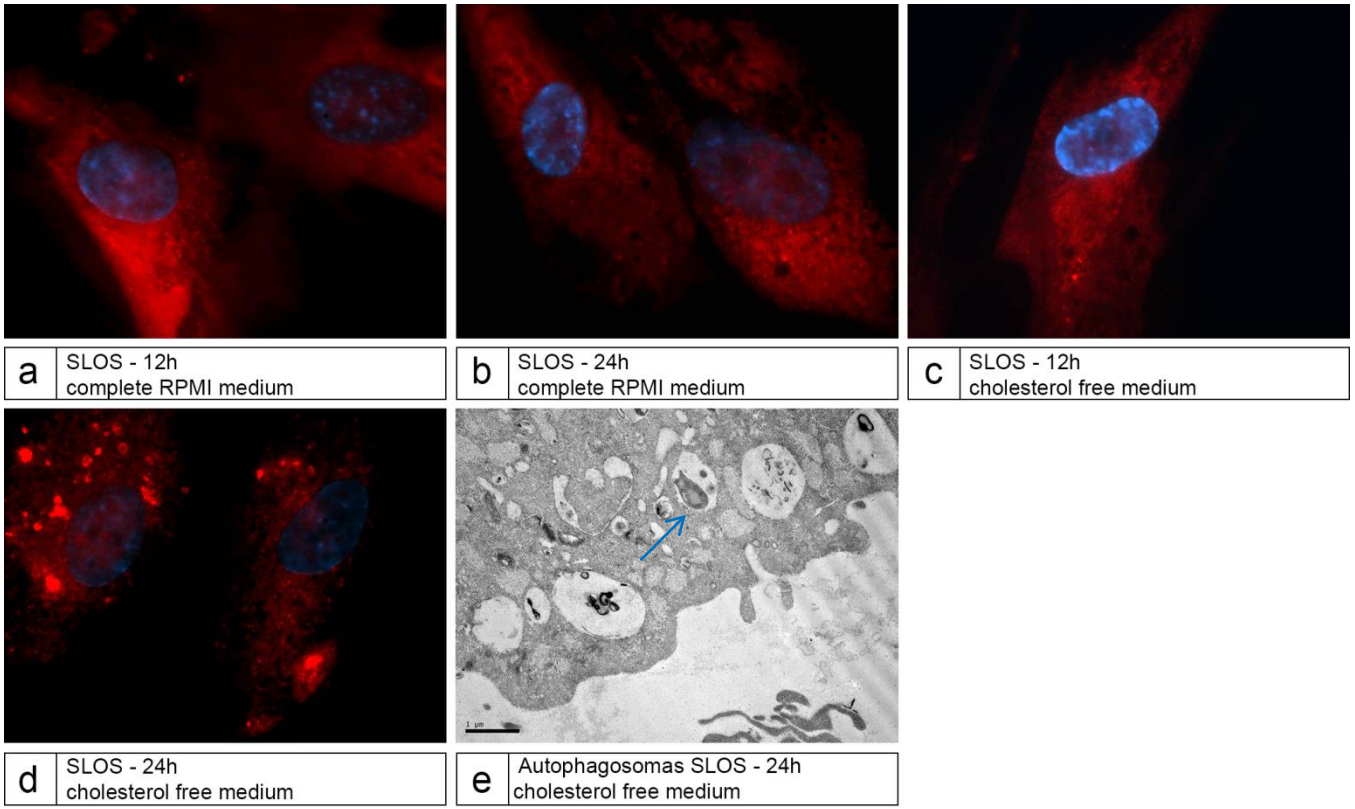
AO



With **Acridine Orange** staining, the normal controls present a small amount of acid vesicular organelles (probably lysosomes) (a) comparing with marked sequential changes that were detected on 7-DHCR deficient cells, in cholesterol depleted medium (b, c, d) .
In the presence of 3-methyladenine the acid vesicular organelles markedly reduced (e and f) , suggesting that cholesterol deficiency can trigger autophagy. These observations were confirmed by flow cytometry quantification of acid vesicular organelles stained with AO.

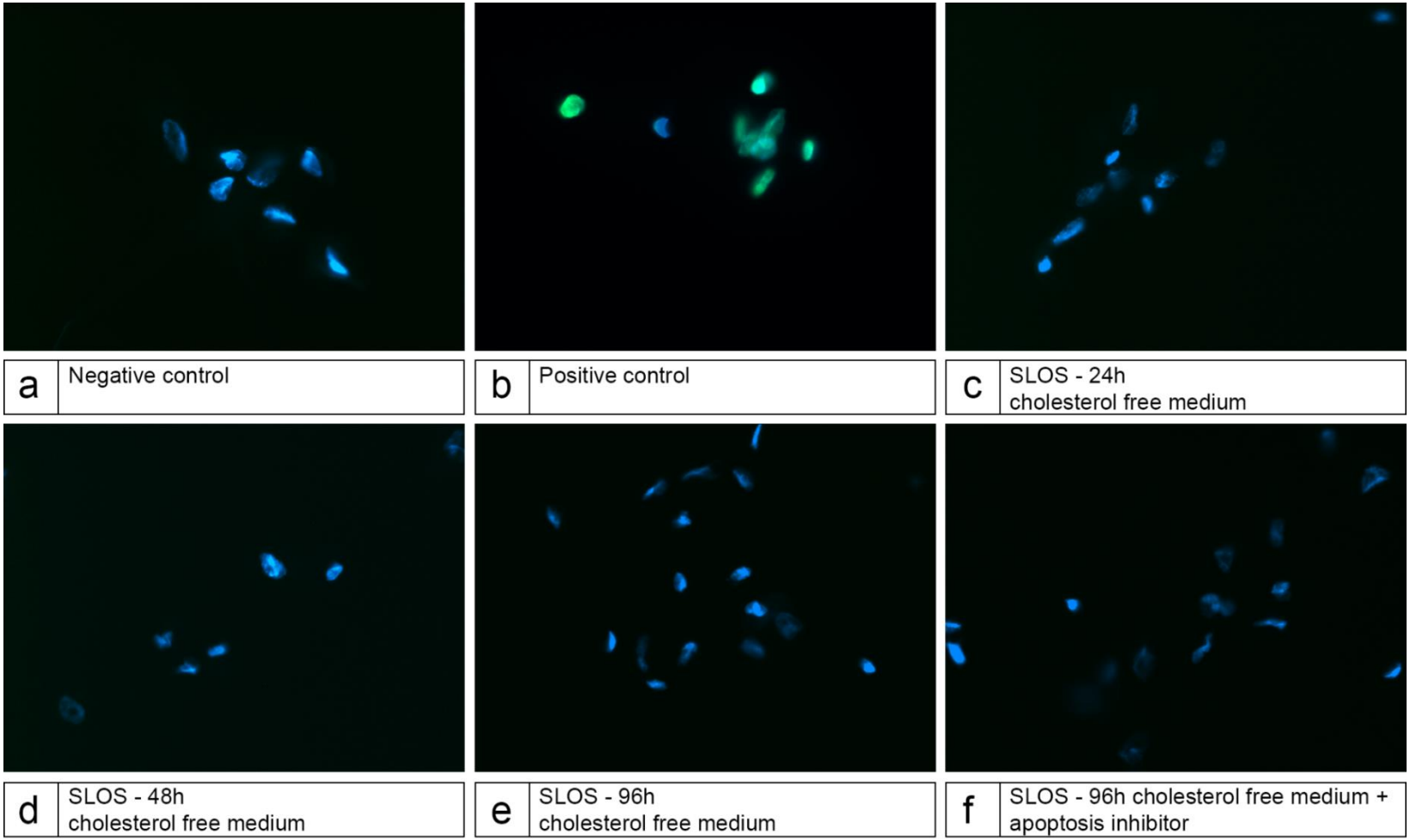
We also performed immunocytochemistry for the autophagosomal marker microtubule-associated protein light chain 3 .

LC3



Immunocytochemistry assay for LC3-II. LC3 protein is an autophagic marker. The autophagosome formation was detected on 7-DHCR deficient fibroblasts between 18 and 24h of incubation by the conversion of LC3-I cytoplasmic (c) to membrane LC3-II (d). Electron microscopy (e) confirmed this data, enabling the visualization of autophagosomes. This change did not occur in cells cultivated on complete RPMI medium that providd exogenous colesterol to the deficient cells (a, b).

We also performed the TUNEL test, a common method for detection of DNA fragmentation resulting from apoptosis.



TUNEL test - Cells were defined as no apoptotic if the nuclear area stains blue and apoptotic if the nuclear area was labeled positively (green color). 7-DHCR deficient fibroblasts incubated 24 (c), 48 (d) and 96 h (e) in a cholesterol free medium, did not evidence DNA fragmentation compatible with apoptosis.

SLOS / Autophagy

One can speculate that **autophagy can be an important mechanism during prenatal and postnatal development of SLOS patients** providing an optimized process for **recycling organelles and liberate cholesterol** (when exogenous delivery and endogenous synthesis are not adequate), with the biological cost of slowing cell proliferation resulting in growth retardation.

In fact, **cholesterol synthesis correlates** strongly with **body weight**, and SLOS children often do not gain weight for many months.

In the other hand **sterol synthesis should adapt to the individual growth process**, and as more cells should be produced during development, more cholesterol should be synthetized in order to satisfy such needs. Many of the **postnatal clinical problems** of patients with SLOS are considered as **direct consequence of the inability to produce large amounts of cholesterol needed for growth**.

It is possible that during these periods of life, autophagy plays an important role promoting tissue turnover and keeping a slower growth velocity. In fact, an **idiopathic hypermetabolism** was identified in some patients that could be related with the **catabolic process of autophagy and this highly conserved program has been shown to act as a pro-survival mechanism** in different physiological and pathological conditions, such as amino acids deprivation or critical steps in development, supporting our hypothesis.

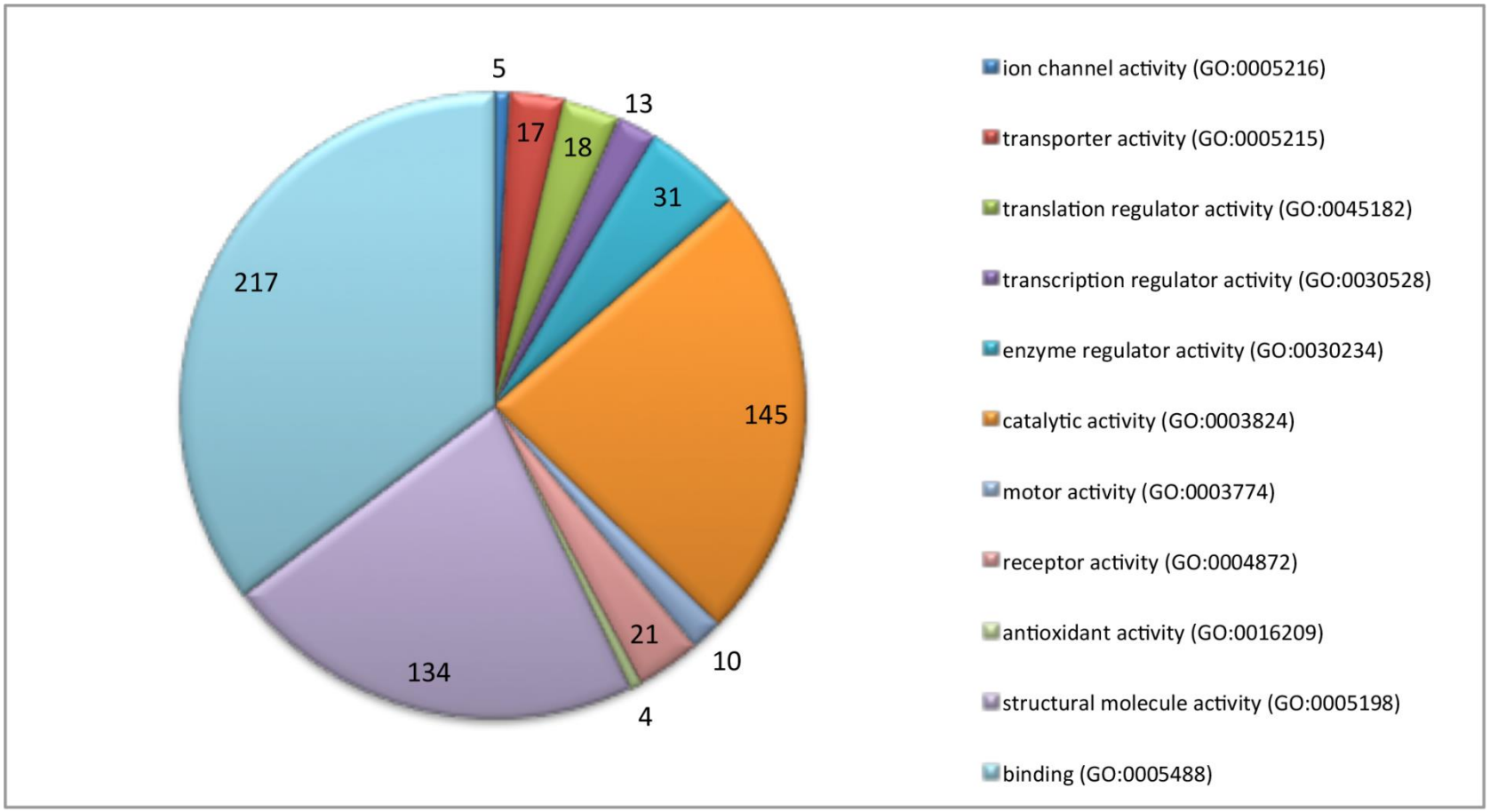
RESULTS
(Proteomic analysis)

Once, activation of autophagy, in the absence of cholesterol seems to be a self-rescue mechanism of the cell, we further investigated **if there was also changes in protein expression** which support surviving cell adaptive modifications.

As **iTRAQ proteomic profiling** is considered a good technique for globally mapping protein signatures from different cell cultures we used such approach in order to evaluate **which proteins were specifically up-regulated and down-regulated in 7-DHCR deficient fibroblasts**.

Our study involved eight distinct fibroblast samples: two from normal controls and two from SLOS patients (7-DHCR deficient), raised both, with and without cholesterol on the culture medium; by adding isobaric tags to the 8 samples and analyzing them together, there was a reduction of the variability associated to the assay.

445 proteins were identified in fibroblasts lysates



Pie chart showing molecular function as a percentage of the 445 identified proteins on fibroblast lysate based on the Panther classification system. The numbers represent the total of proteins corresponding to each section.

As the identification of some proteins based on a single peptide is unreliable such ones were removed from the list, remaining **248 proteins.**

Then, those which presented a huge variability between controls were excluded, because data would not be comparable if controls did not show identical behavior. So, in order to avoid such problems we kept the proteins (**69**) which showed an iTRAQ ratio Cntl1 (C) / Cntl2 (C) = 1.0 ± 10%.

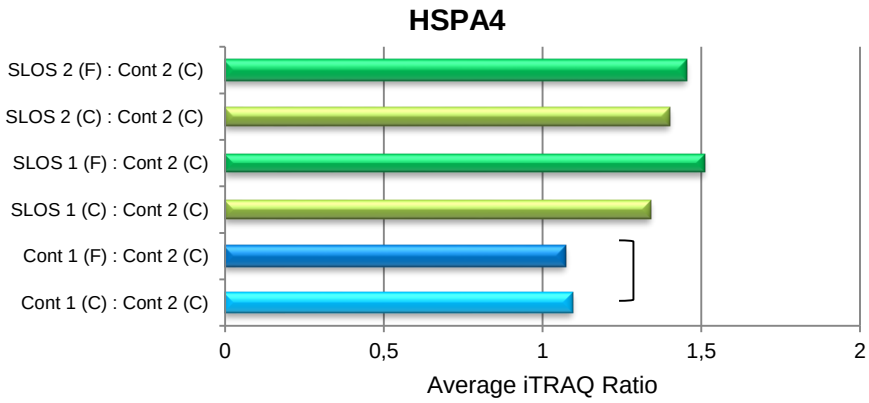
The next step was to pick those with an important variation in 7-DHCR cells (larger than 20%) relatively to controls.

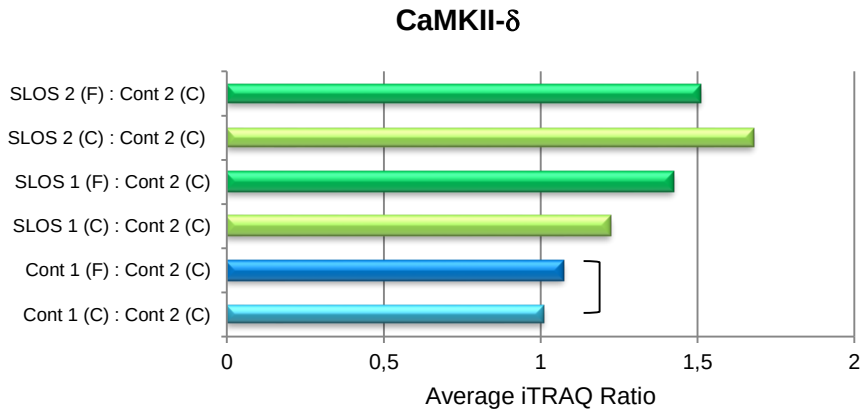
Only **3 proteins** showed an iTRAQ ratio in 7-DHCR deficient cells higher than 1,2 relatively to Cntl 2.

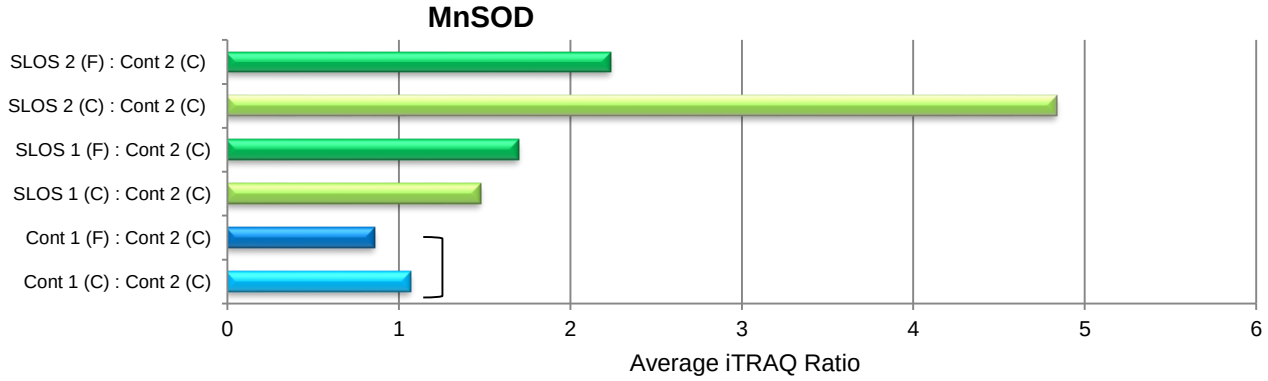
- **Heat shock 70 kDa protein 4** (codified by *HSPA4* gene),
- **Calcium/calmodulin-dependent protein kinase type II subunit delta** (CaMKII-d encoded by *KCC2D* gene)
- **Mitochondrial superoxide dismutase** (MnSOD codified by *SODM*)

No protein was identified in SLOS cells that showed a iTRAQ ratio less than 0,8 in both culture conditions.

Proteins encoded by *HSPA4*, *KCC2D* and *SODM* genes seem to be **hallmarks of SLOS disease** as they are expressed in 7-DHCR deficient fibroblasts in significantly higher amounts than in controls whatever the culture conditions.







Three proteins: heat shock 70 kDa protein 4 (HSPA4), calcium/calmodulin-dependent protein kinase type II subunit delta (CaMKII-d) and mitochondrial superoxide dismutase (MnSOD) were found up-regulatedon on SLOS fibroblasts whatever the culture conditions. The iTRAQ ratios of such proteins from two SLOS patients and controls are represented as bar graphs.

HSPA4 is the mammalian homologue gene of the Atg2 (Autophagy-specific gene 2). The codified protein is a member of the heat shock protein 110 family (HSP110) is induced under stressful conditions, has antiapoptotic properties, and protects cells from oxidative damage. These finding of overexpression of an autophagic specific gene product reinforces the hypothesis of autophagy involvement in the pathogenesis of this inherited metabolic disease.

Ca^{2+}/calmodulin dependent protein kinase CaMKII-d, one of the CaMKII isoenzymes is involved in the regulation of ion channels, cytoskeletal dynamics, gene transcription and cell division and mediates many cellular responses to elevated Ca^{2+} in a wide variety of cells. It was previously found that calcium uptake is increase 3–fold in 7-DHCR deficient fibroblasts compared with control cells and recent studies also showed that a rise of free cytosolic calcium is a potent inducer of autophagy in a process mediated by another CaMKII isoenzyme and AMP-activated protein kinase (AMPK). Thus it is possible that the over expression of CaMKII-d in 7-DHCR deficient cells could also be related with autophagy, reinforcing other data from this study.

It is known that autophagy is regulated by reactive oxygen species (ROS) including superoxide and hydrogen peroxide, through distinct mechanisms, depending on cell types and stimulation conditions. Many stimuli that induce ROS generation also induce autophagy, including nutrient starvation, mitochondrial toxins, hypoxia, and oxidative stress. **MnSOD** destroys superoxide anion radicals produced within the mitochondria and recent studies have shaped the idea that by regulating the mitochondrial redox status and H_2O_2 outflow, MnSOD can act as a fundamental regulator of cellular proliferation and metabolism, thereby assuming roles that extend far beyond its proposed antioxidant functions. There is evidence of increased oxidative stress in 7-DHCR7 deficient cells. So, an increase in MnSOD expression can be a cellular response to intrinsic ROS stress and scavenging superoxide by MnSOD may reduce the stimulating effect of ROS on cell growth.

CONCLUSION

Using a morphological and proteomic approach, we investigated the mechanisms of cell survival under cholesterol deficiency. Specific responses were observed in cholesterol deficient cells.

The dominant proteomic signature of SLOS fibroblasts that emerged from this analysis is an overexpression of heat shock 70 kDa protein 4, CaMKII-d and MnSOD proteins in 7-DHCR deficient cells in complete medium as well as in cholesterol free one. These fibroblasts seems to increase MnSOD expression to combat oxidative stress derived from the increased amount of 7DHC and its derivatives in fibroblasts and thus control cell proliferation, whereas heat shock 70 kDa protein 4 also presents a cytoprotective activity related with oxidative damage and inhibits apoptosis. Both heat shock 70 kDa protein 4 and CaMKII are clearly associated with autophagy in bibliography.

We conclude that the mechanism by which 7-DHCR deficient fibroblasts handles oxidative stress, involves induction of autophagic proteins expression and plays an important role in cell survival.

Furthermore this study provided powerful indications that may be useful to expand the knowledge about the cellular mechanisms involved in SLOS cellular pathophysiology.

Further studies are needed to determine the clinical relevance of these findings; however our study creates a new line of investigation concerning the mechanisms involved in intrauterine and postnatal growth restriction in SLOS.

ACKNOWLEDGMENT

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