Department of Medical-Surgical Sciences and Biotechnologies
Vascular Biology, Atherothrombosis & Mass Spectrometry Lab

Dottorato di Ricerca in Medicina Sperimentale XXVIII ciclo

RELAZIONE DELL'ATTIVITA' SVOLTA NEL CORSO DEL SECONDO ANNO DI DOTTORATO

CURRICULUM:TECNOLOGIE INNOVATIVE IN MEDICINA TRASLAZIONALE

Titolo progetto di tesi: T**HE OXYSTEROL NETWORK IN BIOLOGY AND MEDICINE**

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During the first year of my PhD I studied the relation between oxysterols and cholesterol-5α,6α-epoxide hydrolase (ChEh) in cancer. This study has been conducted in collaboration with Marc Poirot, Head of the Team "Sterol Metabolism and Therapeutic Innovations in Oncology" at the Cancer Research Center of Toulouse (CRCT). At CRCT ChEh has been identified as a target for tamoxifen (one of the major drugs used for the hormonotherapy of estrogen receptor positive breast cancers) and AEBS (microsomal antiestrogen binding site) ligands.

My role in this project was to characterize oxysterols as substrates and products of ChEh by isotope dilution gas chromatography-mass spectrometry, and targeting 9 different oxysterols among which also the two diastereoisomers 5,6α- and 5,6β-epoxycholesterol (EC) and the cholestane-3β,5α,6β-triol (CT) (produced by ChEh).

After treating the MCF-7 cells with tamoxifen, PBPE (AEBS ligand) and dendrogenin A (metabolite of 5,6 α-EC and strong inhibitor of ChEh) we studied the nature of sterol oxidation products; moreover we analyzed the stimulation of the ROS-dependent oxysterols biosynthesis by tamoxifen and PBPE by treating cells with the same concentrations of tamoxifen and PBPE used for the determination of oxysterols, but in the presence of vitamin E as antioxidant.

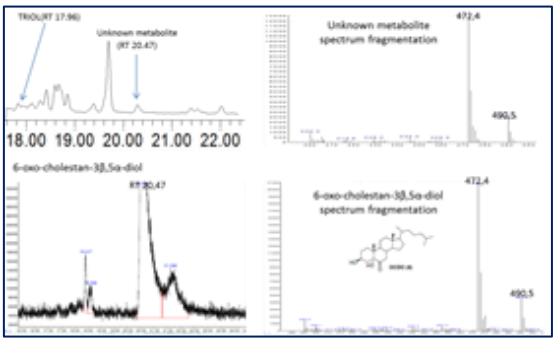
From the data obtained we can say that:

- Tam and PBPE induce the production 5,6-EC diastereoisomers, 5,6α-EC and 5,6β-EC in a 1/3 ratio in MCF-7 cells;
- The production of 5,6-EC diastereoisomers is totally blocked by Vit E suggesting that they are produced through a ROS mediated mechanism;
- The absence of an increase in CT (the product of hydration of 5,6-EC by ChEH), despite the increase in 5,6-EC, is consistent with the inhibition of ChEH by Tam and PBPE in BC cells;

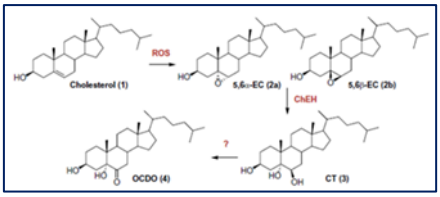
Taken together, these data suggest that Tam and PBPE contributed to the accumulation of 5,6α-EC and 5,6β-EC in MCF-7 cells through a dual mechanism involving a ROS-mediated cholesterol epoxidation and

the inhibition of ChEH. Furthermore our data show that CT is in turn converted into an unknown metabolite potentially acting as a cancer promoter.

In the current, second year of PhD I have worked on the chemistry of the unknown metabolite produced by ChEh upon reaction with CT. I have worked with mass spectrometer in full-scan mode which is useful in determining unknown compounds in a sample because it shows the full mass spectrum of the substance. By comparing the full mass spectrum and the retention time of the unknown metabolite with the mass spectra and retention times of the various oxysterols contained in our database have been able to get the identification of the substance as 6-oxo-cholestan-3β,5α-diol (6oxoCh). Unambiguous identification has been obtained by using authentic standard, and hexa-deuterated standard synthetized de-novo at Poirot's lab.



In conclusion we can say that cholesterol is converted into 5,6-EC diastereoisomers through a process of autoxidation; ChEH transforms 5,6α-EC and 5,6β-EC into cholestane-3β,5α,6β-triol; CT is in turn converted into 6-oxo-cholestan-3β,5α-diol.



During this year I have also dealt with a new study on male fertility in collaboration with the Center of Andrology and Pathophysiology of Reproduction (CAPR) at the SM Goretti Hospital, in Latina.

One of the most important causes of male infertility is oxidative stress induced by an excess of reactive oxygen species (ROS) that impact negatively on the quality and function of sperm; it is estimated that 25% of men suffering from infertility have high levels of ROS in semen; although a controlled production of ROS is required for the maturation, capacitation and the acrosome reaction of the sperm and, thus, for natural fertilization. The involvement of ROS in male infertility stems from their ability to generate chemical deterioration and structural damage to the nuclear DNA, in the proteins and lipids of plasma membranes and mitochondria of spermatozoa. The spermatozoa are particularly sensitive to oxidative damage, due to the massive presence of polyunsaturated fatty acids in their plasma membranes. Cholesterol is another main component of the lipid membranes of sperm cells. The content and distribution of cholesterol in the plasma membranes of spermatozoa are essential for sperm capacitation and acrosomal reaction. Given the abundance of cholesterol, at the level of spermatozoa, and its susceptibility to autoxidation (related to oxidative stress), we were interested to investigate oxysterols as biomarkers of oxidative damage in semen and their potential pathophysiological role in male infertility.

The study includes 134 patients, including 4 groups according to the spermiographic and morphological alteration of spermatozoa, enrolled at CAPR. Semen samples were evaluated immediately after collection at the CAPR following the guidelines of the WHO 2010. Group characteristcs are as follows:

1. Normozoospermic (with normal spermiographic parameters);
2. Asthenoteratozoospermic (with a percentage of the progressive motility and of morphologically normal spermatozoa below the lower limit of reference of the WHO 2010);
3. Oligoasthenoteratozoospermic (with a total number of spermatozoa and with a percentage of the progressive motility and of morphologically normal spermatozoa below the lower limit of reference of the WHO 2010);
4. Normozoospermic , oligoasthenoteratozoospermic, asthenoteratozoospermic with varicocele (pathological varicose dilatation of the testis reflue veins).

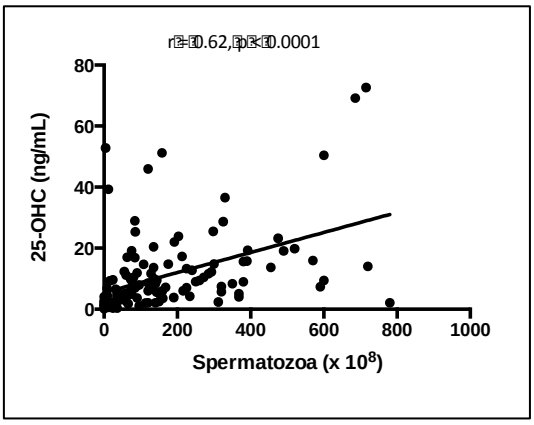
Before proceeding with the oxysterolome analysis of semen samples it was necessary to develop a specific method for this biological fluid. We succesfully developed an isotopic dilution method coupled with gas chromatography-mass spectrometry to monitor the different oxysterol species in the whole semen fluid, inclusive of spermatozoa and seminal plasma.

	7alfa					7beta					5beta,6beta-Epoxy				
	Normo	Oligo	Varico	Asteno	Total	Normo	Oligo	Varico	Asteno	Total	Normo	Oligo	Varico	Asteno	Total
N	26	32	44	25	127	26	32	44	25	127	26	32	44	25	127
Mean	3,33825	2,9183	3,05634	2,81297	3,03136	11,73126	9,89096	10,65046	10,40545	10,63212	9,38644	9,59518	9,89214	9,4193	9,62071
Std. Deviation	1,71523	1,466063	1,314076	0,887722	1,372702	8,197274	7,832174	7,925788	5,224941	7,447265	3,12199	3,134835	6,143948	3,129082	4,375411

	4beta					4alfa					5alfa,6alfa-Epoxy				
	Normo	Oligo	Varico	Asteno	Total	Normo	Oligo	Varico	Asteno	Total	Normo	Oligo	Varico	Asteno	Total
N	26	32	44	25	127	26	32	44	25	127	26	32	44	25	127
Mean	7,96338	7,79934	8,54789	7,2869	7,99139	14,40526	14,89471	13,5073	11,23522	13,59346	5,96385	5,40696	5,47795	5,07535	5,48029
Std. Deviation	2,92072	4,991824	4,523633	4,155365	4,279238	6,77688	12,57371	8,398251	7,69806	9,221668	2,94333	1,44556	2,937823	1,194323	2,352082

	Triol					7keto					27-OH				
	Normo	Oligo	Varico	Asteno	Total	Normo	Oligo	Varico	Asteno	Total	Normo	Oligo	Varico	Asteno	Total
N	26	32	44	25	127	26	32	44	25	127	26	32	44	25	127
Mean	1,70096	1,5863	1,63011	1,6331	1,63416	7,08175	6,30001	6,6673	6,29466	6,58625	2,16982	1,66273	2,03821	1,51736	1,86802
Std. Deviation	0,66595	0,50372	0,494365	0,428087	0,519845	5,057787	2,757358	2,867666	2,063731	3,264128	0,92691	0,838584	1,182272	0,464271	0,962063

	6-Oxo					**25-OH**					
	Normo	Oligo	Varico	Asteno	Total	**Normo**	**Oligo**	**Varico**	**Asteno**	**Total**	Perason correlation
N	26	32	44	25	127	**26**	**32**	**44**	**25**	**127**	spermatozoa number vs. 25-OHC
Mean	4,00951	2,59478	3,79979	3,04325	3,39017	**21,62577**	**2,58963**	**13,48309**	**5,5893**	**10,85139**	r = 0.62, p < 0.0001
Std. Deviation	3,12867	1,957025	3,68361	2,930136	3,077793	**18,46664**	**2,928991**	**11,80525**	**3,170905**	**12,97886**	
						between groups p < 0.0001					



The unexpected high correlation found between the number of spermatozoa and 25-hydroxycholesterol (25-OHC), is extremely important at biomarker level, and raises the question of what is the role of 25-OHC in sperm function and, eventually, in fertility.

25-OHC is produced by Cholesterol 25 hydroxylase (CH25H) that belongs to a family of enzymes that utilize di-iron cofactors to catalyze the hydroxylation of hydrophobic substrates such as cholesterol.

From the data obtained, it could be argued that CH25H could be present in spermatozoa. To verify this hypothesis I separated spermatozoa from the seminal plasma using a Percoll gradient and analyzed the two portions of sperm separately. if the 25-OHC is present in amounts significantly more abundant in spermatozoa, we could say that the enzyme responsible for its synthesis is present in the spermatozoa, otherwise we should do further investigation.

The results show no significant differences in the content of 25-OHC between spermatozoa and the portion of seminal plasma, so we can not confirm the presence of CH25H only in the spermatozoa.

For the last year of my PhD our goals are:

- Locate the CH25H in a portion of the seminal fluid through western blot and immunofluorescence assay;
- Pulse and chase experiments with spermatozoa loaded with deuterated –Cholesterol to confirm the presence of the enzyme 25-hydrolxylase within the spermatozoa;
- Understand the possible role for 25-OHC in the various processes carried out by sperm through test of vitality, motility, etc.

Prof. Luigi Iuliano

Publications 2013-2014

1.Vurusaner B, Gamba P, Testa G, Gargiulo S, Biasi F, Zerbinati C, Iuliano L, Leonarduzzi G, Basaga H, Poli G: Survival signaling elicited by 27-hydroxycholesterol through the combined modulation of cellular redox state and ERK/Akt phosphorylation. Free Radic Biol Med. 2014 Aug 7. pii: S0891-5849(14)00343-8;

2. Ginanni Corradini S, Zerbinati C, Maldarelli F, Palmaccio G, Parlati L, Bottaccioli AG, Molinaro A, Poli E, Boaz M, Serviddio G, Mennini G, Corsi A, Bianco P, Rossi M, Iuliano L: Plasma fatty acid lipidome is associated with cirrhosis prognosis and graft damage in liver transplantation. Am J Clin Nutr. 2014 Jun 25;100(2):600-608

3. Gamba P, Guglielmotto M, Testa G, Monteleone D, Zerbinati C, Gargiulo S, Biasi F, Iuliano L, Giaccone G, Mauro A, Poli G, Tamagno E, Leonarduzzi G: Up-regulation of β-amyloidogenesis in neuron-like human cells by both 24- and 27-hydroxycholesterol: protective effect of N-acetyl-cysteine. Aging Cell. 2014 Jun;13(3):561-72.

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