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DOTTORATO DI RICERCA IN
BIOTECNOLOGIE MOLECOLARI INDUSTRIALI ED AMBIENTALI
XXI CICLO

FATTY ACID-BINDING PROTEINS AS MARKERS OF BRAIN INJURY

S.S.D. Bio/11 Biologia Molecolare

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RIASSUNTO

Le Fatty Acid-Binding Proteins (FABPS) sono proteine intracellulari in grado di legare gli acidi grassi a catena lunga con elevata affinità, regolandone il trasporto all'interno delle cellule. Nell'uomo sono state finora identificate 9 diverse isoforme, ciascuna con un diverso profilo di espressione a livello di tessuti. La proteina heart-type FABP (H-FABP) da anni è considerata uno tra i più sensibili ed efficienti marcatori di danno cerebrale e al miocardio, ma la sua elevata espressione in differenti tessuti non la rende un marker specifico di un particolare disordine. Un'altra isoforma espressa nel cervello, chiamata B-FABP (brain-type FABP), potrebbe invece rappresentare un marcatore specifico di danno cerebrale, essendo la sua espressione limitata al tessuto nervoso.

Questo lavoro ha avuto come scopo la generazione di anticorpi in grado di riconoscere la proteina B-FABP in modo specifico e selettivo.

Il progetto si è articolato in due fasi. Nella prima parte del lavoro la proteina ricombinante B-FABP è stata espressa in *E. coli* e purificata mediante due successive cromatografie. Essa è stata quindi inoculata in conigli New Zealand e in topi BalbC, ottenendo dopo diverso tempo 2 antisieri e 8 diverse cellule ibridoma. Gli anticorpi presenti nel siero e nel surnatante delle cellule ibridoma in coltura hanno evidenziato diversi

gradi di affinità nei confronti delle due isoforme, B-FABP e H-FABP; solamente una cellula ibridoma è stata in grado di produrre un anticorpo specifico e selettivo per la B-FABP ma purtroppo i successivi passaggi di subclonaggio della cellula hanno evidenziato una progressiva diminuzione dell'attività anticorpale, non permettendo l'utilizzo di questo reagente per l'individuazione della proteina stessa in campioni biologici.

Nella seconda parte del lavoro si è quindi cercato di analizzare e superare le problematiche riscontrate nella prima fase mediante un nuovo approccio che, con l'ausilio di software specifici, ha mirato all'identificazione di due regioni potenzialmente antigeniche della proteina stessa. Due peptidi sintetici, di sequenza identica alle regioni identificate, sono stati inoculati in conigli New Zealand con l'ottenimento di due anticorpi policlonali, pAb 2979/2980 e pAb 2981/2982. Questi anticorpi si sono rivelati specifici e selettivi verso l'isoforma B-FABP e non hanno evidenziato reazioni di cross-reazione verso H-FABP.

Si può quindi affermare come l'approccio sperimentale utilizzato abbia contribuito al raggiungimento dell'obiettivo, la produzione di un anticorpo specifico per B-FABP umana. Questo risultato suggerisce possibili scenari nell'utilizzo futuro degli anticorpi, in tecniche ELISA e western blot, per valutare se la

proteina B-FABP possa essere considerata un marcatore ideale, specifico di danno neuronale, in disordini a diversa eziologia (ischemica, infettiva o degenerativa).

ABSTRACT

Fatty acid–binding proteins (FABPs) are abundant intracellular proteins that bind long-chain fatty acids with high affinity. 9 different FABPs, with tissue-specific distribution, have been identified so far. The primary role of all the FABP family members is regulation of fatty acid uptake and intracellular transport. Heart-type FABP (H-FABP) is considered one of the most sensible and efficient markers of heart and brain damage, but to date the cross-reactivity limits its specific use.

Brain-type FABP (B-FABP) might be considered as a good marker of brain damage, being expressed only in the nervous tissue.

The aim of this work was to generate a diagnostic reagent specific for human B-FABP, not cross-reacting with H-FABP.

The work has been articulated in two parts: in the first one, recombinant protein B-FABP was expressed in *E. coli* cells and purified by two subsequent chromatographies. The protein was then utilized as immunogen in New Zealand rabbits and BalbC mice, obtaining two antisera and 8 different hybridoma cells. The antibodies in the rabbit serum and in culture supernatant of hybridoma cells displayed different degrees of affinity for B-FABP and H-FABP; only one hybridoma cell was able to produce antibodies specific

and selective for brain-type FABP, but the antibody low-titer level and the activity decrease after subsequent subcloning steps affected its application on B-FABP detection in biological fluids. To overcome the limits encountered in the first part of work, a computer-assisted approach on B-FABP was employed in order to identify the potentially most antigenic regions of the protein. Two synthetic peptides were produced with the selected sequences and inoculated in rabbits. Two polyclonal antibodies were obtained, pAb 2979/2980 and pAb 2981/2982. They showed specific and selective reactivity for human B-FABP, without cross-reactions with H-FABP.

Taken together, our experimental approach was effective for the generation of specific α-B-FABP antibodies; these results suggest the antibodies obtained could be utilized in ELISA and immunoblot analyses in order to value B-FABP as marker of neurological disorders with ischemic, infective and degenerative etiology.

Abbreviations

ACS: Acute Coronary Syndrome;

BBB: Blood-Brain Barrier;

BSA: Bovine Serum Albumin;

CHF: Congestive Heart Failure;

CSF: CerebroSpinal Fluid;

DHA: DocosaHexaenoic Acid;

ELISA: Enzyme-Linked ImmunoSorbent Assay;

FA: Fatty Acid;

FABP: Fatty Acid–Binding Protein;

FCA: Freund's Complete Adjuvant;

FIA: Freund's Incomplete Adjuvant (FIA);

GST: Glutathione S-Transferase;

HAT: Hypoxanthine-Aminopterin-Thymidine;

HPLC: High-Pressure Liquid Chromatography;

Ig: Immunoglobulin;

IMAC: Immobilized Metal Ion Affinity Chromatography;

IPTG: IsoPropyl-1-Thio-β-D-Galactopyranoside;

KLH: Keyhole Limpet Hemocyanin;

mAb: monoclonal Antibody;

mIgM: membrane-Immunoglobulin M;

MW: Molecular Weight;

NMR: Nuclear Magnetic Resonance;

pAb: polyclonal Antibody;

PCR: Polymerase Chain Reaction;

PDB: Protein Data Bank;

PPAR: Peroxisome Proliferator-Activated Receptor;

PPS: Post-Polio Syndrome;

PUFA: PolyUnsaturated Fatty Acid;

Rec: Recombinant;

RCC: Renal Cell Carcinomas;

RZPD: Deuthsches Ressourcenzentrum fur Genomforschung;

sCJD : sporadic Creutzfeldt-Jakob disease;

SDS-PAGE: Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis;

TNFα: Tumor Necrosis Factor α;

TTR: transthyretin.

Medium composition:

- Luria-Bertani (LB) medium: 1% tryptone,
  0.5% yeast extract,
  0.5% NaCl;

Buffer composition:

- TBE (Tris Borate EDTA): 45 mM Tris-Borate pH 8.0, 1 mM EDTA;
- Buffer A: 20 mM Tris-HCl pH 7.5, 0.5 M NaCl, NaN_3 0.02%;
- Buffer B: 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, NaN_3 0.02%

1.INTRODUCTION

1.1 FABPs: FUNCTION AND STRUCTURE.

Fatty acid–binding proteins (FABPs) are abundant ~15-kDa cytoplasmic proteins expressed in almost all mammalian tissues. They are members of a conserved multigene family that evolved approximately 1000 million years ago by subsequent duplications of an ancestral gene, thereby generating a large number of tissue-specific homologs. The mammalian FABP family includes nine FABPs as well as the cellular retinoid-binding proteins.

The overall gene structure is conserved among all family members and consists of 4 exons separated by 3 introns [1, 2, 3, 4, 5, 6, 7]. Exons are relatively short and the shortest, exon 4, codes for 16–17 amino acids whilst the longest, exon 2, for almost 60 amino acids [8]. The FABP genes contain the canonical TATA box located around 23–30 nucleotides upstream of the transcription start site [1, 2, 3, 7].

Various enhancer elements have also been identified, directing tissue-specific expression. Tissues with high rates of fatty acid (FA) metabolism, such as intestine, liver, adipose tissue, and muscle, have high FABP levels that parallel FA uptake and utilization [9].

The cellular expression of FABPs is regulated primarily at the transcriptional level and is responsive to changes in lipid metabolism. These changes can be induced either by (patho)physiological conditions, such

as ischemia [10], endurance training [11], diabetes [12, 13] and (cardiac) hypertrophy [14, 15], or by pharmacological stimuli such as hypolipidemic drugs [16].

FABP names are assigned according to the tissue where the FABP was first recognized, and are designated by adding "-type," e.g., heart-type FABP (H-FABP), to indicate that the protein may also be expressed in other tissues (Table 1). Many FABPs are prominently expressed in a single tissue or cell type, but some FABPs display a broad tissue distribution. In several cell types, more than one FABP type is expressed, suggesting that these proteins have specialized functions. All FABPs bind one molecule of Fatty Acid (FA) except the liver-type, which binds two.

Gene	Common name	Alternative names	Expression	Chromosomal location		
				Homo sapiens	*Mus musculus*	*Rattus norvegicus*
Fabp1	Liver FABP	L-FABP	Liver, intestine, pancreas, kidney, lung, stomach	2p11	6 C1	4q32
Fabp2	Intestinal FABP	I-FABP	Intestine, liver	4q28–q31	3 G1	2q42
Fabp3	Heart FABP	H-FABP, MDGI	Heart, skeletal muscle, brain, kidney, lung, stomach, testis, aorta, adrenal gland, mammary gland, placenta, ovary, brown adipose tissue	1p32-p33	4 D2.2	5q36
Fabp4	Adipocyte FABP	A-FABP, aP2	Adipocyte, macrophage, dendritic cell	8q21	3 A1	2q23
Fabp5	Epidermal FABP	E-FABP, PA-FABP, mal1	Skin, tongue, adipocyte, macrophage, dendritic cell, mammary gland, brain, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen	8q21.13	3 A1-3	2
Fabp6	Ileal FABP	Il-FABP, I-BABP, gastrotropin	Ileum, ovary, adrenal gland, stomach	5q33.3–q34	11 B1.1	10q21
Fabp7	Brain FABP	B-FABP, MRG	Brain, glia cell, retina, mammary gland	6q22-q23	10 B4	20q11
Fabp8	Myelin FABP	M-FABP, PMP2	Peripheral nervous system, Schwann cell	8q21.3–q22.1	3 A1	2q23
Fabp9	Testis FABP	T-FABP	Testis, salivary gland, mammary gland	8q21.13	3 A2	2q23

Table 1: Family of fatty acid-binding proteins (FABPs); Abbreviations: aP2, adipocyte P2; I-BABP, ileal bile acid-binding protein; MDGI, mammary derived growth inhibitor; MRG, MDGI-related gene; PA-FABP, psoriasis-associated FABP; PMP2, peripheral myelin protein 2. [17].

The members of the FABP family show only moderate primary structure similarity, with amino acid sequence homology varying from 20% to 70% [18, 19]. However, extensive X-ray crystallographic and nuclear magnetic resonance (NMR) analyses have shown that these proteins display a striking tertiary structural similarity [20]. All the members of the family fold as a slightly elliptical β-barrel comprising 10 antiparallel β-strands, with two short α-helices located between the first and second β-strands.

The β-barrel possesses appreciable structural stability, as it is virtually unaffected by chemical modifications, the presence of bulky fluorescent groups, or targeted mutagenesis [21, 22, 23]. The β-strands are organized into two nearly orthogonal β-sheets that wrap around a solvent-accessible ligand-binding cavity. The cavity is centered at the end of the barrel near the helix-turn-helix motif, which is thought to act as a portal for ligand entry and exit [24, 25]. The α-II helix is a key structural element of the putative FA portal and forms long-range interactions with the α-II turn between β strands C and D. The β barrel cavity is two to three times larger than the volume of a fatty acid molecule, and the structures reveal ordered water molecules in the cavity, that are hydrogen bonded to

internal polar residues. Most of the FABPs bind only a single FA, with the carboxylate group oriented inward. L-FABP has the unique property of binding two FAs and other larger hydrophobic molecules [26].

Solution structures of apoFABPs reveal specific regions of disorder in the portal domain, compared to holo-FABP structures [26, 27]. Thus, it is likely that a conformational change in the portal region occurs during FA binding or release. FABP-membrane interactions, or protein-protein interactions, may catalyze this conformational change.

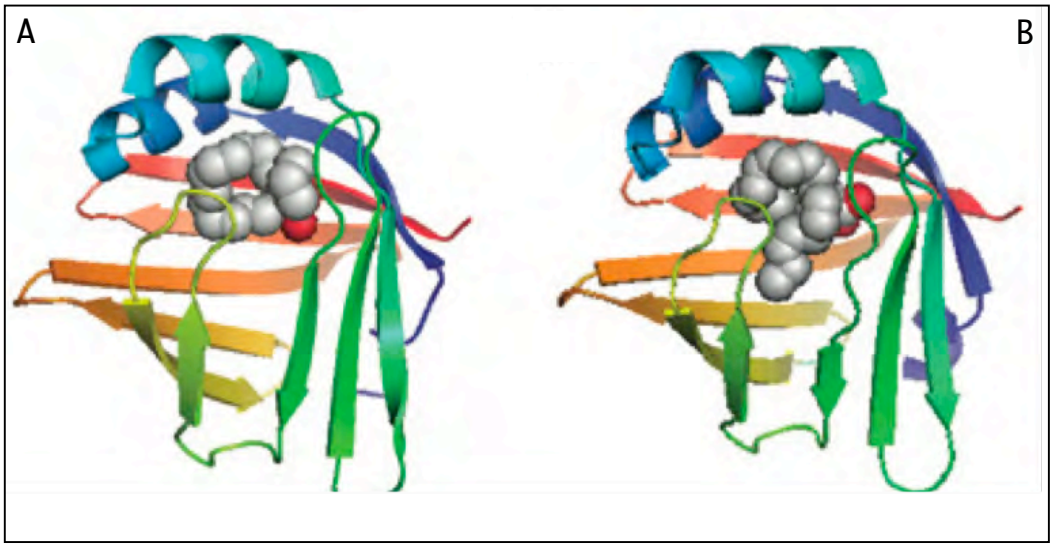

Figure 1: Human brain-type FABP (B-FABP) binds oleic acid in the U-shaped way (PDB code: 1fe3) (A); however, its binding pocket can also accommodate very long-chain docosahexaenoic acid in a helical conformation (PDB code: 1fdq) (B); [17].

All FABPs bind long-chain fatty acids with differences in ligand selectivity, binding affinity and binding mechanism [28] as a result of small structural

differences between isoforms. In general, the more hydrophobic the ligand the tighter the binding affinity – with the exception of unsaturated fatty acids.

It is also possible that the needs of target cells determine the affinity and even selectivity of the major isoform present at different sites. For example, B-FABP is highly selective for very long-chain fatty acids such as docosahexaenoic acid [29]. On the other hand, L-FABP exhibits binding capacity for a broad range of ligands from lysophospholipids to haem [30].

As lipid chaperones, FABPs may actively facilitate the transport of lipids to specific compartments in the cell, such as to the lipid droplet for storage, to the endoplasmic reticulum for signalling, trafficking and membrane synthesis, to the mitochondria or peroxisome for oxidation, to cytosolic or other enzymes to regulate their activity, to the nucleus for lipid-mediated transcriptional regulation, or even outside the cell to signal in an autocrine or paracrine manner (figure 2).

The FABP content in most cells is generally proportional to the rates of fatty-acid metabolism [31].

FABPs are also involved in the conversion of fatty acids to eicosanoid intermediates and in the stabilization of leukotrienes [32, 33].

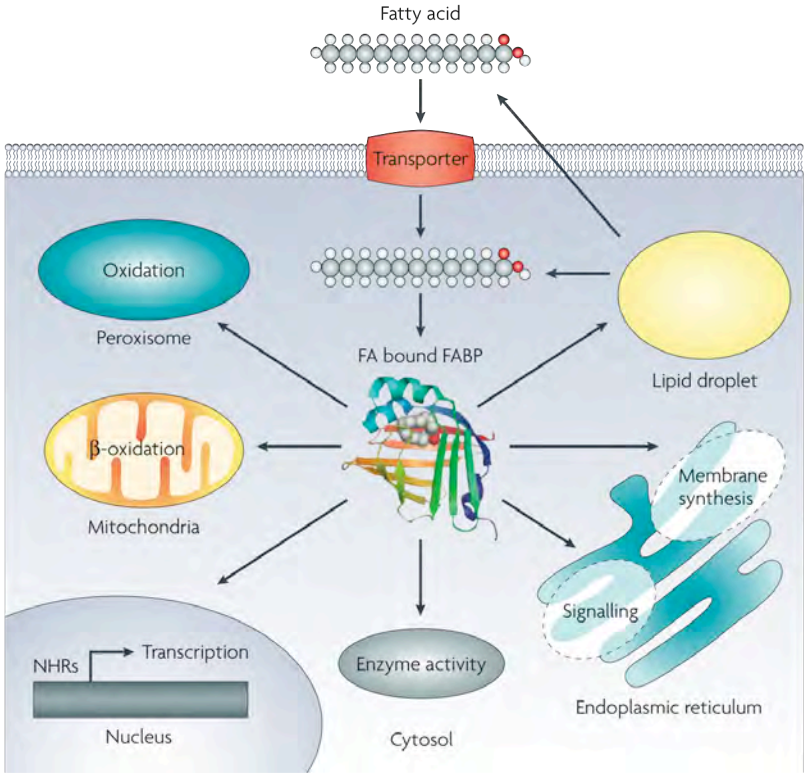

Figure 2: Fatty-acid (FA) trafficking related to the fatty acid-binding proteins (FABPs) in the cell. As lipid chaperones, FABPs have been proposed to play a role in the transport of lipids to specific compartments in the cell: to lipid droplets for storage, to the endoplasmic reticulum for signalling, trafficking and membrane synthesis, to the mitochondria or peroxisome for oxidation, to cytosolic or other enzymes to regulate their activity, to the nucleus for the control of lipid-mediated transcriptional programs via nuclear hormone receptors (NHRs) or other transcription factors that respond to lipids, or even outside the cell to signal in an autocrine or paracrine manner; [17].

1.2 FABPs AND HUMAN PATHOLOGIES.

Reports suggest a link between FABP levels and either increasing or decreasing malignancy. For example, liver and intestinal FABP levels decrease with the

progression of liver and colon cancer, respectively [34, 35]. A-FABP levels are higher in low-grade bladder carcinoma than in high-grade tumours [36], whereas epidermal FABP is expressed at higher levels in prostate cancer compared to prostatic hyperplasia [37]. B-FABP, normally expressed in radial glial cells is also expressed in astrocytoma tumors and in some malignant glioma cell lines, and its expression in astrocytomas is associated with regions of tumor infiltration and recurrence [38].

A high frequency of the Thr54 allele on I-FABP (Ala to Thr substitution) has been detected in obese women and has been correlated with high tumor necrosis factor α (TNFα) levels, high fasting plasma insulin levels, and high leptin levels [39]. TNFα is an inflammatory cytokine expressed in adipocytes that may lead to hypertriglyceridemia by decreasing hepatic lipoprotein lipase activity and increasing de novo FA synthesis [40]; this suggests that I-FABP-Thr54 is associated with obesity and insulin resistance, which may be mediated in part by inflammatory cytokines.

E-FABP is highly expressed in psoriatic skin; indeed, one of its earlier designations was psoriasis-associated FABP. This protein has been shown to interact with an S100 protein called psoriasin or S100A7 [41, 42]. The S100 proteins are calcium-regulated signalling proteins, and both E-FABP and S100A7 are overexpressed in psoriatic skin [43]. A

potential role for E-FABP in cancer metastasis has also been suggested. Differential mRNA display and Northern analysis showed substantial upregulation of the E-FABP gene in prostate and breast cancer cell lines relative to benign cell lines [44]. In human prostate cancer, almost 75% of prostate carcinomas stained positively for E-FABP, compared to fewer than 30% in benign prostatic hyperplasia [37].

H-FABP is considered an efficient early marker for cardiac injury in acute coronary syndromes, but also allows detection of minor myocardial injury in heart failure and unstable angina [45].

1.3 FABPs IN NERVOUS TISSUE.

The brain is rich in fatty acids, primarily in the form of complex lipids. Except for adipose tissue, brain has a higher lipid content than any other organ. Lipids account for about 50% of the dry weight of the brain [46] and the diversity of lipid species is greater in the brain than in any other organ. In addition to the glycerophospholipids and glycerol-ether lipids (plasmalogens) that are normal constituents of all cells and organellar membranes, the brain contains cerebrosides, sulfatides, globosides and gangliosides. The fatty acid composition of the brain is extremely varied, reflecting the wide range of structural and metabolic functions of these complex lipids.

FAs are derived from the local synthesis, breakdown from cerebral lipids, and uptake from the circulation across the blood-brain barrier (BBB) [47]. A number of pathways have been suggested to contribute to fatty acid uptake from blood at the brain capillary membrane. These include receptor-mediated transport of acylated species via lipoprotein receptors [48] and carrier-mediated uptake of intact lysophospholipids [49, 50, 51]. In addition, fatty acids may be cleaved from circulating lipids by the endothelial lipoprotein lipase for direct influx of free fatty acid [52]. Several studies suggest that a primary contributor to brain fatty acid influx is the uptake of free fatty acids following dissociation from plasma proteins [53, 54]. Most unacylated fatty acid in serum circulates noncovalently bound (>99.9%) to plasma proteins, predominantly albumin [55]. Under normal physiologic conditions, the free fraction is estimated to be less than 0.1% in serum [56, 57]. Given that the brain capillary permeability (i.e., blood-brain barrier) to free fatty acid is high, due either to facilitated transport or lipid-soluble passive diffusion [58, 59], a significant fraction of fatty acid that dissociates from albumin in brain capillaries would be expected to enter brain parenchyma [54]. Indeed, a number of vascular injection studies have demonstrated unacylated fatty acid uptake and incorporation in brain [53, 60, 61, 62]. However, dietary studies have suggested that

the overwhelming majority of saturated and monounsaturated fatty acids in adult brain are derived from local synthesis [63, 64]. Therefore, the exact contribution of circulating fatty acids to brain lipid uptake and incorporation remains to be clarified.

Brain tissue contains four FABP types: B-FABP (brain-type FABP), H-FABP (heart-type FABP), M-FABP (myelin-type FABP) and E-FABP (epidermal-type FABP). Each displays a distinct spatio-temporal distribution.

B-FABP was first identified in the brain of rodents with varying tissue expressions during development [7, 65]. It is expressed in neuroepithelial precursor cells of the developing brain and later becomes restricted to radial glial cells and immature astrocytes [66]. H-FABP, which shares a 70% amino acid sequence identity with B-FABP, was initially detected in cardiac myocytes but also was found in the neurons of the grey matter in mouse and rat. In adult brain, H-FABP is predominantly found in neuronal tissue (neuronal cell body) and constitutes 0.01% of the total brain cytosolic protein [67, 65]. In human brain, tissue contents of H-FABP were found to be >10 fold higher than those of B-FABP. H-FABP tissue content was highest in the pons [68], whereas B-FABP tissue content was highest in the frontal lobe.

E-FABP is expressed in astrocytes and in glia of the prenatal and perinatal brain, and also in neurons. E-FABP expression is induced following peripheral nerve

injury, suggesting a role in the regeneration of neurons [19, 66].

M-FABP is only present in the peripheral nerves and is unique in having been shown to be an extrinsic membrane protein, thus its membrane interactions properties are likely to be substantial and may dictate its function in peripheral nerve myelin [69].

FABPs have an important role during embryogenesis and regeneration of neurites and axons, probably supporting cells with adequate amounts of fatty acids originating from blood or formed by *de novo* synthesis, elongation or retroconversion. FAs are indeed essential as components of membrane phospho- and glyco-lipids.

In pathological conditions, such as ischemia and seizure-related brain damage, FABPs may protect cells against deleterious effects of accumulating FA. FABPs may deliver their ligands to nuclear receptors, resulting in the regulation of genes that encode metabolic enzymes, growth factors or other proteins involved in cell growth, adhesion or differentiation. In addition, FABPs may regulate the action of FA on specific ion channels and on neurotransmitter interaction with receptors and in this, or other ways, they may be involved in signal transduction [66].

1.3.1 HUMAN B-FABP (Brain-type FABP).

B-FABP, also known as FABP7, is expressed in various regions of the mouse brain in the mid-term embryonic

stage, but the expression decreases as differentiation progresses [70]. The protein is strongly expressed in radial glia cells of the developing brain, especially in the preperinatal stage, but only weakly in mature glia of the white matter. Neurons of the grey matter express H-FABP and E-FABP but not B-FABP. B-FABP is distinguished from other FABPs by its strong affinity for n-3 polyunsaturated fatty acids, in particular, docosahexaenoic acid (DHA) [29, 71]. DHA is the longest and most highly unsaturated fatty acid found in membranes and it is an important nutrient for the nervous system. Highly unsaturated long-chain fatty acids can alter the structure and function of membranes, increasing their fluidity, elasticity, and permeability, and potentially affecting signal transduction and gene expression [72, 73, 74]. Recent data support a role for DHA in the hyperfluidization of membranes [75].

Pathologically, B-FABP was overexpressed in patients with Down's syndrome and schizophrenia [76, 77]. Interestingly, experiments with B-FABP- deficient mice showed that the animals exhibited altered emotional behavioural responses, decreased prepulse inhibition that is a typical behaviour in schizophrenia, and attenuated neurogenesis in vivo [77, 78].

In the adult brain, B-FABP was localized more numerously to the astrocytes in the amygdala and septal area than to those in the hippocampal area. Analysis of

FA content in the amygdala of adult brain revealed that arachidonic and palmitic acids increased significantly in the mutant mice compared with wild-type. These data indicate that B-FABP is crucially involved in the fear memory and anxiety through its binding with FAs and / or its own direct effects on pertinent metabolism / signaling of FAs [78].

B-FABP may also serve as a useful prognostic marker for astrocytomas, particularly as related to the prediction of tumor spread and recurrence within the brain. In fact, B-FABP expression in malignant glioma cells increases migratory activity. The spread of astrocytoma cells within the brain is performed through the migration of B-FABP – expressing tumor cells that originate from radial glial cells. Manipulation of B-FABP levels in malignant glioma cells has a dramatic effect on their migratory and growth properties [38].

B-FABP gene is expressed in the radial glial cells of the developing central nervous system, as well as in a subset of human malignant glioma cell lines [79, 80]. In contrast, the B-FABP gene is not expressed in several leukemia (myelocytic, lymphocytic and megakaryocytic) cell lines and different carcinoma (alveolar, bladder, colon, prostate, and gastric) cell lines, except for an RCC cell line. B-FABP transcript is a specific marker for renal cell carcinomas (RCC): B-FABP gene is not expressed in the normal tissue but is in the carcinoma tissue, indicating that the B-FABP

transcript is a new marker for RCC. The B-FABP transcript is also detectable in the urine of patients diagnosed with RCC collected before tumor resection, but it is not amplified in their urine after resection [81].

B-FABP is also prominently expressed in the mammary gland, and its overexpression inhibited tumour growth in a mouse breast cancer model [82].

Early pregnancy is known to reduce the risk of breast cancer, as pregnancy results in mammary cell differentiation. It has been suggested that a possible cause of this reduction in breast cancer risk is the reduction in n6/n3 PUFA ratio and increased n3 PUFA content found in the pregnant mammary gland.

Further, in nonpregnant fat-1 transgenic mice, which can convert n6 to n3 PUFA, expression of BFABP resulted in a significantly greater reduction of the n6/n3 PUFA ratio, an increase in DHA levels in mammary tissue, and increased mammary cell differentiation. These results support a role for BFABP in n3 PUFA retention, possibly contributing to the effects of pregnancy on mammary gland differentiation [83].

1.3.2 HUMAN H-FABP (Heart-type FABP).

H-FABP, also known as FABP3, has been isolated from a wide range of tissues, including heart, skeletal muscle, brain, renal cortex, lung, testis, aorta,

adrenal gland, mammary gland, placenta, ovary and brown adipose tissue [28, 84].

Tissue	Part	H-FABP (µg/g ww)
Heart	Epicardial	540
	Midcardial	600
	Endocardial	550
Skeletal muscle		173
Liver		–
Small intestine	Duodenum	3.5
	Jejunum	4.9
	Ileum	3.2
	Colon	2.7
Brain	Frontal lobe	26.3
	Temporal lobe	31.9
	Occipital lobe	21.2
	Striatum	30.6
	Pons	39.5
	Cerebellum	16.2

(–) Not present or not detectable.

Table 2: Overview of reported tissue contents of H-FABP in the heart, skeletal muscle, intestine, liver and brain [45].

The level of H-FABP was influenced by exercise, PPAR-α agonists and testosterone and oscillates with circadian rhythm [30, 85]. In muscle cells, H-FABP was involved in the uptake of fatty acids and their subsequent transport towards the mitochondrial ß-oxidation system. Increased fatty-acid exposure *in vitro* and *in vivo* resulted in elevated H-FABP expression. Conditions with elevated plasma lipids may result in increased H-FABP levels in myocytes, as seen in endurance training [31, 84].

H-FABP is abundant in the myocardium and rapidly released from cardiomyocytes into the circulation after the onset of cell damage. Serum concentration of H-FABP has been proposed as an early biochemical marker of

acute myocardial infarction and a sensitive marker for the detection and evaluation of myocardial damage in patients with heart failure. However, the concentration of H-FABP is significantly influenced by renal clearance and thus has limitations in its usefulness for patients with renal dysfunction [86, 87, 88].

Heart-type FABP was first shown to be released from injured myocardium in 1988 [89], after which several studies have demonstrated that H-FABP is an early and sensitive marker for injured myocardium [90, 91].

H-FABP has proven not only to be an excellent marker for the early, within 6 h, detection of cardiac injury in acute coronary syndromes, but also showed to be sensitive enough for detecting minor myocardial injury in heart failure and displayed promising prognostic values for cardiac events in ACS and CHF [45].

Limitations of the use of H-FABP as a diagnostic plasma marker for myocardial injury are the fact that it is not cardiospecific, the diagnostic window is relatively small, extending to only 24–30 h after onset of chest pain, and its elimination from plasma by renal clearance can cause falsely high values in case of kidney malfunction. However, despite these possible drawbacks, H-FABP has been reported still as the most sensitive plasma marker for myocardial injury. In addition, it has the advantage of rapid detection of re-infarction and the ability to be used as a marker for infarct size quantification by using individually

estimated renal clearance rates. Because H-FABP also is expressed in distal tubular cells, it can be used as biomarker for detection of ischemic injury in kidney perfusates of donors [92, 93, 94, 95].

Interestingly, both H-FABP and GST are good markers for renal toxicity induced by heavy metals [92, 93, 94, 95, 96]. H-FABP also appears to be a marker for brain injury as it is expressed in the gray matter of the brain. Frontal, temporal, occipital lobe, striatum, pons and cerebellum showed 10-fold higher tissue contents of H-FABP compared to B-FABP (Table 2). Unlike B-FABP, H-FABP is detected in the neurons of the gray matter (neuronal cell bodies) and constitutes 0.01% of total brain cytosolic protein [65, 67].

2.MATERIALS AND METHODS

2.1 Expression and purification of human B-FABP in *Escherichia coli.*

2.1.1 Cloning

The plasmid containing the complete cDNA clone of human B-FABP was purchased from RZPD (Deuthsches Ressourcenzentrum fur Genomforschung). *E. coli* cells (DHIOB strain) containing the plasmid vector pCMVsport6 were grown in 3 ml of LB medium overnight at 37°C. Cells were harvested and the vector was purified using the GenElute Miniprep Kit (Quiagen). In order to insert the gene of interest in a different plasmid, it was amplified by PCR using the following primers:

BFABP-for: 5'-GGTGGTGGATCCATGGTGGAGGCTTTCTGTGCT-3'
BFABP-rev:
5'-AATAATAAGCTTGGAACCACGCGGAACCAGTGCCTTCTCATAGTGGCG-3'

GGATCC: Bam HI restriction site;
AAGCTT: Hind III restriction site;
GGAACCACGCGGAACCAG: thrombin cleavage site

A thrombin cleavage site was used for histidine tag removal after protein purification. The histidine tag consists of 6 histidine residues that are added at the

C-terminal portion of the recombinant protein and are coded into the plasmid vector used. This tag is very useful for the following protein purification step using a Nickel-Sepharose affinity column and also for the recombinant protein detection by western-blot analysis.

PCR cycle

1- 3 minutes at 95°C;
2- denaturation: 1 minute at 95°C;
3- annealing: 1 minute at 55°C;
4- extension: 1 minute at 72°C;
5- 10 minutes at 72°C.

Steps 2 to 4 repeated 35 times.

The amplified gene was quantified on a 0.8% agarose gel prepared with TBE buffer and DNA was purified using the Wizard® SVGel and PCR Clean-Up System; it was then digested with Hind III and Bam HI restriction enzymes and ligated to a pQE50 expression vector which was previously digested with the same restriction enzymes and dephosphorylated to prevent self-ligation. A 5:1 molar ratio between the amplified sequence and plasmid vector was used.

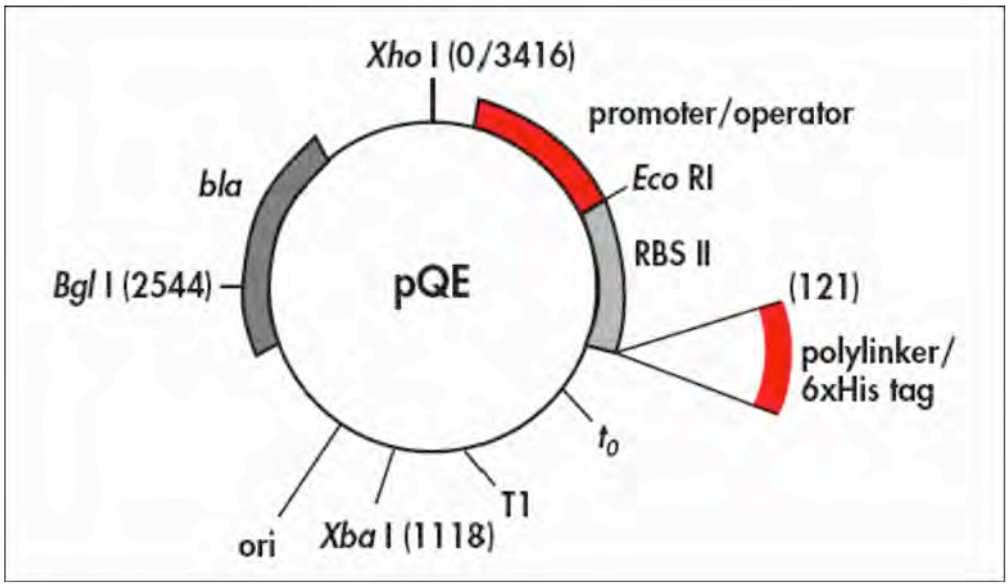

Figure 3: schematic representation of pQE vector.

The construct obtained was transformed into competent *E. coli* cells (XL1-Blue strain) by heat shock, cells were plated on a selective LB-agar plate containing ampicillin 100 µg/ml and grown at 37°C overnight. Bacterial colonies were checked for transformation by colony PCR and by digestion of the purified plasmid. A positive single colony was transferred into 100 ml of selective LB liquid medium and grown at 37°C overnight. Plasmid DNA was recovered by GenElute Miniprep Kit (Quiagen), assessed by DNA sequencing and used to transform 100 µl of *E. coli* cells, SG strain [SG13009 (pREP4)]. The remaining DNA was stored at -80°C. *E. coli* cells were plated on Petri dishes and the resulting colonies tested for protein expression.

2.1.2 Expression

3 colonies were inoculated in 3 ml of selective LB medium. Cultures were grown under agitation at 37°C. Gene expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Expression was allowed overnight at different temperatures (20°C, 28°C, 37°C). *E. coli* cells were harvested by centrifugation (10000g for 10 min) and resuspended in ice-cold buffer A with PMSF as protease inhibitor, then lysed by sonication. Samples from the total lysate and from the supernatant, after centrifugation, were collected and loaded on SDS-PAGE. Protein expression was confirmed by Western-blot analysis using an anti-histidine tag monoclonal antibody (Sigma-Aldrich®).

2.1.3 Purification

1 l culture was grown in a shaking incubator and protein expression was induced by IPTG 0.5 mM when the absorbance at 600 nm reached 0.4; the cells were transferred at 20°C overnight. After centrifugation *E. coli* cells were resuspended in 40 ml of buffer A and sonicated. The lysate was centrifuged and the soluble fraction was directly loaded onto a nickel affinity column (Chelating Sepharose™ Fast Flow, Amersham Biosciences). After washing with buffer A containing 10 mM imidazole, elution was performed with a linear

gradient from 10 mM to 500 mM imidazole. Fractions were analyzed for the presence of B-FABP by SDS-PAGE and western-blot analysis. Eluted fractions containing B-FABP were collected, dialysed against buffer B and incubated overnight at 20°C with thrombin for his-tag removal.

After a subsequent dialysis for removal of thrombin enzyme, the solution containing B-FABP was concentrated to a final volume of 2 ml and loaded onto a Superdex G75 column for a final purification step. Fractions were analysed for the presence of B-FABP by SDS-PAGE, and eluted fractions containing the protein of interest were collected.

2.2 Antibody Production

2.2.1 Overview

Immunogenicity is the ability of a molecule to solicit an immune response. There are three characteristics that a substance must have to be immunogenic: foreignness, high molecular weight (MW) and chemical complexity. Foreignness is required so that the immunized animal does not recognize and ignore the substance as “self”. Generally, compounds from an organism are not immunogenic to that same individual and are only poorly immunogenic to others of the same or related species. The second requirement for the

immunogenicity is high MW. Small compounds, with MW < 1,000 Da as well as many moderately sized molecules are not immunogenic. Most compounds with a MW > 6,000 Da are immunogenic. Compounds smaller than this can often be bound by mIgM on the surface of the B lymphocyte, but they are not large enough to facilitate cross-linking of the mIgM molecules. This cross-linking is commonly called “capping” and consists in the signal for receptor-mediated endocytosis of the antigen. Finally, some degree of chemical complexity is required for a compound to be immunogenic.

Small polypeptides need to be conjugated or cross-linked to larger, immunogenic, carrier proteins to increase immunogenicity. Keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA) are two widely used carrier proteins; poly-L-lysine is also used successfully as a backbone for peptides.

The primary goal of antibody production is to obtain high titer, high affinity antibodies to use experimentally as a diagnostic test. Adjuvants are used to improve or enhance an immune response to antigens. Freund’s Complete Adjuvant (FCA) has been used for many years; it consists of an aqueous solution of antigen emulsified in mineral oil containing heat-killed mycobacterial organisms. The adjuvant activity of FCA is two-fold: first, the oil retains the antigen as a depot at the injection sites, leading to prolonged exposure of the antigen to the immune cells. This

procedure results in a sustained exposure of antigen. Second, the mycobacterial components serve as immunostimulant for both the humoral and cell-mediated immunity, which also facilitates antibody production. The presence of lipid droplets and mycobacterial components at the injection site can cause a granulomatous reaction.

Freund's Incomplete Adjuvant (FIA), which consists of FCA without the mycobacterial component in the oil is commonly used to booster animals given FCA. Alone, FIA is also capable of causing abscess and granuloma formation, but such reactions are generally less severe than those that accompany the use of FCA.

Adjuvants that have less inflammatory complications do exist but are used to a much lesser extent. Alum and aluminum salt solutions stimulate only a humoral response that may be inadequate for weak immunogens, which require cellular mediation for a strong antibody response.

Two types of antibody samples can be used in the study of antibody-related phenomena: polyclonal antibodies and monoclonal antibodies.

Depending on the experimental situation, either a monoclonal or polyclonal approach may be warranted. Each approach offers distinct advantages:

- In the case of polyclonal antibodies, there are clear facilities: they are inexpensive to produce relative to the cost of monoclonal antibody technology. In

addition, large quantities of polyclonal antibodies can be produced from the serum of an immunized animal. Finally, high affinity polyclonal antibodies can be isolated 2-3 months after the initial immunization. This expeditious production facilitates their rapid study. Because polyclonal antibodies contain the entire antigen-specific antibody population, they offer a statistically relevant glimpse into the overall picture of an immune response. A similar viewpoint is considerably more difficult using a monoclonal antibody approach.

- Monoclonal antibodies have certain advantages over polyclonal antibodies. Because of their immortal nature, hybridoma cells can be frozen, thawed and recultured *in vitro.* As a result, for a given monoclonal line, there exists a constant and renewable source of antibodies for study.

2.2.2 Polyclonal Antibodies

Polyclonal antibodies represent the antibodies from multiple clones or B lymphocytes, and therefore bind to a number of different epitopes. For antibody production purposes, these are obtained from the serum of an immunized animal, thereby providing a wide range of antibody types and epitope affinities (to molecules other than the intended antigen as well). For many applications, this diversity of antibodies provides an

advantage by allowing the researcher to detect multiple epitope sites on the protein of interest.

Animals frequently used for polyclonal antibody production include chickens, goats, guinea pigs, hamsters, horses, mice, rats and sheep. However, the rabbit is the most used laboratory animal. Since immune function peaks at puberty and primary responses to new antigens decline with age, young adult rabbits should be used for primary immunization because of their vigorous antibody response. At least two animals per antigen should be used when using outbred animals.

2.2.2.1 Polyclonal Antibody Production Protocol

- Subject: two New Zealand white rabbits;
- Antigen: rec human B-FABP (50-500 µg/immunization);
- Adjuvant: complete Freund's Adjuvant initially, followed by Incomplete Freund's Adjuvant for all subsequent injections;
- Procedure: 0.5 ml of protein was combined with 0.5 ml of FCA, mixed thoroughly to form a stable emulsion that was injected beneath the rabbit skin (subcutaneously) in the area around the shoulders and intra-muscularly into the large muscle of the rear legs;
- Bleeds: blood was collected from the central ear artery and allowed to clot and retracted at 37°C

for 1 hour. The clotted blood was then refrigerated overnight at 4°C before the serum was decanted and clarified by centrifugation at 5000 rpm for 20 minutes.

Antibody Production Schedule:

Week 0	Bleed	Pre-immune serum collection	5 ml serum/rabbit
Week 0	Immunize	Complete Freund's Adjuvant	/
Week 4	Bleed	#1- Production bleed	25 ml serum/rabbit
Week 5	Immunize	Incomplete Freund's Adjuvant	/
Week 8	Bleed	#2- Production Bleed	25 ml serum/rabbit
Week 9	Bleed	#3- Terminating Bleed	100 ml serum/rabbit

2.2.3 Monoclonal Antibodies

Monoclonal antibodies, by contrast, represent a single B lymphocyte generating antibodies to one specific epitope. Two primary advantages arise with such an application. First, by fusing the lymphocyte with an immortal myeloma cell line, one can create a hybridoma

cell line capable of producing virtually unlimited quantities of a specific antibody. Second, the antibodies produced are identical and specific only to the epitope of interest. In particular, this is an advantage in applications requiring controlled manufacturing procedures, such as in clinical diagnostic tests or in therapeutic treatments directed towards humans.

Production of monoclonal antibodies involves *in vivo* and *in vitro* procedures. Before antibody production by either method, hybrid cells that will produce the antibodies are generated. The generation of mAb-producing cells requires the use of inbred mice. The procedure yields a cell line capable of producing one type of IgG antibody protein for life long. A tumor from this "immortal" cell line is called hybridoma.

2.2.3.1 Monoclonal antibody production protocol

- Subject: BalbC mice;
- Antigen: rec human B-FABP (5-50 µg/immunization);
- Adjuvant: complete Freund's Adjuvant only for the first injection;
- Procedure:
    - Mice immunization and selection of mouse donors for the generation of hybridoma cells: mice were immunized with the antigen that was prepared for injection by

emulsifying the antigen with Freund's adjuvant. Mice were immunized every 2 weeks. When a sufficient antibody titer was reached in serum, immunized mice were euthanized. The spleen was removed and the cells used for fusion with myeloma cells NSO.

- Screening of mice for antibody production: after several weeks of immunization, mice blood samples were obtained for the measurement of serum antibodies by enzyme-linked immunosorbent assay (ELISA). If the antibody titer was high, cell fusion was performed. If the titer was too low, mice were boosted until an adequate response was achieved, as determined by repeated blood sampling. If the antibody titer was sufficiently high, mice were boosted by injecting antigen without adjuvant intraperitoneally 3 days before fusion and 2 weeks after the previous immunization. Then the mice were euthanized and their spleens removed for in vitro hybridoma cell production.
- Preparation of myeloma cells: fusing antibody-producing spleen cells, which have a limited life span, with cells derived from an immortal lymphocyte tumor (myeloma) results in a hybridoma that is capable of

unlimited growth. Myeloma cells are immortalized cells that are cultured with 8-azaguanine to ensure their sensivity to the hypoxanthine-aminopterin-thymidine (HAT) selection medium used after cell fusion. A week before cell fusion, myeloma cells were grown in 8-azaguanine. Cells should have high viability and rapid growth. The HAT medium allows only the fused cells to survive in culture.

- Fusion of myeloma cells with immune spleen cells: single spleen cells from the immunized mouse were fused with the previously prepared myeloma cells. Fusion was accomplished by co-centrifugation freshly harvested spleen cells and myeloma cells in polyethylene glycol, a substance that causes cell membranes to fuse. Only fused cells would grow in the selection medium. The cells were then distributed to 96 well plates containing feeder cells derived from saline mouse peritoneal washes. Feeder cells are believed to supply growth factors that promote growth of the hybridoma cells.
- Cloning of hybridoma cell lines: small clusters of hybridoma cells from the 96 well plates were grown in tissue culture followed

by selection for antigen binding or grown by the mouse ascites method.

2.3 Antigenic peptide sequences: computer-assisted selection

In order to obtain some antibodies able to recognize human B-FABP and not cross-reacting with other isoforms or proteins, it was decided to produce synthetic peptides to utilize them as antigens, for the generation of antibodies in rabbits. Indeed, new antibodies would be able to recognize also the protein, being the peptide sequence a part of the whole protein. The steps to produce an effective antibody included:

1. Designing the peptide sequence based on the protein sequence;
2. Synthesizing the peptide;
3. Preparing the immunogen by coupling the peptide to a carrier protein;
4. Immunizing the host animal;
5. Obtaining the antiserum and/or isolating the antibody.

The human B-FABP sequence was studied with a computer-assisted approach. The first goal was to identify the sequences that had a higher-than-average probability of producing an effective antigen.

For the choice of the sequences, a BLASTp (www.expasy.org/tools/blast/) was performed on the human, rabbit and murine proteome to find proteins with a high percent identity to human B-FABP. Ten high score proteins were taken, and their sequences were aligned with Clustal W2, a general purpose multiple sequence alignment program for DNA or proteins (www.ebi.ac.uk/tools/clustalw2/index.html).

At this point, the choice of epitope sequences was performed following these criteria:

1. Percentage identity of the sequence: the epitope must be specific for a single protein, so it must be constituted of residues different from other similar proteins.
2. solvent-exposition: the epitope must be solvent-exposed to be accessible to the antibody. The general features of protein structure that correspond to these criteria are turn or loop structures and areas of high hydrophilicity.

A structural study was also performed on the three-dimensional structure of the target protein with the Pymol software (Pymol 0,99rc6) on PDB files (www.rcsb.org), comparing different structure models obtained by NMR or X-ray diffraction techniques, displaying which residues were solvent-exposed and which were buried.

2.4 Antigenic peptide sequences: synthesis, conjugation and antibody production protocol in rabbits

Peptide synthesis, peptide conjugation to a carrier protein and polyclonal antibody production were performed by Pacific Immunology (www.pacificimmunology.com);

After chemical synthesis, all peptides were analyzed by Mass Spectrometry and HPLC. The peptides were conjugated to KLH and inoculated in rabbits.

2.4.1 Polyclonal Antibody Production Protocol

- Subject: 4 New Zealand white rabbits (PAC 2979; 2980, 2981; 2982);
- Antigen: peptide 1/peptide 2;
- Immunogen: the peptides were conjugated to KLH.
- Adjuvant: complete Freund's Adjuvant initially, followed by Incomplete Freund's Adjuvant for all subsequent injections;
- Procedure: 0.5 ml of protein solution was combined with 0.5 ml of FCA, mixed thoroughly to form a stable emulsion that was injected beneath the rabbit skin (subcutaneously) in the area around

the shoulders and intra-muscularly into the large muscle of the rear legs;

- Bleeds: blood was collected from the central ear artery and allowed to clot and retract at 37°C for 1 hour. The clotted blood was then refrigerated overnight at 4°C before the serum was decanted and clarified by centrifugation at 5000 rpm for 20 minutes.

Antibody Production Schedule:

Week			Rabbit 1	Rabbit 2
0	Bleed	Pre-immune serum collection	5 ml	5 ml
0	Immunize	Complete Freund's Adjuvant	/	/
3	Immunize	Incomplete Freund's Adjuvant		
6	Immunize	Incomplete Freund's Adjuvant		
7	Bleed	Production bleed	25 ml	25 ml
9	Bleed	Production bleed	25 ml	25 ml
10	Immunize	Incomplete Freund's Adjuvant		
11	Bleed	Production bleed	25 ml	25 ml
13	Bleed	Production bleed	25 ml	25 ml
14	Bleed	Final bleed part 1	25 ml	25 ml
14	Bleed	Final bleed part 2	45 ml	40 ml

Serum from the third bleed was subjected to affinity purification, with the peptides coupled to a column in order to capture only those antibodies that recognized the peptide sequence, obtaining pAb 2979/2980 and pAb 2981/2982.

3.RESULTS

3.1 Expression and purification of human B-FABP in *Escherichia coli*

The gene codifying the human B-FABP was cloned efficiently in pQE50 vector and the construct utilized for transforming *E. coli* cells (SG strain). Protein expression was induced by addition of IPTG 0.5 mM to cultures growing in LB selective medium. Three different temperatures were tested for induction overnight: 20°C, 28°C and 37°C. *E. coli* cells were then harvested by centrifugation (10000g for 10 min) and resuspended in ice-cold buffer A with PMSF as protease inhibitor, then lysed by sonication. After centrifugation, samples from the total lysate and from the supernatant, were collected and analyzed by SDS-PAGE. Protein expression was confirmed by western-blot analysis using an anti-histidine tag monoclonal antibody (Sigma-Aldrich™).

A soluble protein expression was obtained with IPTG induction at 20°C overnight: 1 l culture was grown and the soluble protein fraction was loaded onto a nickel affinity column. The elution pattern from the IMAC column showed two separate peaks: the protein of interest was present in the second, corresponding to an imidazole concentration of 190 mM.

With overnight induction the yield was 16 mg of recombinant B-FABP per litre of shaking culture of bacteria.

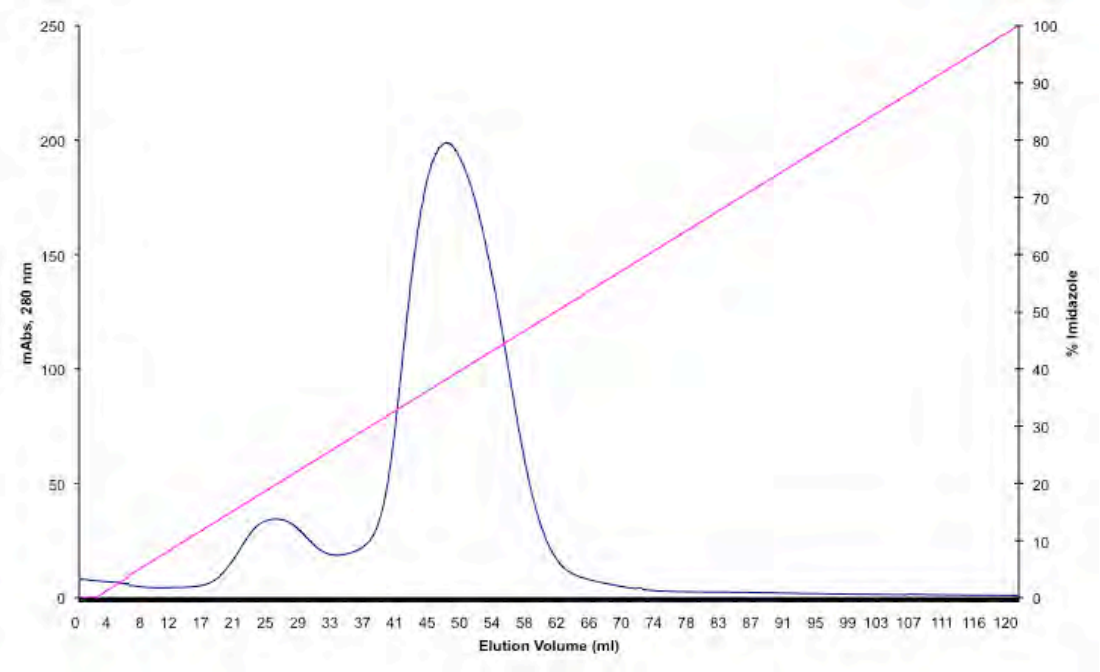

Figure 4: Elution profile of B-FABP from the Nickel Sepharose affinity column. Blue line corresponds to UV absorbance and pink line to imidazole gradient in percentage. B-FABP is found in the second peak.

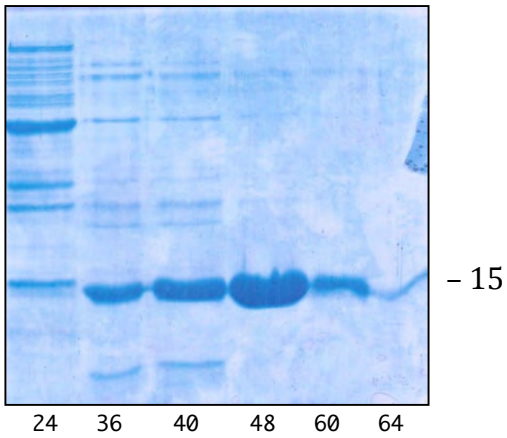

Figure 5: SDS-PAGE analysis (15%) of fractions eluted from the Nickel Sepharose affinity column, showing recombinant B-FABP. Approximate molecular weight is in kilodaltons.

Proteolytic cleavage of the hexa-histidine tag by thrombin enzyme resulted in a complete tag removal.

A subsequent purification step was performed with a Superdex G75 column. The eluted protein is more than 90% pure, as indicated by SDS-PAGE.

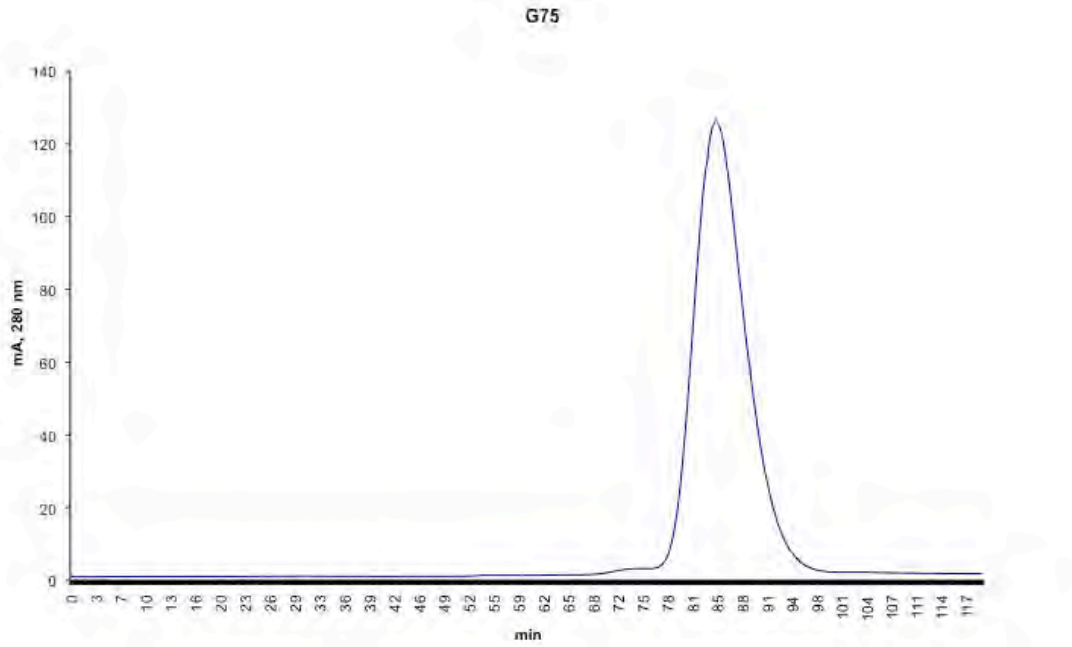

Figure 6: Elution profile of B-FABP from gel-filtration chromatography. Blue line corresponds to UV absorbance.

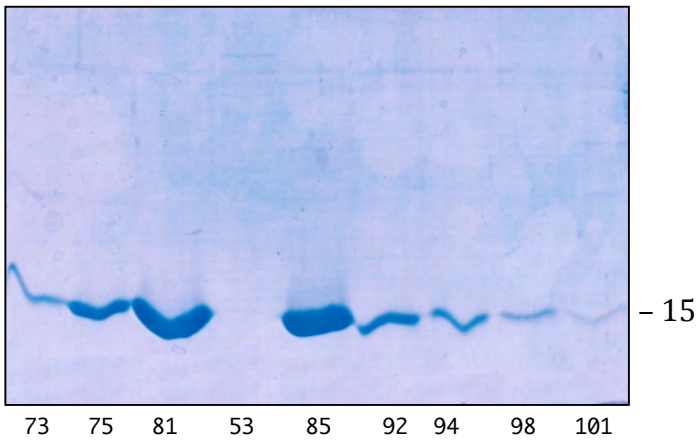

Figure 7: SDS-PAGE analysis (15%) of fractions eluted from gel-filtration chromatography showing recombinant B-FABP. Approximate molecular weight is in kilodaltons.

3.2 Antibody Production

All the antibodies generated were tested for their ability to recognize human B-FABP by ELISA and western

blot analysis. Because of the high degree of identity between human brain- and heart-FABP sequences, and since they are both expressed in the nervous system, the hypothetical cross-reaction of antibodies with rec human H-FABP was evaluated. (Rec H-FABP was kindly provided by Dr. Pelsers, Maastricht University, The Netherlands).

3.2.1 Polyclonal Antibody Production

Rec human B-FABP was inoculated in New Zealand rabbits, obtaining after 9 weeks two antisera that were tested for their ability to recognize rec human B-FABP and rec H-FABP. The antisera were able to recognize the two proteins, in both ELISA and in western blot analysis.

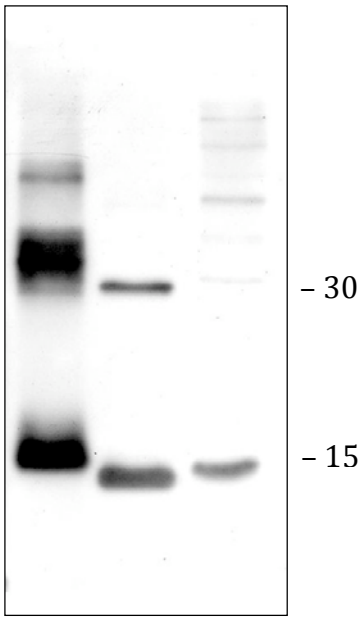

Figure 8: An immunoblot analysis of rec B-FABP, rec H-FABP and brain homogenate 10%. The membrane was probed with rabbit antiserum (1:1000). Approximate molecular weights are in kilodaltons.

3.2.2 Monoclonal Antibody Production

The high degree of sequence homology between murine and human B-FABP indicated that most of the protein sequences were not potentially immunogenic.

Since after immunization low levels of antibody titer were obtained, the protocol was prolonged. After hybridoma cell creation, the cell populations were cultured *in vitro* and clones named: 1C6, 1E1, 2D7, 2F9, 3A5, 3G9, 4E2 and 4F6. The culture supernatants of hybridoma cells were subjected to a first screening to test their ability to recognize antigen (rec B-FABP) and to cross-react with rec H-FABP.

First, an Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed for determining the affinity for the two FABP forms of the antibodies produced by the 8 hybridoma cells.

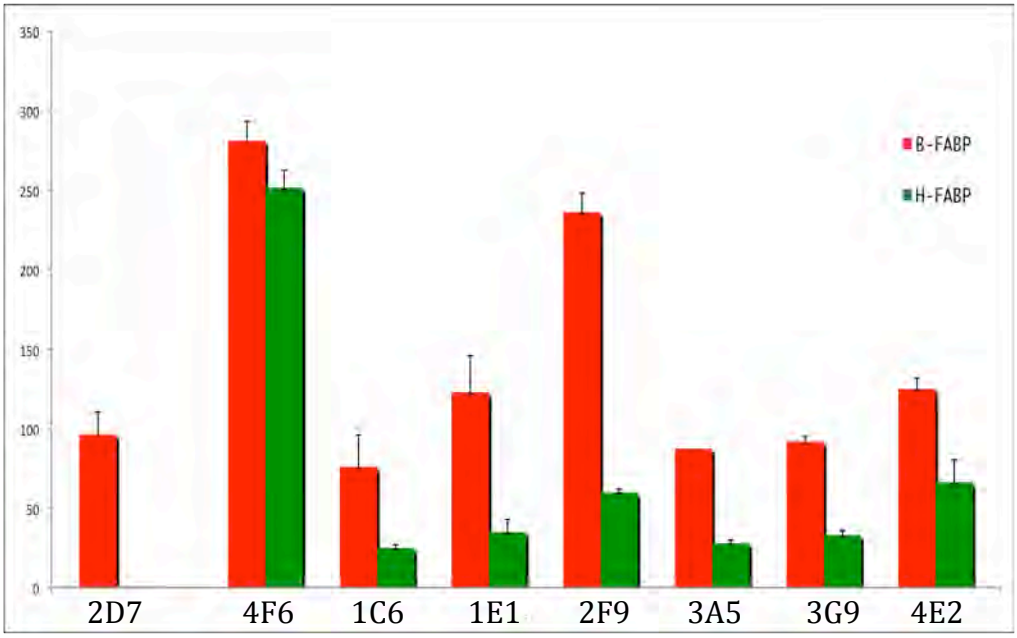

Figure 9: Enzyme-Linked ImmunoSorbent Assay measuring affinity of culture supernatant of 8 hybridoma cells for rec B-FABP, rec H-FABP. Values are expressed in mA at 450 nm. The measurement was done with 6 replicates of each sample.

In this test, the recombinant protein (B-FABP/H-FABP) was coated on an ELISA microplate and incubated with the culture supernatant. The results showed that only 2D7 was able to recognize selectively B-FABP, with a weak signal of 95 mA at 450 nm.

On the contrary, 4F6 showed a big affinity for both isoforms, whereas other culture supernatants of hybridoma cells cross-reacted with B-FABP and H-FABP at different levels.

Since the aim of this project was to obtain a specific antibody for human B-FABP, able to detect this protein in a biological sample, an ELISA sandwich assay was performed. ELISA assay was based on 2D7 as capture antibody (specific) and 4F6-biotinylated as revelation antibody. Rec human B-FABP was used as sample. Unfortunately, no signal was detected in any experiment.

Thus, another ELISA sandwich test was performed utilizing 2D7 as capture antibody and purificated rabbit antiserum as revelation antibody. After this analysis, rec B-FABP was not detectable. The same tests were performed using 2D7 with the other culture supernatants of hybridoma cells, but also these combinations did not reveal B-FABP presence.

Thus, it was valued 2D7 ability to recognize the two FABP forms by immunoblot analysis, preceded by an

electrophoretical separation of recombinant protein by SDS-PAGE.

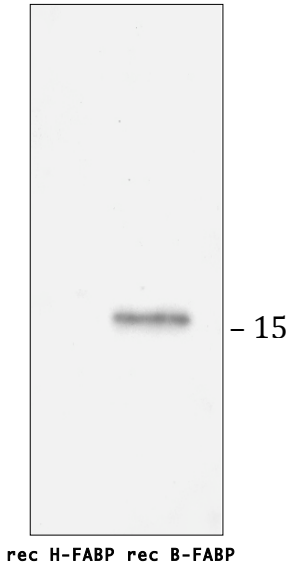

Figure 10: Immunoblot analysis of rec B-FABP and rec H-FABP. The membrane was probed with 2D7 (1:10). Approximate molecular weight is in kilodaltons.

2D7 recognized only recombinant B-FABP specifically by western blot analysis and by ELISA. 2D7 was able to recognize although weak, only brain-type FABP. This experiment confirmed the specificity and selectivity of 2D7 that was therefore probed for detecting brain-type FABP in cerebrospinal fluid (CSF).

Cerebrospinal fluids from patients affected with different neurological pathologies [dementia, sCJD (sporadic Creutzfeldt-Jakob disease) and PPS (post-polio syndrome] were utilized; CSF from healthy people age-matched were used as controls.

The samples were loaded on a SDS-PAGE and blotted onto a pvdf membrane: 2D7 was utilized as primary antibody. Unfortunately, samples incubated with this culture supernatant were negative.

Although 2D7 was specific for B-FABP, it did not present the characteristics needed for its utilization by western blot and ELISA analysis. The 2D7-cloning step, for the isolation of a single immortal cell producing mAb 2D7 encountered some difficulties: some fusions were strongly positive at first screen, however after subsequent subcloning the activity gradually decreased.

Since other culture supernatants of 7 hybridoma cells in preliminary ELISA tests had shown different degrees of affinity for the 2 isoforms, it was decided to use them in western blot analysis: the attention focused first on 4F6, because of strong signal obtained in ELISA test.

Thus, a pvdf membrane blotted with rec B-FABP and rec H-FABP samples was probed by 4F6: the signal was detectable in both lanes, corresponding to rec B-FABP and rec H-FABP.

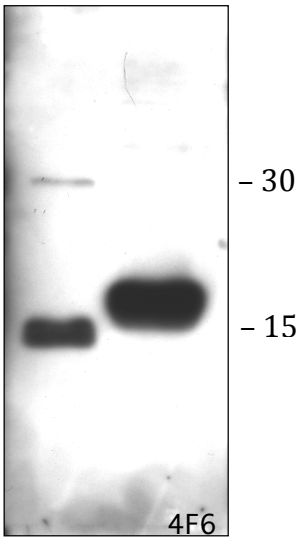

Figure 11: Immunoblot analysis of rec B-FABP and rec H-FABP. The membrane was probed with 4F6 (1:50). Approximate molecular weight is in kilodaltons.

Though 4F6 was not selective for brain-type isoform, it demonstrated to produce high antibodies levels, giving raise to strong signal by western blot analysis. Thus, for detecting brain-type FABP in cerebrospinal fluid (CSF) of patients affected with neurological pathologies (dementia, sporadic Creutzfeldt-Jakob disease and post-polio syndrome), this culture supernatant was probed. CSF from healthy people age-matched were used as controls.

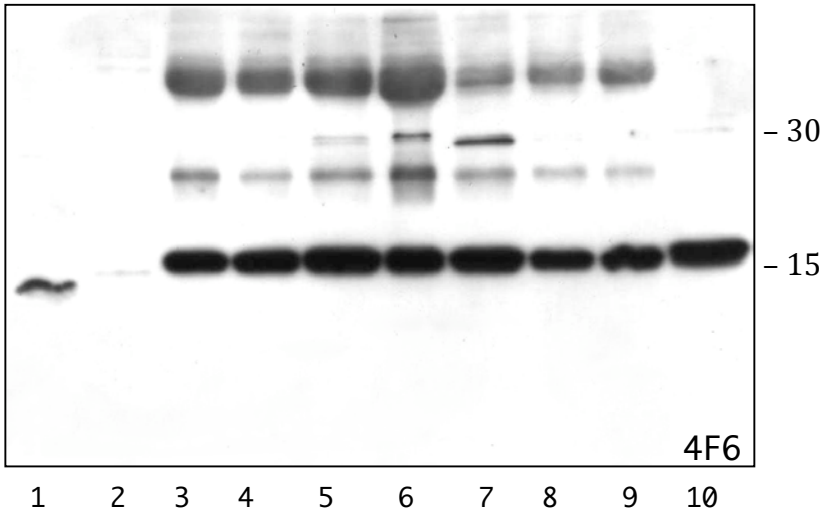

Figure 12: An immunoblot analysis of rec B-FABP, rec H-FABP and CSF. The membrane was probed with 4F6 (1:50). [1=rec H-FABP; 2,10= rec B-FABP; 3,4= CSF from healthy people (controls); 5,6= CSF from patients with dementia; 7,8= CSF from patients with sCJD; 9= CSF from patients with PPS]. Approximate molecular weights are in kilodaltons.

A positive ~ 15-kDa band was detected in samples corresponding to CSF from controls and from patients affected with neurological diseases. The electrophoretic migration profile of this ~ 15-kDa protein was comparable to the rec B-FABP one. Though the strong signal displayed, no differences were shown

among samples. It was decided to verify if the protein band observed in immunoblot analysis would correspond to another protein, highly represented in the cerebrospinal fluids, with a molecular weight ~ 15/16-kDa, human transthyretin (TTR). This protein, of 15.8 kDa, is a serum and a cerebrospinal fluid carrier of the thyroid hormone thyroxine (T4). TTR also acts as a carrier of retinol (vitamin A) through an association with retinol binding protein.

Human B-FABP and TTR did not exhibit significant similarity, but this not excluded a possible cross-reaction of 4F6 for transthyretin.

In order to verify if this hypothesis was correct, an immunoblot analysis was performed on rec TTR sample (rec human TTR was kindly provided by Dr. M. Perduca, University of Verona, Italy).

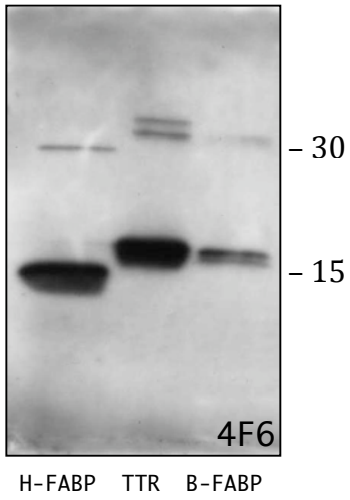

Figure 13: Immunoblot analyses of rec B-FABP, rec H-FABP and rec TTR. Membrane was probed with 4F6 (1:50). Approximate molecular weight are in kilodaltons.

As expected, 4F6 recognized rec TTR by immunoblot analysis.

To confirm that the ~ 15/16-kDa protein band, visualized in the previously CSF immunoblot, corresponded to TTR, 4 CSF samples were incubated with 4F6 and with anti-TTR antibody (Prealbumin, DAKO); the cerebrospinal fluids were obtained from 2 healthy people age-matched, from a patient affected with dementia and from a patient affected with sCJD.

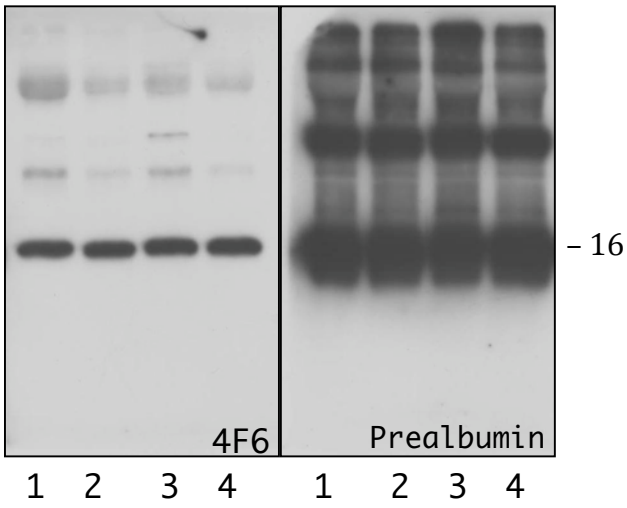

Figure 14: Immunoblot analyses of 4 CSF. The membrane were probed with 4F6 (1:50) and Prealbumin antibody (DAKO, 1:1000). [1,2= CSF from healthy people (controls); 3= CSF from patient with dementia; 4= CSF from patient with sCJD]. Approximate molecular weights are in kilodaltons.

The results confirmed what supposed, 4F6 and Prealbumin antibody recognize the same protein, as shown observing the signal pattern around area of 16 kDa.

The intensity of signal obtained was the same in control and in pathological CSF samples. At the moment it is not possible to determine whether the protein

band visualized on membrane would reflects the presence of either proteins, or only the TTR one.

At this point an ELISA analysis was performed for determining the degree of affinity of 4F6 for TTR. Likewise, the other 7 culture supernatants of hybridoma cells were tested in order to exclude or confirm cross-reactivity for transthyretin.

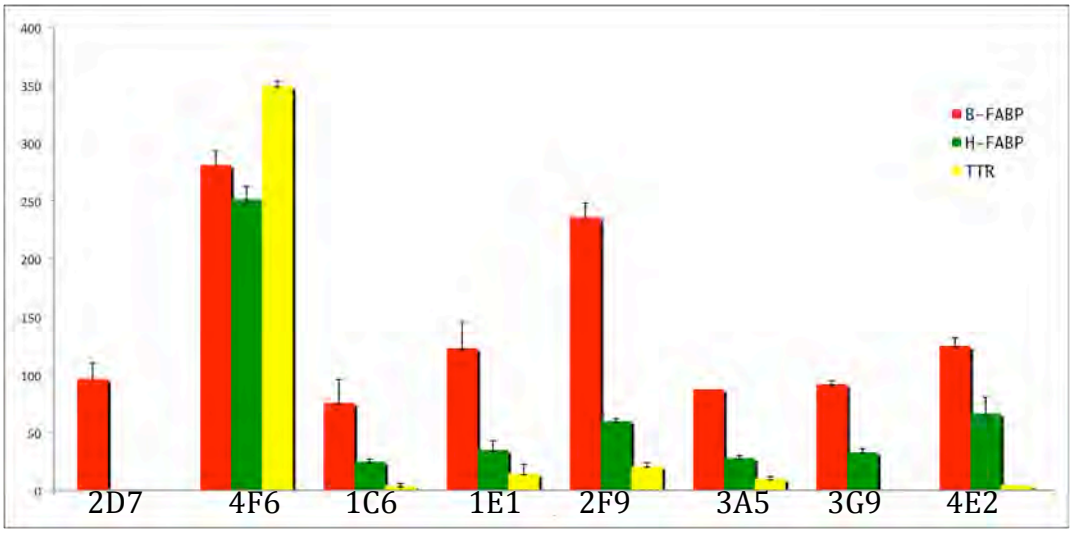

Figure 15: ELISA measuring affinity of 8 culture supernatants of hybridoma cells for rec B-FABP, rec H-FABP and rec TTR. Values are expressed in mA at 450 nm. The measurement was done with 6 replicates of each sample.

ELISA proved 4F6 cross-reaction for TTR.

This finding confirmed that, though 4F6 gave raise to a strong signal both in immunoblot and in ELISA analyses, it could not be utilized for detection of B-FABP in CSF, because of its lack of selectivity and evidence of B-FABP recognition in cerebrospinal fluid.

To be noted, 2D7 did not cross-react with transthyretin, confirming its specificity and selectivity for B-FABP.

About 1C6, 1E1, 2F9, 3A5, 3G9 and 4E2, as previously described in the first ELISA test, they showed a different degree of affinity for the 2 FABP forms; moreover, 1C6, 1E1, 2F9, 3A5 and 4E2 cross-reacted with TTR contrary to 3G9 not exhibiting cross-reaction with transthyretin but with H-FABP. An immunoblot analysis was performed on rec FABPs and TTR samples utilizing the culture supernatants of the 6 hybridoma cells, to value their specificity and selectivity utilizing a different technique than ELISA. No signal was detectable in membranes probed with 1C6, 1E1, 2F9, 3A5, 3G9 and 4E2.

In order to circumvent cross-reactions problems, because of high homology between human and murine B-FABP, and human brain-type and heart-type FABP, it was decided to generate anti-human B-FABP antibodies inoculating in rabbits a synthetic peptide with a sequence corresponding to a part of the B-FABP molecule.

3.3 Analysis of Antigenicity of human B-FABP

The human B-FABP sequence was analysed with a computer-assisted approach. The first goal was to identify the sequences that had a higher-than-average probability to be highly antigenic.

For the choice of the sequences, a BLASTp (www.expasy.org/tools/blast/) search was performed on the human, rabbit and murine proteome to find proteins with a high percent identity to human B-FABP.

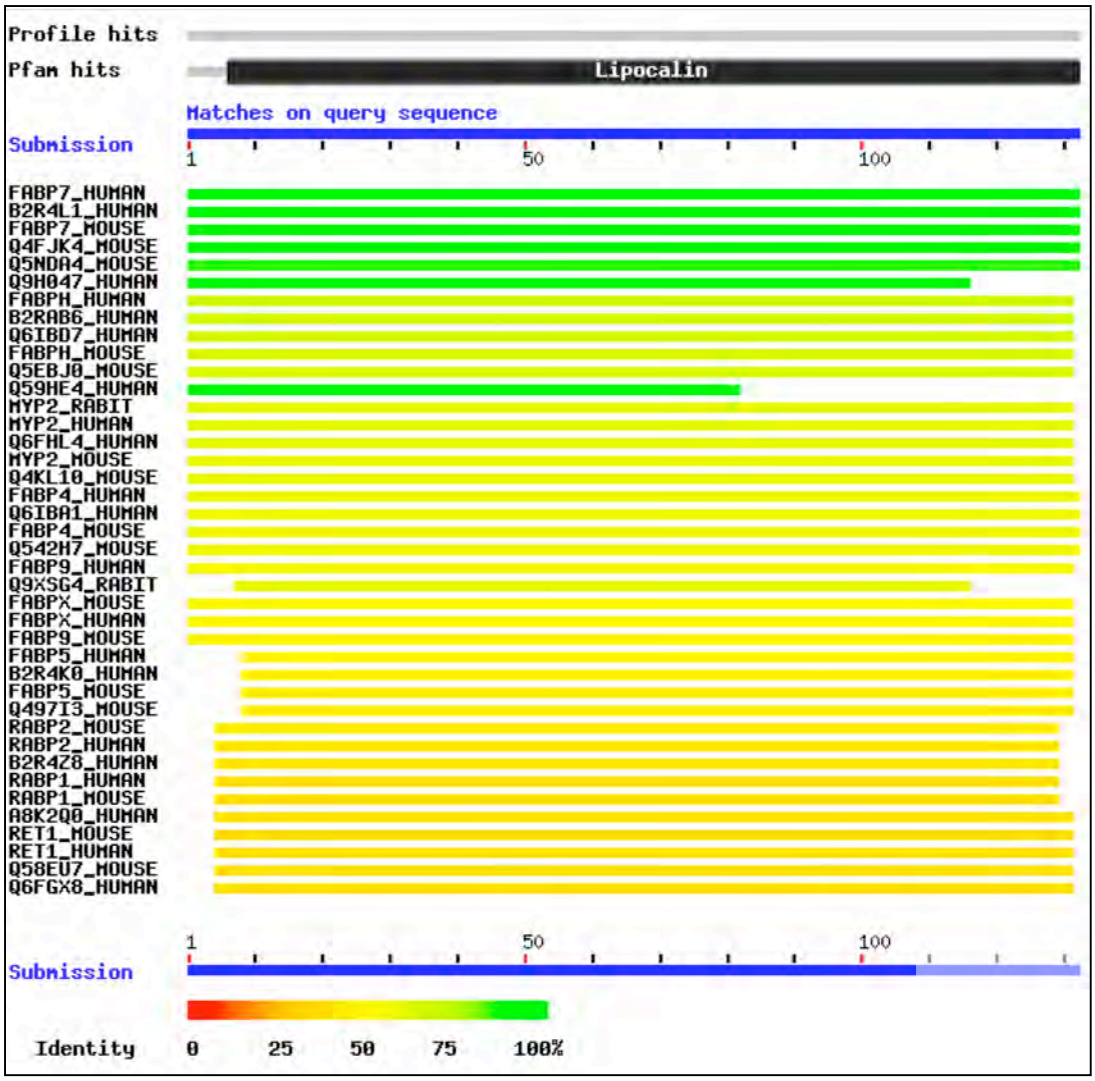

Figure 16: BLASTP-query against the UniProt Knowledgebase (Swiss-Prot + TrEMBL; Taxonimoc Group: Homo Sapiens, Mus Musculus, Oryctolagus cuniculus).

Ten high score proteins were selected (discarding fragments), and their sequences were aligned with Clustal W2, a general purpose multiple sequence alignment program for DNA or proteins (www.ebi.ac.uk/tools/clustalw2/index.html).

```
CLUSTAL 2.0.10 multiple sequence alignment


sp|P02691|MYP2_RABIT        MSNKFLGTWKLVSSENFDDYMKALGVGLATRKLGNLAKPNVIISKKGDII 50
sp|P02689|MYP2_HUMAN        MSNKFLGTWKLVSSENFDDYMKALGVGLATRKLGNLAKPTVIISKKGDII 50
sp|P24526|MYP2_MOUSE        MSNKFLGTWKLVSSEHFDDYMKALGVGLANRKLGNLAKPTVIISKKGDYI 50
sp|Q0Z7S8|FABP9_HUMAN       MVEPFLGTWKLVSSENFEDYMKELGVNFAARNMAGLVKPTVTISVDGKMM 50
sp|P15090|FABP4_HUMAN       MCDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNMIISVNGDVI 50
sp|P04117|FABP4_MOUSE       MCDAFVGTWKLVSSENFDDYMKEVGVGFATRKVAGMAKPNMIISVNGDLV 50
sp|O15540|FABP7_HUMAN       MVEAFCATWKLTNSQNFDEYMKALGVGFATRQVGNVTKPTVIISQEGDKV 50
sp|P51880|FABP7_MOUSE       MVDAFCATWKLTDSQNFDEYMKALGVGFATRQVGNVTKPTVIISQEGGKV 50
sp|P05413|FABPH_HUMAN       MVDAFLGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDIL 50
sp|P11404|FABPH_MOUSE       MADAFVGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDTI 50
                            * : * .****..*::*::*** :**.:* *::..:.**.  *. .*  :

sp|P02691|MYP2_RABIT        TIRTESTFKNTEISFKLGQEFEETTADNRKTKSIITLERGALNQVQKWDG 100
sp|P02689|MYP2_HUMAN        TIRTESTFKNTEISFKLGQEFEETTADNRKTKSIVTLQRGSLNQVQRWDG 100
sp|P24526|MYP2_MOUSE        TIRTESAFKNTEISFKLGQEFDETTADNRKAKSIVTLERGSLKQVQKWDG 100
sp|Q0Z7S8|FABP9_HUMAN       TIRTESSFQDTKISFKLGEEFDETTADNRKVKSTITLENGSMIHVQKWLG 100
sp|P15090|FABP4_HUMAN       TIKSESTFKNTEISFILGQEFDEVTADDRKVKSTITLDGGVLVHVQKWDG 100
sp|P04117|FABP4_MOUSE       TIRSESTFKNTEISFKLGVEFDEITADDRKVKSIITLDGGALVQVQKWDG 100
sp|O15540|FABP7_HUMAN       VIRTLSTFKNTEISFQLGEEFDETTADDRNCKSVVSLDGDKLVHIQKWDG 100
sp|P51880|FABP7_MOUSE       VIRTQCTFKNTEINFQLGEEFEETSIDDRNCKSVVRLDGDKLIHVQKWDG 100
sp|P05413|FABPH_HUMAN       TLKTHSTFKNTEISFKLGVEFDETTADDRKVKSIVTLDGGKLVHLQKWDG 100
sp|P11404|FABPH_MOUSE       TIKTQSTFKNTEINFQLGIEFDEVTADDRKVKSLVTLDGGKLIHVQKWNG 100
                            .::: .:*::*:*.* ** **:* : *:*: ** : *: . : ::*:* *

sp|P02691|MYP2_RABIT        KETTIKRKLVDGKMVVECKMKGVVCTRIYEKV- 132
sp|P02689|MYP2_HUMAN        KETTIKRKLVNGKMVAECKMKGVVCTRIYEKV- 132
sp|P24526|MYP2_MOUSE        KETAIRRTLLDGRMVVECIMKGVVCTRIYEKV- 132
sp|Q0Z7S8|FABP9_HUMAN       KETTIKRKIVDEKMVVECKMNNIVSTRIYEKV- 132
sp|P15090|FABP4_HUMAN       KSTTIKRKREDDKLVVECVMKGVTSTRVYERA- 132
sp|P04117|FABP4_MOUSE       KSTTIKRKRDGDKLVVECVMKGVTSTRVYERA- 132
sp|O15540|FABP7_HUMAN       KETNFVREIKDGKMVMTLTFGDVVAVRHYEKA- 132
sp|P51880|FABP7_MOUSE       KETNCTREIKDGKMVVTLTFGDIVAVRCYEKA- 132
sp|P05413|FABPH_HUMAN       QETTLVRELIDGKLILTLTHGTAVCTRTYEKEA 133
sp|P11404|FABPH_MOUSE       QETTLTRELVDGKLILTLTHGSVVSTRTYEKEA 133
                            :.*   *   . :::        ...* **:
```

Figure 17: Clustal 2.0.10. multiple sequence alignment.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	sp|O15540|FABP7_HUMAN	132	2	sp|P51880|FABP7_MOUSE	132	87
1	sp|O15540|FABP7_HUMAN	132	3	sp|P05413|FABPH_HUMAN	133	66
1	sp|O15540|FABP7_HUMAN	132	4	sp|P11404|FABPH_MOUSE	133	64
1	sp|O15540|FABP7_HUMAN	132	5	sp|P02691|MYP2_RABIT	132	59
1	sp|O15540|FABP7_HUMAN	132	6	sp|P02689|MYP2_HUMAN	132	59
1	sp|O15540|FABP7_HUMAN	132	7	sp|P24526|MYP2_MOUSE	132	58
1	sp|O15540|FABP7_HUMAN	132	8	sp|P15090|FABP4_HUMAN	132	56
1	sp|O15540|FABP7_HUMAN	132	9	sp|P04117|FABP4_MOUSE	132	56
1	sp|O15540|FABP7_HUMAN	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	53
2	sp|P51880|FABP7_MOUSE	132	3	sp|P05413|FABPH_HUMAN	133	62
2	sp|P51880|FABP7_MOUSE	132	4	sp|P11404|FABPH_MOUSE	133	65
2	sp|P51880|FABP7_MOUSE	132	5	sp|P02691|MYP2_RABIT	132	56
2	sp|P51880|FABP7_MOUSE	132	6	sp|P02689|MYP2_HUMAN	132	56
2	sp|P51880|FABP7_MOUSE	132	7	sp|P24526|MYP2_MOUSE	132	54
2	sp|P51880|FABP7_MOUSE	132	8	sp|P15090|FABP4_HUMAN	132	53
2	sp|P51880|FABP7_MOUSE	132	9	sp|P04117|FABP4_MOUSE	132	53
2	sp|P51880|FABP7_MOUSE	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	51
3	sp|P05413|FABPH_HUMAN	133	4	sp|P11404|FABPH_MOUSE	133	85
3	sp|P05413|FABPH_HUMAN	133	5	sp|P02691|MYP2_RABIT	132	62
3	sp|P05413|FABPH_HUMAN	133	6	sp|P02689|MYP2_HUMAN	132	62
3	sp|P05413|FABPH_HUMAN	133	7	sp|P24526|MYP2_MOUSE	132	60
3	sp|P05413|FABPH_HUMAN	133	8	sp|P15090|FABP4_HUMAN	132	64
3	sp|P05413|FABPH_HUMAN	133	9	sp|P04117|FABP4_MOUSE	132	64
3	sp|P05413|FABPH_HUMAN	133	10	sp|Q0Z7S8|FABP9_HUMAN	132	55
4	sp|P11404|FABPH_MOUSE	133	5	sp|P02691|MYP2_RABIT	132	60
4	sp|P11404|FABPH_MOUSE	133	6	sp|P02689|MYP2_HUMAN	132	60
4	sp|P11404|FABPH_MOUSE	133	7	sp|P24526|MYP2_MOUSE	132	58
4	sp|P11404|FABPH_MOUSE	133	8	sp|P15090|FABP4_HUMAN	132	67
4	sp|P11404|FABPH_MOUSE	133	9	sp|P04117|FABP4_MOUSE	132	63
4	sp|P11404|FABPH_MOUSE	133	10	sp|Q0Z7S8|FABP9_HUMAN	132	55
5	sp|P02691|MYP2_RABIT	132	6	sp|P02689|MYP2_HUMAN	132	94
5	sp|P02691|MYP2_RABIT	132	7	sp|P24526|MYP2_MOUSE	132	87
5	sp|P02691|MYP2_RABIT	132	8	sp|P15090|FABP4_HUMAN	132	70
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5	sp|P02691|MYP2_RABIT	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	69
6	sp|P02689|MYP2_HUMAN	132	7	sp|P24526|MYP2_MOUSE	132	87
6	sp|P02689|MYP2_HUMAN	132	8	sp|P15090|FABP4_HUMAN	132	66
6	sp|P02689|MYP2_HUMAN	132	9	sp|P04117|FABP4_MOUSE	132	68
6	sp|P02689|MYP2_HUMAN	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	67
7	sp|P24526|MYP2_MOUSE	132	8	sp|P15090|FABP4_HUMAN	132	64
7	sp|P24526|MYP2_MOUSE	132	9	sp|P04117|FABP4_MOUSE	132	65
7	sp|P24526|MYP2_MOUSE	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	65
8	sp|P15090|FABP4_HUMAN	132	9	sp|P04117|FABP4_MOUSE	132	91
8	sp|P15090|FABP4_HUMAN	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	63
9	sp|P04117|FABP4_MOUSE	132	10	sp|Q0Z7S8|FABP9_HUMAN	132	62

The human B-FABP sequence was studied looking for streches of 10-15 residues interesting for a low degree of similarity compared to those of other proteins, aligned by Clustal W. Ten high score proteins are members of the FABP family, so it was important to detect some antigenic regions, specific for human

brain-type isoform, in order to produce a strong immune response in the animal inoculated, obtaining specific antibodies.

Since three-dimensional structure of human B-FABP is known, a structural study was performed by using Pymol software (Pymol 0,99rc6) on PDB files (www.rcsb.org) of human B-FABP, comparing different structure models obtained by NMR (Nuclear Magnetic Resonance) or X-ray diffraction techniques.

X-ray crystallographic and NMR analyses showed that all the members of the family fold as a slightly elliptical β-barrel comprising 10 antiparallel β-strands, with two short α-helices located between the first and second β-strands.

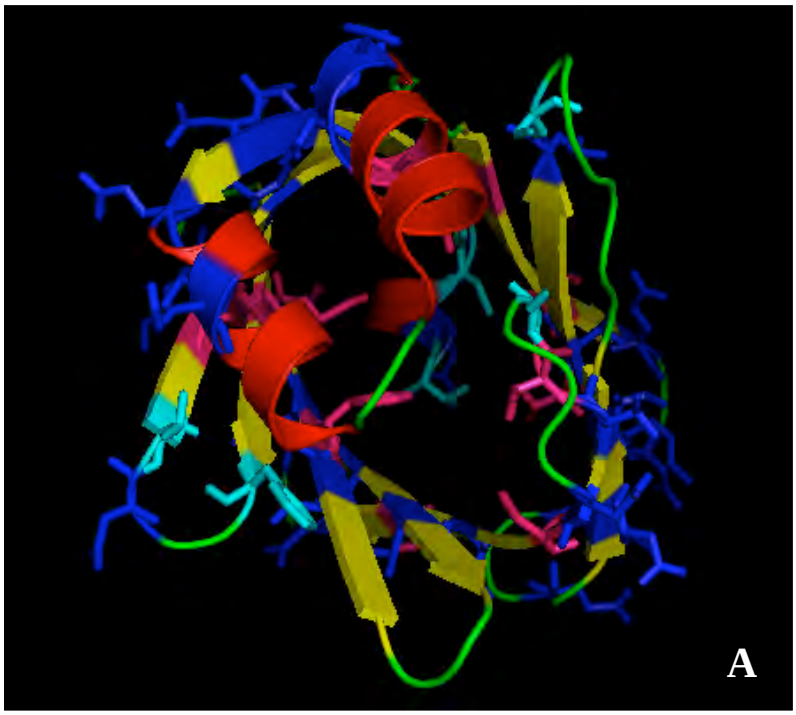
The β-strands are organized into two nearly orthogonal β-sheets that wrap around a solvent-accessible ligand-binding cavity. The cavity is centered at the end of the barrel near the helix-turn-helix motif, which is thought to act as a portal for ligand entry and exit The α-II helix is a key structural element of the putative FA portal and forms long-range interactions with the α-II turn between β strands C and D.

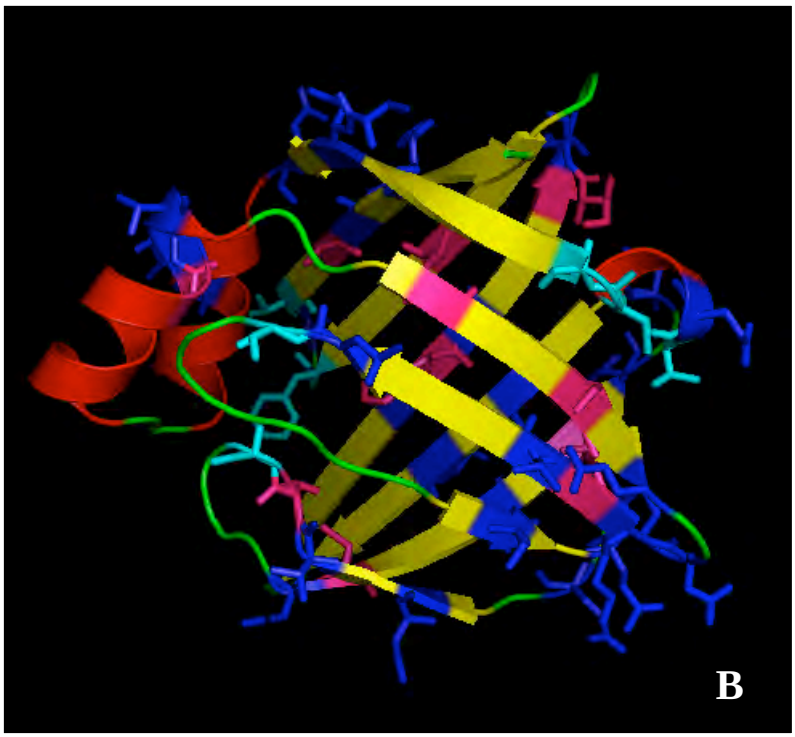
This structural study was performed to value which regions of brain-type FABP, characterized by low degree of similarity to respect to other FABPs, were solvent-exposed and which were buried. In fact, the epitope must be solvent-exposed to be accessible to the antibody. The general features of protein structure

that correspond to these criteria are turn or loop structures and areas of high hydrophilicity.

B-FABP structure models were visualized by Pymol software (1FDQ and 1JJX PDB codes), and the protein backbone was coloured by secondary structure: helix in red, loops in green and sheets in yellow.

All residues side chains of B-FABP, similar and different from those of other FABPs on the basis of the alignment performed by Clustal W, were displayed on three-dimensional model of B-FABP, valuating their degree of exposition to the solvent. These residues side chains were displayed and coloured: the solvent-exposed residues in blue, the partially solvent-exposed in light blue and the buried in pink.





Figures 18-19: B-FABP 3D strucure models were visualized (A: 1FDQ and B: 1JJX PDB codes; Pymol software). The typical FABP fold, ß-barrel, was shown. Residues were coloured by secondary strucure: helix (red), loop (green), sheet (yellow); residues side chains different from those of similar proteins were displayed and coloured: solvent-exposed residues (blue), partially solvent-exposed residues (light blue), buried residues (pink).

Based on sequence similarity and exposition degree to the solvent of B-FABP residues, the multiple sequence alignment of 7 higher score proteins could be so represented:

1. MVEAFCATWKLTNSQNFDEYMKALGVGFATRQVGNVTKPTVIIS
2. MVDAFCATWKLTDSQNFDEYMKALGVGFATRQVGNVTKPTVIIS
3. MVDAFLGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIE
4. MADAFVGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIE
5. MSNKFLGTWKLVSSENFDDYMKALGVGLATRKLGNLAKPNVIIS
6. MSNKFLGTWKLVSSENFDDYMKALGVGLATRKLGNLAKPTVIIS
7. MSNKFLGTWKLVSSEHFDDYMKALGVGLANRKLGNLAKPTVIIS

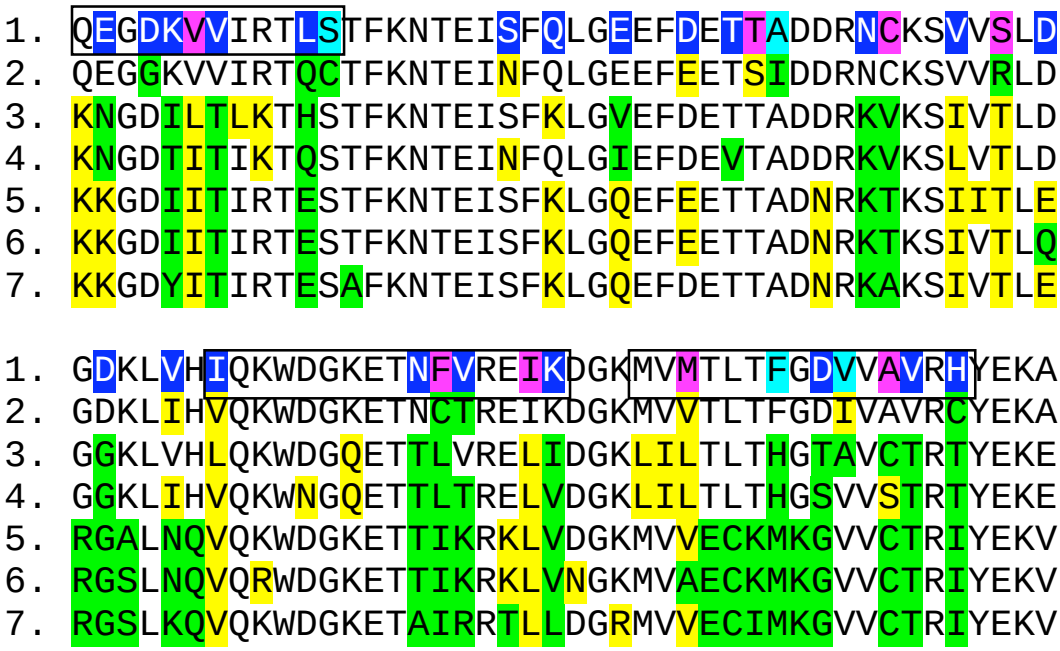


Figure 20: Multiple sequence alignment of 7 higher score proteins (see fig alignment). Based on sequence similarity and exposition degree to the solvent of B-FABP residues, the protein sequence could be so represented: residue similar to target (yellow), residue different from target (green), solvent-exposed residues (blue), partially solvent-exposed residues (light blue), buried residues (pink). In rectangles, 3 sequences chosen for peptide synthesis. Sequences: 1= sp O15540 (FABP7_HUMAN, target), 2= sp P51880 (FABP7_MOUSE), 3= sp P05413 (FABPH_HUMAN), 4= sp P11404 (FABPH_MOUSE), 5= sp P02691 (MYP2_RABIT), 6= sp P02689 (MYP2_HUMAN), 7= sp P24526 (MYP2_MOUSE). Rectangles identify the 3 sequences chosen for chemical synthesis.

Based on sequence similarity and exposition degree to the solvent of B-FABP residues, three sequences were identified:

- Sequence 1: NH_2-QEGDKVVIRTLS-COOH (residues 45-56);
- Sequence 2: NH_2-IQKWDGKETNFVREIK-COOH (residues 95-110);
- Sequence 3: NH_2-MVMTLTFGDVVAVRH-COOH (residues 114-128).

3.4 Polyclonal Antibody Production

After an evaluation for antigenicity by the Pacific Immunology company, two were considered highly antigenic sequences (45-56 and 95-110), while the remaining one (114-128) was discarded on the basis of the algorithms used (not known). The 2 peptides were synthesized chemically, analyzed by Mass Spectrometry and HPLC.

After the synthesis, the 2 peptides were conjugated to KLH and inoculated in New Zealand rabbits. The peptides were soluble in inoculation solution and did not show problems linked to low solubility. After 13 weeks, 4 sera were obtained (serum codes: 2879, 2980, 2981 and 2982).

Serum from the third bleed was subjected to affinity purification, where peptides were coupled to a column in order to capture only those antibodies that recognized the peptide sequence, obtaining two polyclonal antibodies: pAb 2979/2980 and pAb 2981/2982. pAb 2979/2980 and 2981/2982 were utilized in western blot and ELISA analyses in order to evaluate their affinity for rec B-FABP, rec H-FABP and rec TTR.

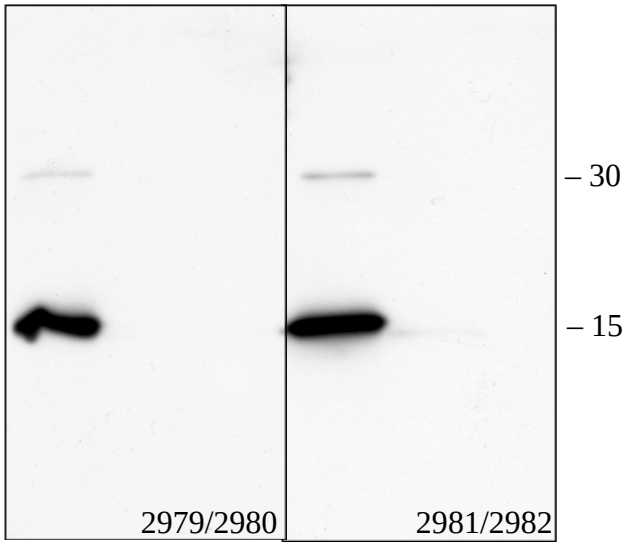


Figure 21: Immunoblot analyses of rec B-FABP, rec H-FABP and rec TTR. The membranes were probed by 2979/2980 (1:500) and 2981/2892 (1:500). Approximate molecular weights are in kilodaltons.

Both antibodies recognized the whole protein, B-FABP.
pAb 2979/2980 gave raise to a strong signal in the lane corresponding to rec B-FABP with absence of any signal in correspondence of rec H-FABP and rec TTR. This antibody was specific and selective for B-FABP.
pAb 2981/2982, as pAb 2979/2980, recognized with high affinity rec brain-type FABP but showed a weak cross-reaction with rec H-FABP.
Both antibodies did not recognize transthyretin.
In Enzyme-Linked ImmunoSorbent Assay (ELISA), for determining the affinity for the recombinant proteins of pAb 2979/2980 and 2981/2982, rec B-FABP, rec H-FABP and rec TTR were coated on a ELISA microplate and were incubated with the 2 antibodies.

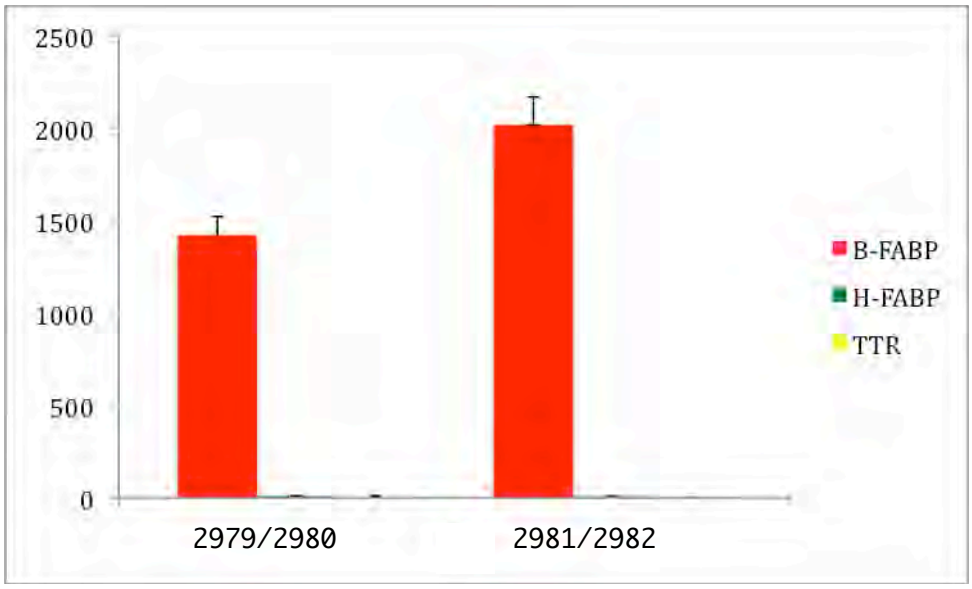


Figure 22: ELISA measuring affinity of polyclonal 2979/2980 and 2981/2982 antibodies for rec B-FABP, rec H-FABP and rec TTR. Values are expressed in mA at 450 nm. The measurement was done with 6 replicates of each sample.

The two polyclonal antibodies recognized specifically and selectively rec B-FABP, not displaying cross-reaction with the other 2 proteins.

4.DISCUSSION AND CONCLUSIONS

The work done during these years has been articulated in 2 different parts, sharing a common objective: the generation of a diagnostic reagent specific for human B-FABP.

The strategy was based on immunizing animals with rec human B-FABP or with two synthetic peptides conjugated to KLH carrier.

As described before [1.3], brain-type FABP is expressed in human brain whereas heart-type FABP is expressed at high levels in cardiac myocytes and in nervous tissue.

H-FABP sequence displays 70% degree of identity with B-FABP sequence and, in addition, the two proteins display a common three dimensional structure, which is a limiting factor for generating antibodies able to distinguish B-FABP from H-FABP, giving raise to cross-reaction problems.

As H-FABP is considered a good marker of brain and heart injury, as B-FABP could represent a more sensitive and specific marker of nervous tissue damage, since its expression is confined to brain cells. In order to value the effectiveness of this hypothesis, an antibody specific for B-FABP was generated, to determine the levels of this protein in biological fluids from patients affected with neurological disorders.

In the first part of project, 2 antisera and 8 different mouse hybridoma cells were obtained by immunizing New Zealand rabbits and BalbC mice with

recombinant human B-FABP.

Rec B-FABP was expressed efficiently in *E. coli* cells and purified by two subsequent column chromatographies, with a high yield; the protein was inoculated in animals but the standard protocols for obtaining a good immune response were prolonged, especially for mice. In fact, the high degree of sequence identity between murine and human B-FABP indicated that most of the protein sequences was not immunogenic because of a self protein. Also, low molecular weight of the protein could have affected antibody generation, being immunization easier with high molecular weight proteins. The analysis of antibody fractions obtained from rabbit serum and culture supernatant of hybridoma cells proved that they did not display the characteristics needed for being considered a specific antibody for B-FABP. Specifically, the two antisera were able to recognize rec B-FABP but displayed a cross-reaction for rec H-FABP, by ELISA and western blot analyses; since they were not selective for brain-type isoform, a possible role as revelation antibody in an ELISA sandwich experiment was thought but only after the isolation of a selective and specific capture antibody for B-FABP.

The 8 culture supernatants of hybridoma cells displayed different affinity for the two FABP forms and this could be due to different reasons: a more detailed description about 2D7, 4F6 and other 6 culture

supernatant behaviour is so needed.

2D7 supernatant displayed selectivity and specificity for brain-type FABP, by ELISA and immunoblot techniques on recombinant protein samples, but when it was tested for detection of B-FABP in cerebrospinal fluid samples, an absence of signal was obtained.

This result could probably be due to multiple factors: 2D7 supernatant antibody-titer was too low for this kind of detection, mainly if, as hypothized, B-FABP concentrations in CSF were in range of pg/ml. Also, the impossibility to purify the corresponding 2D7-producing mAb could have affected the results of test performed, especially in case of ELISA sandwich tests.

4F6 culture supernatant behaviour was different, displaying a high antibody-titer but a strong cross-reactivity for H-FABP and transthyretin proteins, probably due to the recognition of both linear and conformational epitopes on other proteins than target.

1E1, 1C6, 2F9, 3A5, 3G9 and 4F9 supernatants displayed variable degree of cross-reaction phenomena for H-FABP and TTR and no one was specific for brain-type isoform. They probably recognized a conformational epitope, as demonstrated by absence of signal in immunoblot analysis, when samples were denaturated by SDS and lost their three-dimensional structure.

Thus, the antibodies generated in the first part of this work were able to recognize B-FABP but no one

could be utilized in an immunoblot or ELISA analysis if not correlated to a selective antibody for B-FABP.

The results obtained in this part allow to focus the attention on specific problems deriving from use of B-FABP rec proteins as immunogen and to plan how to solve problems.

In order to circumvent cross-reactions problems, affecting the use of antibodies obtained from animals immunized with rec protein, it was decided to generate antibodies against two synthetic peptides, whose sequence was chosen after a structural and sequence study on human B-FABP, for the individuation of the more antigenic stretches of residues that presented specific characteristics: low degree of identity with the homologous proteins sequence, based on alignment, and high exposition to the solvent, based on three-dimensional proteins structure. The polyclonal antibodies obtained utilizing the peptides as immunogen, pAb 2979/2980 and pAb 2981/2982, were specific for B-FABP and did not cross-reacted with H-FABP and transthyretin.

These results, obtained only after a sequence and structure study on B-FABP, confirmed the importance of computational analysis that allowed focusing on specific regions that were predicted to be more antigenic.

The applications of the two polyclonal antibodies, specific for human B-FABP, are multiple: they could be

probed on biological fluid samples, as cerebrospinal fluid, urine or serum by immunoblot analysis for detection of B-FABP in patients affected with neurological disorders in order to value the presence/absence of B-FABP. Since the detection limit of western blot analysis is in range of ng/protein, the development of a more sensitive test, ELISA sandwich, could help to improve the detection of low levels of antigen in analyzed samples.

The importance of different studies on B-FABP levels in biological fluids in different neurological pathologies could demonstrate whether this protein might be considered as a good marker for brain damage, allowing an early detection of acute brain injury with the final target of an early treatment.

H-FABP is considered one of the most sensible and efficient markers of heart and brain damage, but to date the cross-reactivity limits its specific use.

B-FABP could overcome this limitation, representing a specific marker of axonal damage, being expressed prevalently in the brain.

The production of a distinct reagent for B-FABP is certainly the final goal of this study, since it acts as a marker for neuronal and axonal damage.

With this perspective it would be possible to study different neurological disorders, with ischemic, infective and degenerative etiology to value the efficiency of B-FABP as marker of cell damage.

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PAPERS AND MEETING COMMUNICATIONS

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SUPPLEMENT

Increased levels of CSF heart-type fatty acid-binding protein and tau protein after aneurysmal subarachnoid hemorrhage

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Summary
Background Heart-type Fatty Acid-Binding Protein (H-FABP) and tau protein (τ) have been shown to be novel biomarkers associated with brain injury and, therefore, they could represent a useful diagnostic tool in patients with subarachnoid hemorrhage (SAH). The goal of this study was to measure H-FABP and τ in cerebrospinal fluid (CSF) following SAH to test the hypothesis that a relationship exists between SAH severity and H-FABP/τ values.
Methods Twenty-seven consecutive SAH patients admitted to our ICU were studied. Serial CSF samples were obtained in every patient starting on the day of SAH and daily for up to 2 weeks post-SAH. H-FABP/τ levels were measured by enzyme-linked immunosorbent assay.
Results Patients with severe SAH showed significantly higher peak levels of H-FABP and τ compared to mild-SAH patients (FABP: $p=0.02$; τ: $p=0.002$). In addition the peak concentrations of H-FABP and τ in CSF from SAH patients correlated significantly with Glasgow Coma Scale motor score (H-FABP: Spearman $r=-0.52$, $p=0.006$; τ: Spearman $r=-0.63$, $p=0.0004$). Based on outcome at discharge from the hospital, patients were categorized into

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survivors and non-survivors. Peak concentrations of both proteins in the non-survivors group were significantly higher than in the survivors.
Conclusions H-FABP and τ CSF levels are proportional to SAH severity and may be novel biomarkers that can be used to predict the severity of outcome following clinical SAH.

Keywords Aneurysmal subarachnoid hemorrhage · Biomarkers · Heart-type fatty acid-binding protein · Tau protein

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a severe condition due to the rupture of an intracranial aneurysm in the subarachnoid space. SAH is an important cause of premature death and disability worldwide, affecting approximately 10/100,000 hospitalized patients every year [1, 2]. Studies on mortality and morbidity of SAH in Italy report a mortality rate of 25% within 6 months from the first bleeding and severe neurological disability in 15% of the survivors [2].

The severity of initial brain damage is one of the most important factors associated with the outcome following SAH [3]. Global brain ischemia associated with acute increases in intracranial pressure (ICP) and/or focal brain ischemia due to tissue compression are two major mechanisms of initial brain damage following SAH [3]. The identification of biomarkers, known to be released during brain ischemia following SAH could therefore represent an aid for the clinician.

Recently, Heart-type Fatty Acid-Binding Protein (H-FABP), a small cytoplasmic lipid-binding protein, with a molecular weight of 15 kDa, was found to be increased in patients with mild traumatic brain injury [4] and those with ischemic and hemorrhagic stroke [5]. Following acute ischemic stroke, an acute increase in plasma H-FABP was significantly associated with the severity of the neurological deficit and the functional outcome [6]. H-FABP in the brain is predominately localized in the neuronal cell body, and following cellular damage, is rapidly released into the blood. CSF levels of H-FABP following SAH are unknown and we wondered whether this protein might therefore have the potential to be a rapid diagnostic marker of injury severity following human SAH.

Tau protein (τ), a phosphorylated microtubule-associated protein, has been recognized as a nonspecific marker of axonal injury [7]. Following brain damage τ is released into the extracellular space and may be increased in the cerebrospinal fluid (CSF). In a study conducted on 17 SAH patients, it was reported that the increased CSF τ observed following SAH was correlated with both injury severity and clinical outcome [8]. In the present study we assessed CSF levels of both H-FABP and τ in SAH patients to determine whether a direct relationship exists between expression of these proteins and SAH severity.

Materials and methods

Patient population The study was approved by the Local Research Ethics Committee of the Ospedale Maggiore Policlinico, Milano. Written informed consent was obtained from the patient or, in the comatose patients, from the next of kin. Twenty seven consecutive patients admitted to our neuro-critical Intensive Care Unit (ICU) with a diagnosis of SAH were enrolled.

Clinical Management The severity of SAH, on admission and before surgery/endovascular treatment, was recorded according to the World Federation of Neurological Surgeons (WFNS) grading scale [9].

Admission CT scan was classified using the Fisher scale [10]. Management goals included the early clipping/coiling of the aneurysm (in our Center, 77% of ruptured intracranial aneurysms are secured within the first 24 hours) and, where indicated, surgical evacuation of the intracranial hematoma. Hydrocephalus was drained using an intraventricular catheter and intracranial pressure (ICP) was monitored in all patients with the goal to maintain ICP levels below 20 mmHg, and cerebral perfusion pressure (CPP) above 70 mmHg. In addition a jugular bulb catheter was inserted in all comatose patients to allow determination of jugular saturation of oxygen (SjO_2) and calculation of $AjDO_2$ differences. Outcome criteria included survival recorded at discharge from the hospital.

CSF sampling CSF samples (5 ml) were collected beginning on day 1 and thereafter twice daily (8 A.M./ 8 P.M.). CSF samples were treated with an anticoagulant, centrifuged and then frozen and kept at - 80 degrees Celsius. Samples were analyzed using specific ELISA kits for H-FABP and τ as described [11].

Groups From the analysis of serial CSF samples, the maximum (peak) values were determined in each patient for both proteins. To explore the relationship between CSF H-FABP/τ and injury severity, patients were grouped and dichotomized according to the WFNS score of mild SAH (WFNS 1–3) and severe SAH (WFNS 4–5). However, since GCS can vary from 12 to 3 amongst patients with WFNS 4 and 5, we investigated the relationship between the motor component of GCS (mGCS) and CSF levels of H-FABP and τ. To explore the relationship between CSF

H-FABP/τ and outcome, patients were also categorized into survivors and non-survivors assessed at hospital discharge.

Statistical analysis ICP data are presented as mean ± standard error of the mean. H-FABP and τ (whose distribution was not normal) are presented as median values (+ range). Mann Whitney test was used to assess the relationship between peak H-FABP/τ values and WFNS, and outcome at discharge. Correlation and linear Spearman regression analyses were used to determine the relationship between CSF biomarkers and injury severity (assessed with the mGCS). A "p" value of < 0.05 was accepted as significant.

Results

Patients The clinical features of the patients are presented in Table 1. Mean age was 56 (range 30–78) and 23 patients (85%) were female. Seventeen patients (63%) were in poor clinical conditions on admission (WFNS 4–5). Anterior circulation aneurysms were present in 18 (69%) patients. On admission CT, 2 patients were Fisher scale grade 3 (localized clots and/or vertical layers of blood 1 mm or greater in thickness), and 25 were grade 4 (diffuse or no subarachnoid blood with intracerebral or intraventricular clots). Aneurysms were treated with an endovascular approach in 20 patients (74%) and with surgical clipping in 4 patients. In 3 patients with irreversible brain damage (GCS 3 and absence of upper brainstem reflexes after hydrocephalus drainage), aneurysm closure was not performed. Intracranial pressure was monitored in 23 patients (85%).

CSF H-FABP/τ concentrations following SAH All but 2 patients had at least one pathologically elevated concentration of H-FABP CSF values (median 6607 pg/ml; range 603–100000 pg/ml) compared to published reference

t1.1 **Table 1** Characteristics of patients with subarachnoid haemorrhage

t1.2 Patient Code n°	Age	Sex	WFNS*	Fisher	Aneurysm location	Aneurism treatment	Timing (hours)	mean ICP mmHg	max ICP mmHg
t1.3 **1**	56	F	5	4	MCA	Coil	<24	34	66
t1.4 **2**	51	F	5	4	PCA	Coil	<24	77	110
t1.5 **3**	48	F	5	4	ICA	NT	-	68	91
t1.6 **4**	65	M	5	4	VA	NT	-	44	117
t1.7 **5**	39	F	5	4	ACoA	Coil	24–72	56	63
t1.8 **6**	68	F	5	4	Unknown*	NT	-	50	51
t1.9 **7**	73	F	5	4	ACoA	Coil	24–72	10	80
t1.10 **8**	68	F	4	4	ACoA	Coil	<24	12	39
t1.11 **9**	58	F	4	4	MCA	Clip	<24	14	24
t1.12 **10**	78	F	4	4	Peric.	Clip	<24	9	20
t1.13 **11**	38	F	1	3	PCA	Coil	<24	26	36
t1.14 **12**	56	F	5	4	PICA	Coil	<24	16	32
t1.15 **13**	47	F	1	4	ACoA	Coil	<24	-	-
t1.16 **14**	54	F	4	4	Basilar A	Coil	<24	13	28
t1.17 **15**	62	F	5	4	MCA	Clip	<24	12	18
t1.18 **16**	73	F	1	4	ACoA	Coil	<24	-	-
t1.19 **17**	37	F	1	3	PCoA	Coil	<24	-	-
t1.20 **18**	63	F	2	4	Basilar A	Coil	24–72	9	18
t1.21 **19**	63	F	1	4	SCA	Coil	<24	-	-
t1.22 **20**	30	F	1	4	ICA	Coil	24–72	18	26
t1.23 **21**	41	M	2	4	PICA	Coil	<24	11	25
t1.24 **22**	67	F	4	4	MCA	Coil	<24	11	22
t1.25 **23**	45	F	4	4	ACA	Coil	>72	14	16
t1.26 **24**	68	F	1	4	PCA	Coil	<24	10	25
t1.27 **25**	63	M	5	4	ACoA	Clip	<24	22	76
t1.28 **26**	49	F	3	4	ACA	Coil	<24	12	25
t1.29 **27**	42	M	5	4	ACA	Coil	<24	14	30

t1.30 **Legend:** WFNS, World Federation of Neurological Surgeons grading scale. MCA = Middle cerebral artery, AcoA = Anterior communicating artery, PcoA = Posterior communicating artery, PCA = Posterior cerebral artery, ACA = Anterior cerebral artery, PICA = Posterior inferior cerebellar artery, SCA = Superior cerebellar artery, Peric. = Pericallosal artery, VA = Vertebral artery, ICA = Internal carotid artery. * No aneurysm found or patient died before ancillary investigations could be performed. Clip = surgical treatment, Coil = endovascular treatment, NT = no treatment, Timing = timing of treatment from SAH, ICP = intracranial pressure.

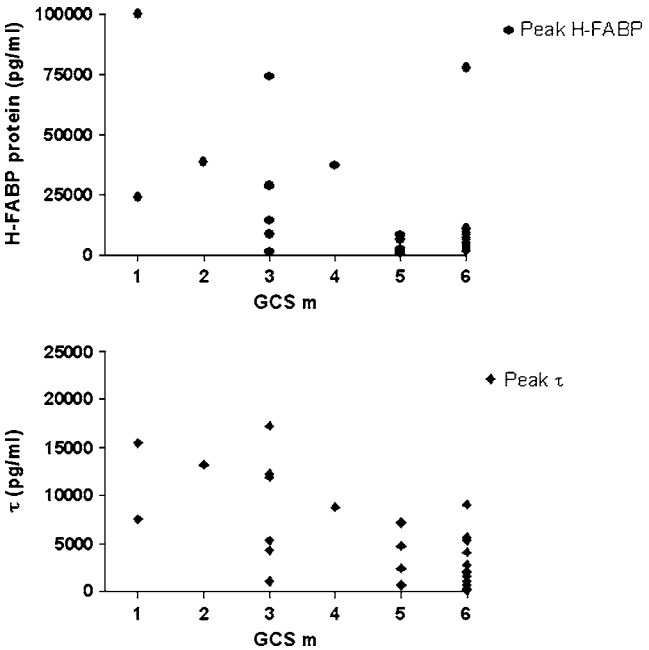

Fig. 1 Relationship between CSF mGCS and peak H-FABP (upper panel) and τ (lower panel) levels. H-FABP: Spearman r=−0.52, p=0.006; τ Spearman r=−0.63, p=0.0004

population (CSF H-FABP of 1729 pg/ml: [12]). Similarly, all but 3 patients had at least one pathologically elevated concentration of CSF τ (median 4322 pg/ml; range 146–17222 pg/ml) compared to published reference population (CSF τ normal values up to 672 pg/ml: [7]).

Relationship between H-FABP/τ values and degree of brain damage following SAH Patients with severe SAH (WFNS 4–5) showed significantly higher peak H-FABP (median 14388 pg/ml; range 603–100000 pg/ml) and τ (median 7194 pg/ml; range 710–17222 pg/ml) levels compared to mild-SAH (H-FABP median 3622 pg/ml; range 1800–8873 pg/ml; Mann Whitney test p=0.02. τ median 1572 pg/ml; range 146–5627 pg/ml; Mann Whitney test p=0.002). Additionally, we investigated the relationship between mGCS and CSF biomarkers. The peak concentrations of H-FABP and τ in SAH CSF correlated significantly with mGCS (H-FABP: Spearman r=−0.52, p=0.006; τ: Spearman r=−0.63, p=0.0004) (Fig. 1).

CSF H-FABP and τ concentration in Survivors versus Non-Survivors Based on outcome at hospital discharge, patients were categorized into survivors and non-survivors (Table 2). The peak concentration of H-FABP (28793 pg/ml with range of 1800–100000 pg/ml) in the non-survivors group was significantly higher than that recorded from survivors: 3925 (603–37127) pg/ml (Mann Whitney: p=0.005). Similarly the peak concentration of τ was 9066 (719–17222) pg/ml in the non-survivors group, it was significantly higher than the peak concentration in survivors: 2260 (146–12296) pg/ml (Mann Whitney: p=0.008).

Influence of Surgery In case of surgical clipping of the aneurysm (4 patients) CSF sampling occurred after surgery. To address the influence of surgery on CSF H-FABP and τ levels, we repeated all analyses after exclusion of these 4 patients. The relationship between peak concentrations of H-FABP and τ with WFNS, mGCS and outcome at discharge remained significant, suggesting that CSF protein increases are associated with SAH and not with surgical manipulation.

t2.1 **Table 2** Survivors versus non-survivors

t2.2	Characteristic	All	Survivors	Non-survivors	Significance
t2.3	H-FABP (pg/ml)	6607 (603–100000)	3925 (603–37127)	28793 (1800–100000)	0.005
t2.4	tau (pg/ml)	4322 (146–17222)	2260 (146–12296)	9066 (719–17222)	0.008
t2.5	Age, years	56 (30–78)	56 (30–73)	63 (39–78)	ns
t2.6	Sex (F:M)	23: 4	16: 2	7: 2	ns
t2.7	WFNS	4 (1–5)	3.5 (1–5)	5 (1–5)	0.038
t2.8	mGCS	5.5 (1–6)	6 (3–6)	3 (1–6)	ns (p=0.06)
t2.9	Fisher	4 (3–4)	4 (3–4)	4 (4–4)	ns
t2.10	Number	27	18	9	
t2.11	H-FABP (pg/ml)	6607 (603–100000)	3925 (603–37127)	28793 (1800–100000)	0.005
t2.12	tau (pg/ml)	4322 (146–17222)	2260 (146–12296)	9066 (719–17222)	0.008
t2.13	Age, years	56 (30–78)	56 (30–73)	63 (39–78)	ns
t2.14	Sex (F:M)	23: 4	16: 2	7: 2	ns
t2.15	WFNS	4 (1–5)	3.5 (1–5)	5 (1–5)	0.038
t2.16	mGCS	5.5 (1–6)	6 (3–6)	3 (1–6)	ns (p=0.06)
t2.17	Fisher	4 (3–4)	4 (3–4)	4 (4–4)	ns
t2.18	Number	27	18	9	

t2.19 **Legend:** The median (+ range) is shown. Ns = not significant; WFNS = World Federation of Neurological Surgeons; mGCS = Glasgow Coma Scale motor score.

Discussion

In this study we observed that, following SAH, (i) a significant increase of CSF concentrations of H-FABP and τ occurs and (ii) a direct relationship exists between the degree of brain damage, evaluated with WFNS scale, mGCS and outcome at discharge, and CSF levels of H-FABP and τ. Several studies have reported that alterations in CSF H-FABP [5, 12, 13] and τ [7, 8, 14, 15] levels are associated with cellular brain damage in patients with acute neurological disorders and degenerative disease. SAH is characterized by a loss of structural integrity of glial and neuronal cells and release of cell-specific proteins into the CSF. The majority of patients in our study showed increased levels of H-FABP (92%) and τ (89%) compared to normal references. Perhaps more importantly, the degree of H-FABP and τ increase reflected the clinical condition of the patient. In addition, we observed higher levels of these proteins in non-survivors when compared to survivors. Among non-survivors, the cause of death was acute brain injury in 5 cases (median mGCS=3; range 1–3), and vasospasm associated delayed cerebral ischemia (DCI) in the other 4 patients (median mGCS=6; range 3–6). Even though the relationship between DCI and CSF H-FABP/τ levels was not the aim of our study, high levels of these proteins in this sub-group of patients suggest that the relationship of H-FABP/τ with intracranial secondary insults should be investigated.

The latency between time of SAH and H-FABP and τ elevation is an important variable that was not determined in the present study, future directions for our work will be to address the temporal relationship of FABP/τ changes and the occurrence of secondary ischemia. Furthermore, we limited our analysis to the CSF, particularly since τ is a neuronal protein that cannot be detected in plasma. Conversely, H-FABP is not brain-specific and can increase in plasma following extracranial injuries [16, 17]. It would therefore be important to investigate the relationship between CSF and plasma H-FABP levels following SAH to discriminate between H-FABP increases due to brain damage and plasma CSF contamination due to the initial bleeding. Moreover in this pilot study we only investigated the relationship between H-FABP/τ changes and the outcome at time of discharge. Additional work is needed to establish whether high H-FABP/τ values are related or not to functional outcome 6 month after SAH, and to address which role, if any, these proteins might play as predictive factors for late morbidity and mortality following SAH. Despite these limitations, our findings provide new evidence that H-FABP and τ in the CSF increase substantially in patients following SAH and that a direct relationship between injury severity and H-FABP and τ levels exist

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MOLECULAR CHARACTERIZATION OF LOW MOLECULAR MASS C-TERMINAL FRAGMENTS IN DIFFERENT CREUTZFELDT-JAKOB DISEASE SUBTYPES

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Content:

Background
In sporadic Creutzfeldt-Jakob disease (sCJD) the clinical variability has not been fully explained by molecular studies relating two major types of PrP27-30 with unglycosylated peptides of 21 (type 1) and 19 kDa (type 2) and the amino acid methionine or valine at position 129. In a previous work, by using two-dimensional immunoblot we identified distinct N-terminal truncated forms of prion protein in different sCJD subtypes.

Objectives
In the present study, we searched on low molecular mass PrP^{Sc} fragments (below 10kDa) which might correlate with the phenotypic variability observed in different sCJD molecular subtypes.

Methods
Brain homogenates of sCJD subjects were separated by mono- and two-dimentional electrophoresis and immunoblotted by using anti-PrP antibodies directed to N- and C-terminus epitopes.

Results and discussion
We biochemically characterized by mono- and two-dimensional analyses novel C-terminal PK-resistant fragments migrating at ~5.5 kDa and with an isoelectric point around 4. These fragments were found in almost all different sCJD subtypes with minor variabilities among subjects. These data show the presence of multiple PrP^{Sc} conformations in sCJD and, in addition, shed new light on the correlation between sCJD phenotypes and disease-associated PrP molecules.

Poster, Prion 2008, Madrid, Spain, October 8-10, 2008.

Dottoressa LAURA CRACCO

Nel corso dei tre anni del Dottorato di Biotecnologie Molecolari, Industriali ed Ambientali, la Dr.ssa Laura Cracco ha svolto attività di ricerca presso la Sezione di Neurologia Clinica del Dipartimento di Scienze Neurologiche e della Visione dell'Università di Verona.

Ha partecipato a tutte le attività formative e di aggiornamento scientifico tenute in tale sede prendendone attivamente parte, ed ha focalizzato i propri interessi nel campo delle malattie neurodegenerative. In particolare, la Dr.ssa Laura Cracco ha svolto attività di ricerca orientata alla generazione di anticorpi specifici e selettivi verso la proteina B-FABP umana, per rendere possibile lo studio dei livelli di tale proteina nei fluidi biologici, ipotizzando un possibile ruolo di questa proteina come marker di danno neuronale acuto.

Durante i tre anni di dottorato la Dr.ssa Laura Cracco ha lavorato attivamente alle diverse fasi operative del progetto, applicando sia tecniche di biologia molecolare, sia biochimiche che strutturali. Ha espresso e purificato la proteina di interesse in modo ricombinante, in un sistema procariotico; ha quindi valutato la sensibilità e la specificità dei reagenti prodotti da animali immunizzati mediante diverse tecniche di analisi proteomica quali ad esempio l'analisi in elettroforesi mono e bi-dimensionale, sia su campioni di proteina ricombinante che su fluidi biologici, al fine di fornire ulteriori dettagli circa le differenti affinità degli anticorpi in esame verso l'antigene di interesse. La dottoressa Cracco ha inoltre effettuato studi di sequenza e strutturali sulla B-FABP umana e sulle proteine ad elevata identità di sequenza con la stessa, per l'identificazione delle regioni potenzialmente piu' immunogeniche. Dopo la generazione di anticorpi policlonali in coniglio, la Dott.ssa Cracco ha svolto numerose analisi western blot e ELISA volte allo studio delle proprietà dei reagenti stessi, per l'identificazione della proteina B-FABP in campioni di fluido cerebrospinale, in pazienti affetti da malattia di Creutzfeldt-Jakob (sCJD), sclerosi multipla, post-polio e altre patologie neurologiche.

In questi anni si è inoltre occupata dell'esame diagnostico per la rivelazione delle proteine 14-3-3 nel fluido cerebrospinale e si è dedicata con grande entusiasmo, assieme al suo gruppo di lavoro, all' attività di ricerca orientata alla caratterizzazione e alla tipizzazione della Proteina Prionica Patologica (PrP^{Sc}) utilizzando un approccio biochimico.

Durante questi anni di lavoro la Dr.ssa Cracco ha prodotto contributi scientifici che sono stati presentati dalla candidata in congressi nazionali ed internazionali.

La Dr.ssa Cracco Laura ha sempre dimostrato serietà, entusiasmo ed intelligenza nello svolgimento di tutte le attività di ricerca. E' diventata un ricercatore di buone qualità tecniche e del tutto autonomo, in grado di gestire problematiche complesse di progetti di ricerca. Per le sopracitate capacità la Dr.ssa Cracco Laura può legittimamente aspirare al titolo di Dottore di Ricerca.

Prof. Salvatore Monaco