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Early Predictive Biomarkers for Hypertension Using Human Fetal Astrocytes

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ABSTRACT

Background: Hypertension (HTN) is a major risk factor for Cerebrovascular and cardiovascular diseases, causing premature deaths. Hence, there is an urgent need for predictive biomarkers to detect HTN before its onset.

Methods

- **Cell Culture Technique:** Growth of HFAs, followed by conversion of normal (A2) HFAs intoreactive (A1) type, by using ATP solution.
- **Confocal Microscopy:** Quantification of Glial Fibrillary Acidic Protein (GFAP) in A1 and A2 HFAs, by using antibody ab 7260 with AF 488.

Proteomics Methos

- Extraction of proteins from A1 and A2 HFAs, using Single-pot, Solid-phase-enhanced Sample Preparation (SP3) protocol.
- Liquid Chromatography- Mass Spectrometry techniques for obtaining the lev-els of calcium-gated proteins in A1 and A2 cell-solutions.

Results

- Micrographs obtained by Leica Microsystem indicated thicker filaments in A1 HFAs compared to A2 HFAs.
- Confocal results showed higher levels of GFAP in A1 HFAs (P> 0.05).
- Proteomics data analysed by PEAKS software revealed that proteins, such as GFAP were higher in reactive HFAs.

Conclusions: The results showed higher levels of GFAP in A1 HFAs. Therefore, early detection of GFAP in human body fluids could be a reliable predictive biomarker for HTN.

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Introduction

Scientific studies have established that hypertension needs to be reduced in order to control cardiovascular and cerebrovascular diseases [1]. At present, many laboratories around the world are exploring a plethora of biomarkers, as early indicators of hypertension [2,3]. Several kinds of biomarkers have been identified for the detection of hypertension. Some of the well-known biomarkers of cardiovascular diseases, including hypertension are triglycerides, C-reactive protein, fibrinogen, serum albumin, uric acid, homocysteine and intracellular adhe-sion molecule-1(ICAM-1). Currently, all the identified biomarkers for HTN are simply classi-fied according to their damaging effects (Table 1) [4].

Table 1: Predictive Values of Novel Biomarkers for Hypertension [4]

Biomarker	Adjusted Relative Risk (95% Confidence Interval)
Triglycerides	0.99 (0.94-1.05)
C-reactive protein Fibrinogen Interleukin 6	1.39 (1.32-1.47)
Fibrinogen	1.45 (1.34-1.57)
Interleukin 6	1.27 (1.19-1.35)
BNP or NT-proBNP	1.42 (1.24-1.63)
Serum albumin	1.2 (1.1-1.3)
ICAM-1	1.11 (0.75-1.64)
Homocysteine	1.05 (1.03-1.07)
Uric acid	1.09 (1.03-1.16)

Abbreviations: BNP, brain-type natriuretic peptide; NT-proBNP, N-ter- minal prohormone of BNP; ICAM-1, intercellular adhesion molecule 1.

Either these biomarkers are produced because of HTN or due to the diseases associated with HTN. Therefore, this study focused on the interaction between two endogenous chemicals, which are, orexin (a neuropeptide) and a type of glutamate N-Methyl-D-Aspartate (NMDA), and the resultant molecules produced from the increased influx of calcium ions through NMDA receptors (NMDARs). These molecules such as calpain, Jacob, cathepsin, calpastatin, and mitogen activated protein kinases (MAPKs), may act as predictive biomarkers for HTN, i.e., indicating a person's risk of developing this disease in the future. Additionally, enzymes such as Src-kinases potentiate the interaction between post- and presynaptic NMDARs, thus affecting blood pressure [5]. Another study reported that blood pressure is mainly dysregulated due to the expression of different subunits in pre- and post-synaptic NMDA receptors [6].

Astrocytes are positioned between the brain's blood vessels and neurons and are considered to be extremely sensitive to the depletion of arterial blood pressure. A recent study suggested that astrocytes monitor cerebral perfusion and control systemic circulation to maintain brain blood flow [7]. When the blood supply to the brain is reduced, astrocytes release a chemical signal to nearby neurons that raises blood pressure, restoring blood flow and oxygen supply to the brain [8]. Thus, astrocytes perform a balancing role between brain perfusion and neuronal activities, leading to neurotransmitter-evoked activation of astrocytic receptors to mobilize internal calcium [9]. Elevation in astrocytic calcium, in-turn, triggers the release of chemical transmitters, including glutamate, ?-aminobutyric acid (GABA), ATP, histamine, and acetyl-choline from astrocytes, causing sustained modulatory actions on neighbouring neurons [10]. Thus, there is a calcium-dependant bidirectional signalling pathway between astrocytes and neurons [11], which opens up the possibility of astrocytic involvement in the modulation of calcium-induced molecules such as calpain, Jacob, calpastatin, cathepsin and MAPKs, which could be considered as potential predictive biomarkers for HTN.

Calpain is a calcium-dependant cytosolic proteolytic enzyme with different isoforms [12], such as μ-calpain and m-calpain, which are activated by the synaptic and extra-synaptic NMDA receptors respectively [13, 14]. Scientific studies have indicated that the activation of μ-calpain is important for cellsurvival, while the stimulation of m-calpain initiates toxic effects and cell death [15]. Under stressful conditions, the excessive release of corticotropin-releasing hormone (CRH) activates NMDA receptors, resulting in an influx of Ca^{2+} molecules, which enhance the activity of m-calpain. Thus, a vicious cycle of excitotoxicity continues, resulting in cell-death [16]. As orexin is one of the well-known endogenous neuropeptides, which directly and/or indirectly stimulates extra synaptic NMDAR, through m-calpain, hence the later, may be considered as a predictive biomarker for HTN.

Another significant calcium-dependent protein, Jacob reveals the origin of the NMDA receptor-signal and defines the communication from the synapse to the nucleus [17]. When synaptic NMDAR was stimulated, then the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylate the protein, Jacob [16]. Conversely, the activation of extra-synaptic NMDARs fails to phosphorylate Jacob, instead helps in the translocation of Jacob to the nucleus. The phosphorylated or non-phosphorylated state of Jacob determines whether it promotes cell survival and enhances synaptic plasticity, or induces cell death [17]. These diverse functions require the regulation of gene expression, making synapse-to-nucleus communica-tion a key element controlled by Jacob [18]. Activation of synaptic NMDARs induces the expression of cell survival and plasticity genes, while the activation of extra-synaptic NMDARs primarily drives the expression of cell-death genes, such as caspases. Overexpression of Jacob results in the gene expression that induces neurodegeneration, whereas suppression has the opposite effect [19]. Therefore, a balanced expression of Jacob protein can play a pivotal role in protecting the neurons from the future damaging effects, making Jacob a po-tential therapeutic target for HTN.

Unfortunately, to date, most proteomic research aimed at discovering biomarkers, has failed to incorporate adequate biomarker validation studies in independent sample sets. These are necessary steps in translating potential biomarkers into clinical practice. Recent development of sophisticated mass spectrometry-based quantitation of multiple proteins has enabled the validation of large sets of candidate biomarkers in plasma, and cell-solutions.

The literature indicated that A1 astrocytes and their filaments contain higher concentration of glial fibrillary acidic protein (GFAP) as compared to A2 astrocytes [20], and our earlier experiments using spontaneously hypertensive rats (SHRs) and Wistar Kyoto (WKY) normo-tensive rat brain slices also indicated a higher concentration of GFAP in SHRs [21]. Therefore, the purpose of this study was twofold: firstly, to demonstrate that the protein profile of reactive A1 type HFAs is clearly different from A2 HFAs, and secondly, to show that the functional characteristics of A1 type HFAs i.e. reactive astrocytes represent hypertensive conditions.

Materials and Methods

- Protocol for the Cell Culture of Primary Human Foetal Astrocytes:
- **Primary Human Fetal Astrocytes (HFAs):** Obtained from the Biobank Macquarie University, NSW. Cells were kept for 14 days in quarantine for mycoplasma testing at UTS. The cells were maintained in a 10ml/T75cm2 flask of RPMI media (4.5g/L glu-cose, L-glutamine and 25 mM HEPES buffer) containing 10% heat-inactivated Foetal Calf Serum (FCS) [22].
- **Cell Maintenance:** After establishing that the cells were mycoplasma-free, they were seeded at 4x106 cells/T75 cm2 tissue culture flasks and cultured for two weeks in RPMI media containing 10% FCS at 37oC, in a 5% CO_2 humidified incubator.
- **Synchronization:** After achieving 95 to 100% confluency, normal HFAs were trans-ferred into two T75 flasks, labelled as A1 (reactive) and A2 (normal). They were syn-chronised by shifting the serum concentration to 1% instead of 10% FCS in the media for 24 hrs before the experiment to avoid false-positive proteomic results due to the proteins from 10% FCS [23].
- **Experiment Preparation:** On the day of the experiment, media in both flasks was replaced with 10ml of RPMI+ 10% FCS, with an additional 100μl of 1mM ATP in A1-labelled T75 flask only, to convert the cells into reactive HFAs. The cells in the A2 flask, which had no ATP, were used as control/ or non-reactive HFAs [23].
- **Cell Harvesting:** After 48 hours of incubation at 37°C, cells were washed with 5 ml of Phosphate buered saline (PBS), followed by the addition of 3 ml of trypsin (to remove adherent cells) and incubated at 37°C in a humidified incubator for four minutes. Cells were harvested and washed three times with 5 mL of PBS to remove traces of trypsin, then centrifuged to

remove extra water/moisture from the cells before freezing. Cell-pellets were snap-frozen in liquid nitrogen and stored at -80°C for the proteomics experiments using Single-Pot, solid-phase-enhanced sample preparation (SP3) protocol, for quantitative protein extraction from biological samples [24].

Ethical Approval: HFAs Obtained from Biobank Macquarie University, NSW, were approved by the UTS Human Research Ethics Committee. Approval number: UTS HREC REF NO. ETH17-1883.

Extraction of Proteins: Pellets of reactive (A1) and normal/control (A2) HFAs were prepared as described in the cell culture protocol, followed by protein extraction SP3 protocol [24].

Chemicals

Reagents for Protein Reconstitution

BSA (Sigma, 00A2153), SDS (Bio-Rad, 1610302), Triton X-100 (Sigma, T8787), NP-40 (Merck-Millipore, 492016-100ML), Tween 20 (Sigma, P1379), Deoxycholate (Sigma, 3970), DTT (Bio-Rad, 1610611), EDTA (Thermo Fisher, 15575020), NaCl (Sigma, S7653), Glycerol (Sigma, G5516), HEPES sodium salt (Sigma, H3375), NaOH (Sigma, S8045), Sodium hy-droxide (Sigma, S8045), EDTA-free protease inhibitor (Sigma,04693132001), Iodoacetamide IAA;Bio-Rad1632109).

Reagents for SP3 Sample Preparation

Sera-Mag Speed Beads (GE Healthcare, 45152105050250), Sera-Mag Speed Beads (GE Healthcare, 65152105050250), Absolute ethanol (Sigma, 34852), Ammonium bicarbonate (Sigma, A6141), Trypsin + rLysC mix (Promega, V5073), complete protease inhibitor, EDTA-free (Sigma, 4693132001), Benzonase Nuclease (Sigma, E8263).

Protein Reconstitution

Equipment

Safe-Lock, 1.5-mL tubes, (Eppendorf, 022363204), Benchtop centrifuge capable of holding 1.5-mL tubes and achieving 20,000g relative centrifugal force (Thermo Fisher, 75002431), ThermoMixer capable of holding 1.5-mL tubes (Eppendorf, 5382000023).

SP3 Processing and Protein Digestion

Magnetic rack capable of holding 1.5-mL tubes. We use Magnet Sphere 12-position rack from Promega (Z5342) or any compatible magnetic rack. Bath sonicator (VWR, 89375-454) for bead disaggregation after SP3 processing.

SP3 Protocol for Proteomics (modified version)

Before mass spectrometric analysis, proteins of interest were extracted from A1 and A2 HFAs using the SP3 protocol [24].

Preparation of Reconstitution (Lysis) Buffer as per SP3 Protocol

Reconstitution buffer was freshly prepared by mixing 100µl of each of the following chemi-cals: 10% SDS, 10% Triton X-100, 10% NP-40, 10% Deoxycholate, and 10% Glycerol. Ad-ditionally, 10µl of 5M NaCl, 20µl of HEPES (pH 8.0), and 470 µl of water was added to the mixture. Astrocyte pellets of A1 and A2 HFAs were resuspended in 50 µl of lysis buffer prepared according to the SP3 protocol for complete protein extraction by heating at 90°C for 10 minutes [24]. One µl of Tris 2-carboxyethyl phosphine (TCEP) was added to reduce the disulfide bonds, and 1 µl of Acrylamide monomer was used to alkylate the mixture. After an hour of incubation at the room temperature, the alkylation reaction was stopped by adding 1 µl of Dithiothreitol (DTT). The Bicinchoninic Acid Assay (BCA) (Thermo Fisher Scientific) was performed to determine the volume of cell-solution that would contain ~40 µg of total proteins in A1 and A2 HFAs samples. The cell-solution was diluted to a final volume of 48µl by adding double-distilled water and 2µl of hydrophilic magnetic beads to the samples, giving a total volume of 50µl of protein solution. To induce binding of the proteins to the beads, 50 µl of 100% ethanol was added to the mixture and vortexed for 5 minutes. Hydrophilic magnetic beads, coated with carboxylate functional groups, capture the proteins, making it easier to wash the cells in absolute alcohol. To enhance surfactant removal, three rinses of the cells with 80% ethanol were performed, and the supernatant with unbound compounds was discarded in an appropriate waste container. A freshly prepared, 100 µl solution of 200 mM ammonium bicarbonate was added to the beads carrying attached proteins, which were finally extracted in the aqueous phase. Trypsinization of proteins was achieved by incubating the samples overnight with 1µl of trypsin at 37°C, making a 1:40 trypsin to protein ratio. Further desalting and cleaning of the peptides were performed using an extended protocol called the STop And Go Extraction (STAGE) protocol [25]. The supernatant containing the peptides was vacuum centrifuged using a Thermo Speed Vac to evaporate the water, and proteins were resuspended in 25µl of Mass spectrometer loading Solvent A, which is a mixture of 2% Ace-tonitrile + 0.1% Trifluoroacetic acid.

Bicinchoninic Acid Assay (BCA) Protein Assay Procedure

Using 96-well microplates with bovine serum albumin (BSA) standard and diluted cell solu-tions by 1:1 & 1:10, covered plates were incubated at 37°C for 30 minutes. The plates were read on the Tecan Infinite 200 Pro spectrophotometer at a wavelength 562nm using Magellan software. Using the data obtained, a standard curve (Figure 1) was plotted and used to de-termine the volume of cell solution to be used for the protein extraction of unknown samples of the astrocytes.

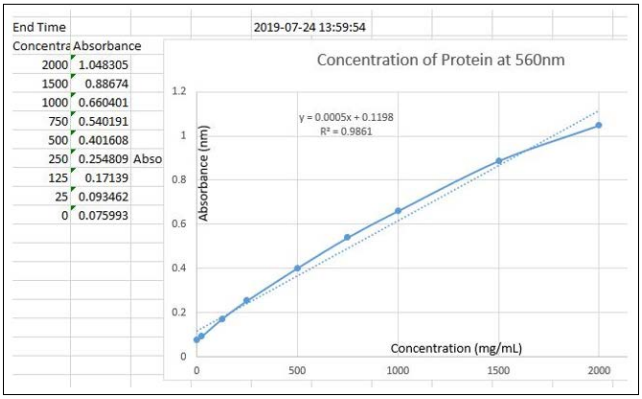

Figure 1: Standard curve plot using BCA protocol

Desalting and Cleaning of Trypsin-Digested HFA Samples by Stop and Go Extraction (STAGE) Tips [25]

This protocol is used to desalt and clean up the peptides resulting from the enzymatic digestion of protein samples. A large-sized Styrenedivinylbenzene-reverse phase sulfonated (SDB-RPS) disk was placed in a covered petri dish, and a 47mm disk core was employed in this protocol as a solid phase extraction sorbent. [Note: These sorbents have a high affinity for polar organic compounds, reducing extraction time and solvent use for the analytical preparation of aqueous environmental samples]. Literature indicates that A1 astrocytes and their filaments contain higher concentrations of GFAP compared to A2 astrocytes [20]. Similarly, our initial immunohistochemistry studies using anti-

GFAP antibodies on astrocytes of spontaneously hypertensive rats (SHRs) and their normal counterparts, Wistar Kyoto (WKY) rats, also demonstrated a higher concentration of GFAP in SHRs [21]. Therefore, the purpose of this study was twofold: firstly, to demonstrate that the protein profile of reactive A1 type HFAs is clearly different from A2 HFAs, and secondly, to show that the functional characteristics of A1 type HFAs, i.e., reactive astrocytes, represent hypertensive conditions.

Making Stage Tip

Using a flat-ended needle, the 47mm SDB-RP core was pushed carefully inside a 2ml pipette-tip, keeping 2-3 mm above the end of the tip.

Preparing Collection Tubes

Eppendorf tubes of 2ml size with lids on were used to make a hole into the middle of the lid, using a rotary cutting tool with all the required precautions. The pipette tip with SDB-RPS disk was inserted into the Eppendorf tube through the hole.

Reagents

10% v/v trifluoroacetic acid (TFA) was made in double distilled water, then 1% of this TFA was made in 90% Isopropanol and dilute it in 1:10 in water. Elution solvent (made fresh), by mixing 700µl of CAN, 71 µl of NH4OH stock and 229µl of water.

Desalting and Washing the Samples

Acidified the sample by adding 10µl of 10% Trifluoroacetic acid TFA and centrifuged for 5 minutes at maximum speed. Using one STAGE tip per sample, the sample was pipetted into the top of the tip and centrifuged at 5000rpm for 1 minute. Centrifugation was repeated if all the liquid had not passed through. To clean the sample, 60 µl of Isopropanol/ 1% TFA was pipetted into the STAGE tip and centrifuged at 5000 rpm for 2 minutes. Repeated centrifuging was performed to clear all the salts and contaminants from the bound peptides and the liquid in collection tube was discarded.

Setting up STAGE Tip in Atovial Insert

One autovial per sample was used for each collection tube. A yellow spacer was placed be-tween STAGE tip and collection tube to hold the STAGE tip in the centre of the autovial in-sert.

Elution of Peptides

Sixty µl of elution solvent (containing 1M of ammonium hydroxide plus 70% Acetonitrile) was pipetted into the STAGE tip and microfuged for a few seconds to drag the liquid onto the disk and this was subsequently incubated on the bench for 10 minutes to complete the reaction. The STAGE tip with the autovial insert was centrifuged at 5000 rpm for 5 minutes, this step was repeated to allow all the fluid to pass through the disk into the autovial insert, which contains all the peptides.

Removing all the liquid from the Peptides

The STAGE tip was discarded at this point and collection tubes with autovial inserts con-taining samples were placed into the Speed Vac (Thermo Speed Vac Concentrator DNA 120) to vacuum centrifuge until all the liquid was removed. Autovial inserts were removed from the Speed Vac and the springs were attached back to the bottom of the inserts, before placing them into the autovials (Figure 2).

Twenty-five µl of MS Loading Solvent A (a mixture of 2% Acetonitrile + 0.1 % Trifluoroa-cetic acid) was pipetted into each auto vial inserts with samples ready to be injected and ana-lysed on mass spectrometer.

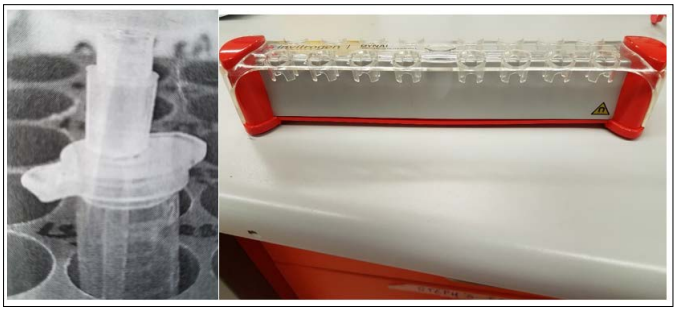

Figure 2: This figure shows a 2ml yellow pipette-tip STAGE tip (right panel), inserted through a hole drilled into the top of a 2ml collection tube. The pipette tip with SDB-RPS disk was inserted into the Eppendorf tube through the hole. Cell solution was pipetted from the top into yellow tip and peptides were allowed to react with the SDB-RPS disk for 10 minutes. When sample-vials containing magnetic beads were placed on the magnetic rack (left panel), the beads start moving to one side of the vial, making the protein molecules safely at-tached to the beads and fluid with contaminations was discarded appropriately.

Proteomics Data of Reactive (A1) and Normal (A2) Human Foetal Astrocyte Samples, Using Liquid Chromatography – Mass Spectrometry (LC/MS/ MS) Technique

LC/MS/MS, Optimization

Optimization of the protein extraction before (Figure 3) and after optimization (Figure 4)

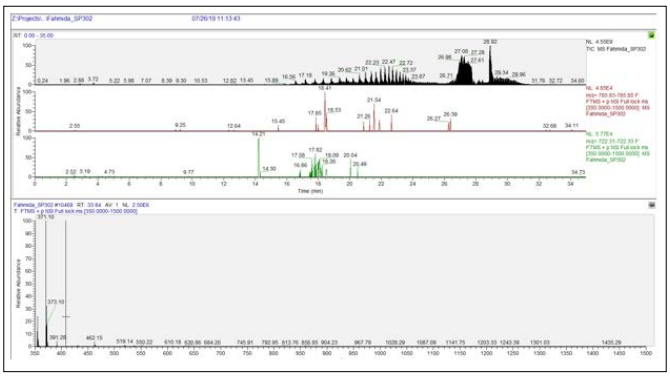

Figure 3: First set of Chromatograms obtained after LC-MS analysis of A1 and A2 astrocytes. Regularly seen peaks suggested that surfactant-like substances are present in the sample. Low profusion indicated that less than 2ug/µL is present in the sample.

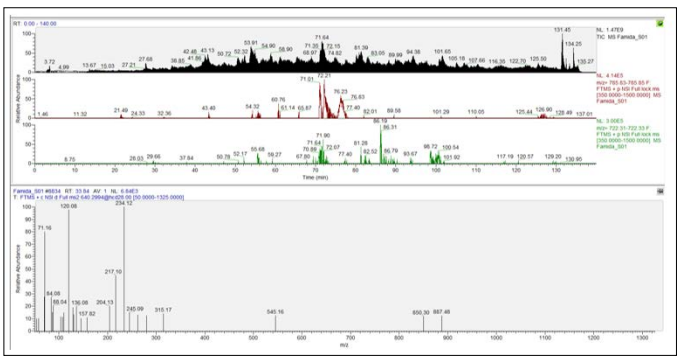

Figure 4: In this figure we were looking at the optimized peaks of proteins, which were 10-fold lower than expected value. After analyzing the chromatograms, following steps were performed

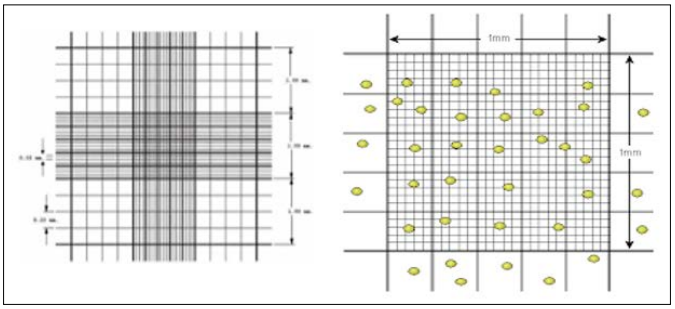

Figure 5: Counting of the astrocytes (1X108 per sample) using hemocytometer. Equal parts of cell-solution and Trypan Blue were mixed and 15-20µl of the mixture was injected care-fully between the cover glass and haemocytometer using a P-20 Pipetman. The goal was to have approximately 100 to 200 cells/square. The total number of cells were counted in all four outer squares and divided by four to get the mean number of cells/squares. Since 1 cm3 is equivalent to approximately 1 ml.

Percentage of cell viability= [Total Viable cells (Unstained) / Total cells (Viable +Dead) X 100.

Viable Cells/ml = Average viable cell counts per square x Dilution Factor x 104

Briefly, after adding a small amount of trypan blue, the cell suspension was transferred onto a hemocytometer and both viable and non-viable astrocytes were counted under an inverted microscope in the phase contrast mode at 100X magnification.

All viable HFA cells were used for protein estimation. 1% sodium deoxycholate (SDC) in-stead of reconstitution buffer was used as lysis buffer.

Using PEAKS software (Bioinformatics solutions Inc), proteins were quantified based on peptide features detected from LC-MS data by integrating the area under the curve (AUC+E6).

Triplicates of Each Set of A1 and A2 Samples (n=9) were Carefully Analysed Using Ei-ther One of the Two Filters (Figure 6):

- Peaks Peptide Score (-10lgP) threshold, only peptides with a score above this thresh-old were used to quantify the identified proteins separating low scoring proteins as unique peptide count.
- False discovery rate (FDR) threshold (1 %)

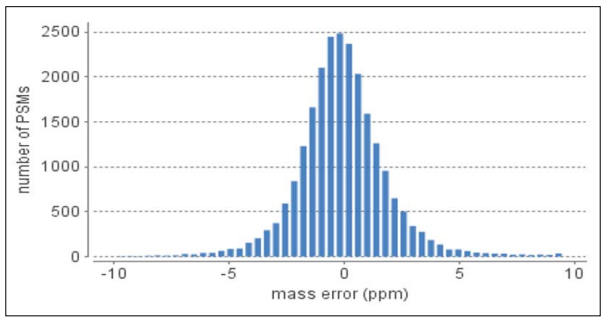

Figure 6: Experimental Error Chart, indicating the minimal error (1%), in peptides-spectrum matches (PSMS) against part per million (ppm), using PEAKS software. It shows the best eluted peptides close to the middle were used for our study and the insignificant small peaks were separated as low protein unique count

Venn diagram

A Venn diagram (Figure 7) shows all possible logical relations between a finite collection of different sets. These diagrams depict elements as points in the plane and sets as regions inside closed curves. A Venn diagram consists of multiple overlapping closed curves, usually circles, each representing a set Normal (A2) and Reactive (A1) astrocytes [26].

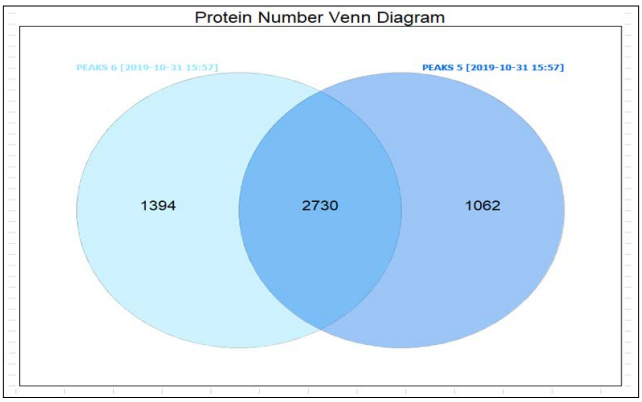

Figure 7: Comparison of the number of proteins detected in Human Foetal Astrocytes (HFAs); reactive (A1) and normal (A2) astrocyte-samples. Venn diagram, showing all possible logical relations.

Statistical Analysis

All data were expressed as the mean± standard error mean (SEM). Differences between two groups were determined using paired t tests and were considered significant at $p<0.05$. All statistical analyses were conducted using GraphPad Prism version 8.0.1 (244), GraphPad Software.

Results

Confocal

Photomicrographs of both WKY and SHR were viewed by brightfield optics (Figure 8).

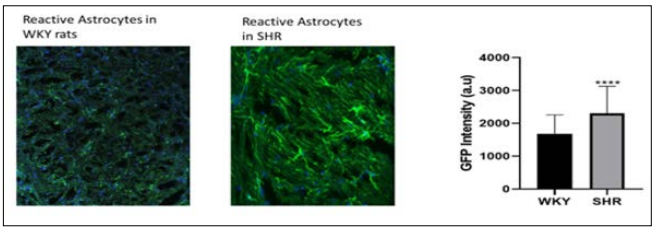

Figure 8: Using Nikon A1 microscope, confocal images of astrocytes from WKY and SHR brain stem slices immunostained with anti-glial fibrillary acidic protein (GFAP) antibody ab 7260, con-jugated with Alex Fluor 488. Statistical analysis of GFAP intensity was obtained using Graph Pad Prism, version 8.0.1, indicating the upregulation of filament proteins in reactive SHR as-trocytes. $P \leq 0.0001$

Proteomics

Our proteomic experiments obtained from both types of HFAs indicated many potential can-didates as predictive biomarkers for hypertension. At this stage we are focusing on certain specific proteins, such as GFAP (Figure 9), m-Calpain (Figure 10), Calpastatin (Figure 11), Cathepsin (Figure 12), Mitogen Activated Protein Kinase (MAPK) (Figure 13), as they are associated with NMDA and Orexin interfaces. Most of the predictive biomarkers chosen from the LC/MS/MS experiments are calcium binding proteins. Both cytoplasmic and nuclear calcium plays an important

role in the gene expression and pro survival effect of synaptic stimulation, which is disrupted by the activation of extra synaptic NMDAR.

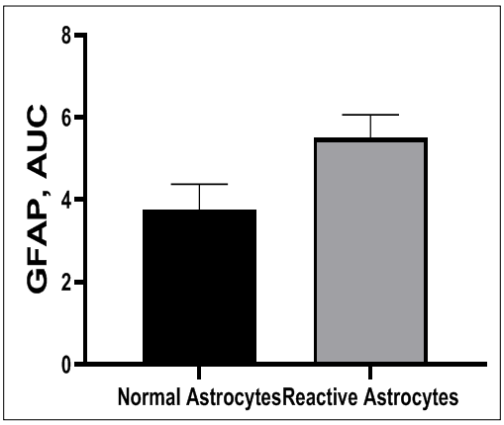

Figure 9: Shows the higher levels of Glial Fibrillary Acidic Protein (GFAP) in reactive astrocytes (n=9) than normal astrocytes (n=9), measured by LC/MS/MS and using PEAKS software. Area under the curve (AUC) values are shown as means ± SEM, Reactive cells = 4.39 ± 0.4 and Normal cells = 3.02 ± 0.3 respectively. Statistically analysed by GraphPad Prism, with a value *P < 0.05. Higher levels of GFAP is due to the accumulation of filament proteins, which is a hallmark of reactivity in astrocytes.

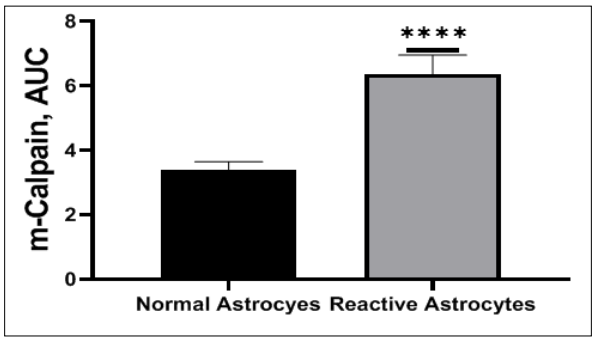

Figure 10: Exhibits elevated levels of m-Calpain in the reactive astrocytes AUC value= 6.37 ± 0.6 (n=9) whereas normal astrocytes AUC value = 3.40 ±0.2 (n=9). Statistically analysed by GraphPad Prism, significant by the value ****P < 0.0001. An increased expression of m-Calpain, which is a calcium-dependant proteolytic enzyme indicates a greater excitotoxicity due to the activation of extra synaptic NMDA receptors in the reactive astrocytes.

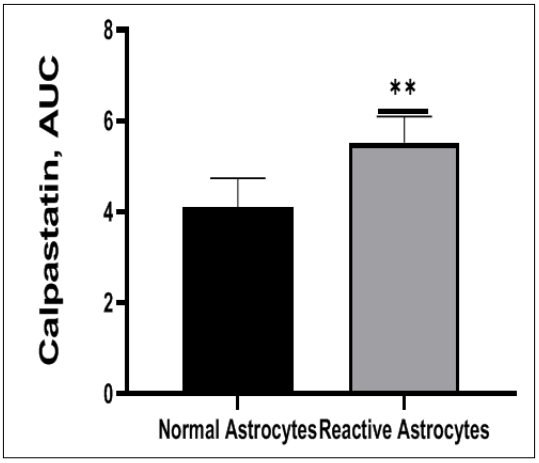

Figure 11: Demonstrates the greater concentration of Calpastatin in reactive astrocytes AUC value= 5.52 ± 0.5 (n=9) whereas in normal astrocytes AUC value= 4.09 ±0.6 (n=9). Statisti-cally analysed by GraphPad Prism, significant by the value **P < 0.01. Calpastatin is an en-dogenous inhibitor of calcium ion-activated enzymes such as Calpain. Thus, it plays a positive role in controlling the HTN and could be used for therapeutic purposes.

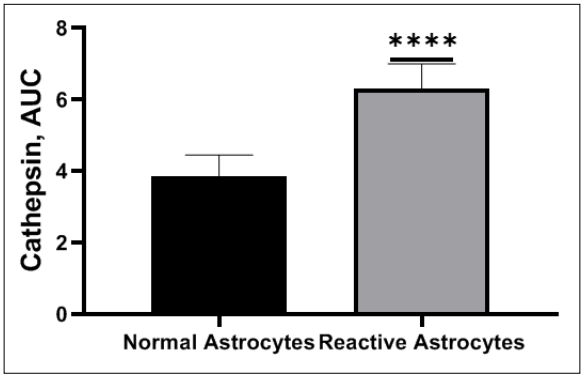

Figure 12: Shows the higher level of Cathepsin in reactive astrocytes AUC value = 6.31 ± 0.7 (n=9) whereas normal astrocytes AUC value = 3.86 ±0.4 (n=9). Statistically analysed by GraphPad Prism, significant ****P < 0.0001. Cathepsins are lysosomal proteases and excess of these molecules are known to be involved in the pathophysiology of cardiovascular system through MAPK signalling pathway, leading to HTN. Hence, early detection of cathepsins could be a promising predictive biomarker for HTN.

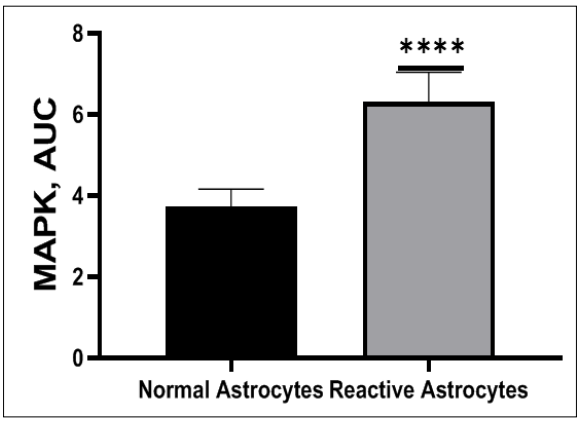

Figure 13: Reveals an elevated concentration of Mitogen-activated protein kinase (MAPK) in reactive astrocytes AUC = 6.31 ± 0.7 (n=9) as compared to normal astrocytes AUC = 3.72 ±0.4 (n=9). Statistically analysed by GraphPad Prism, significant by ****P < 0.0001. Extra synaptic NMDA receptors are known to activate m-Calpain, which initiates a cascade of excitatory enzymes such as MAPK, extracellular signal-regulated kinase (ERK) and caspases leading to apoptotic effects on the cells.

The objective of this study was to identify and estimate the protein-peptide molecules (in A1 and A2 human foetal astrocytes) as predictive biomarkers for hypertension. Therefore, our first step was to prepare reactive astrocytes from normal ones by using ATP. The result con-firms the elevated reactivity in SHR as compared to WKY astrocytes, verifying that the reac-tive astrocytes mimic hypertensive condition.

Calcium- induced protein molecules such as GFAP, Calpains, Calpastatin, Cathepsin and Mi-togen Activated Protein Kinase (MAPK) (Figures 9-13) have been identified in our results obtained after LC/MS/Ms analyses, using PEAKS software.

Discussion

Our results show significantly higher level of the isoform m-Calpain, which is mostly respon-sible for excitotoxicity in reactive HFAs. Calpains are a calcium-dependant cytosolic proteolytic group consist of 15 isoforms. The widely studied isoforms are calpain 1 (also known as μ-calpain) and calpain 2 (also known as m-calpain), are encoded by the CAPN1 and CAPN2 genes, respectively.

These studies are like earlier studies [27] indicating that μ-calpain and m-calpain are activated by the synaptic and extra-synaptic NMDA receptors, respectively. Under stress like conditions, extrasynaptic NMDAR excitation induces calcium influx activating neuronal m-calpain. This, in turn, activates the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases (ERKs) signalling pathway, leading to apoptosis.

Similarly, both calpains are found in the endothelial cells, where they can be activated by growth factors such as vascular endothelial growth factor (VEGF), resulting in the activation of PI3K/AMPK/Akt/eNOS pathway. This leads to the production of nitric oxide and subsequently angiogenesis. Studies also indicate that calpains can exit the cell and act on the extracellular substrates, such as collagen-fibronectin, to modulate cell activity. Therefore, cal-pains are considered target proteins for various cardiovascular diseases, including hypertension [28].

Our investigation indicates significantly greater expression of calpastatin in A1 than in A2 cells. Calpastatin is the most specific endogenous calpain-inhibitor. It binds to calpain 1 and 2 in response to calcium ions to prevent activation of calpains. Calpastatin has four inhibitory domains, each capable of binding to one calpain molecule. Each inhibitory domain has 3 sub-domains: A, B, and C. Subdomain A and C bind to calpain domain IV and VI, respectively. Protecting its own active site, calpastatin binds around the active sites of the calpains [29].

A recent study demonstrates that the treatment of endothelial cells with VEGF, down-regulates calpastatin expression, leading to increased calpain's degenerative activities. The combined activity of calpain and calpastatin results in maximum modulation of calpain and its downstream signalling in response to VEGF treatment. Although calpastatin has a high molecular mass and is membrane impermeable, limiting its use as a pharmacological tool, a study on a mouse model has shown that calpastatin overexpression protects against cardio-vascular remodelling.

Our data reveal a significantly higher expression of cathepsin in A1 than in A2 cells. Cathepsins are an extensive family of cysteine proteases with broad proteolytic activity in lysosomes and endosomes, with 35 known isoforms.

There are two distinct cathepsin L proteases, encoded by the human cathepsin L1 and cathep-sin V, which participate in inflammatory disorders, regulate apoptosis, antigen processing, and extracellular matrix remodelling.

Evidence suggests that cathepsin L plays an important role in hypertension- induced vascular modulation. It has also been reported that cathepsin L/V regulates MEK phosphorylation in an Angiotensin II-induced hypertension model. Angiotensin II induces the G-protein-coupled receptor (GPCR). activation of the MAPK cascade, which consists of p38, JNK, and ERK.

Studies have also reported that cathepsin L directly participates in atherosclerosis by degrad ing elastin and collagen. Thus, cathepsins play a key role in extracellular matrix (ECM) re-modelling and have been implicated in the development and progression of many cardiovascular diseases, including hypertension [30].

Our research demonstrates an extensively greater level of MAPK molecules in A1 than A2 HFAs. MAPK /ERK pathway is a chain of proteins in the cell that communicates a signal from a receptor on the cell surface to the DNA in the cell nucleus. The MAPK pathway, including Ras, RAF, MEK and ERK signalling molecules, plays a significant role in the regulation of gene expression, cellular growth, and survival. Abnormal MAPK signalling may lead to increased or uncontrolled cell proliferation and resistance to apoptosis.

A recent study [31] found that MEK-ERK is phosphorylated in the arteries of hypertensive patients and in a mouse model, promoting the proliferation of the human arterial smooth muscle cells (HASMCs) in vitro, thus leading to degenerative conditions.

All these enzymes we studied in human astrocytes models are potential biomarkers. Further studies are required to identify the most promising predictive biomarkers for hypertension.

Our initial findings, based on proteomic results, indicated that identifying ways to preserve reactive astrocytes to augment their protective functions or both may lead to novel approaches for reducing secondary tissue damage and improving functional outcome leading to hypertension.

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