REPORT

Report of the Fellow who availed Fellowship / Training under Human Resource Development for Health Research.

1.	Name and designation of Fellow	: Dr RITU SINGH
2.	Address	: Professor, Deptt of Biochemistry , LHMC , New Delhi
3.	Type of Fellowship and period	: DHR-ICMR Short term(one Month) Training in foreign institute
4.	Duration of fellowship	: 17th feb 2015 to 16th March , 2015
5.	Frontline area of research in which Training /research was carried out	: Genomics in atherosclerosis
6.	Name & address of mentor and host institute	: Dr Sampath Parthasarathy , Professor of Medicine and Assoc. Dean for research College of Medicine, 6900 Lake Nona Blvd, Orlando, 32827, Florida ,USA

7. **Highlights of work conducted**

i) **Technique/expertise acquired (Give in about 150 words)**

The techniques acquired include mRNA expression Analysis , flow-cytometry , GC-MS , calcium staining for aortic tissues, DNA microarrays basic protocol and insight into statistical evaluations of its data and maintenance of cell culture lines

1)**Maintenance of cell lines**: Cell lines are procurred from ATCC cell lines.They are supplied in DMSO. They can be adherent cell types like epithelial cells or in suspension like mmocytes. Adherant cell lines require treatment with trypsin before utilization . Cells are centrifuged to extract pellets and remove media. For storage , they are are re-suspended in DMSO and cryopreserved in liquid Nitrogen at -80.For working, they are put in the appropriate media for specific cell line in TC-75 tubes and sub-cultured on 6 well plates

2) **mRNA expression in epithelial cell lines** : Extraction of total mRNA from intestinal epithelial cell lines , confirming purity of mRNA by nano-drop , synthesizing cDNA using

Reverse transcriptase super script 111 first strand synthesis super mix for qPCR , evaluation of mRNA expression for MCP-1, PON-1,ALP-1,ApoA1 and IL-6 .

2) **Blood analysis by flowcytometry** after adequate preparation and RBC cell lysis .The fluorophore was attached to appropriate antibody for NK cells, T cells and B cells using beads as controls. The scatter plot was evaluated . The machines used were Beckman flowcytometer

3) **Gene Knock out mice were used in study of mRNA expression in atherosclerotic aorta** : 14 month old PON1 and ApoE gene double knockout mice were sacrificed as per standard procedure and atherosclerotic aorta dissected out and preserved in liquid nitrogen . Before assay , it was homogenized, total mRNA extracted using trizol reagent from ambion .cDNA was synthesized using super-mix by invitrogen and further quantified by qPCR super-mix for icycler .Appropriate primers for MMP_9 and MCP-1 were used .

4) **Calcium staining of aortic tissues** by von kossa stain using aqueous silvernitrate solution and sodium thiosulphate and counter staining by nuclear fast red .Alizarin Red S staining was also done

5) **GC-MS for study of lipids:** after derivitization of lipids into volatile phase of hexane ,GCMS gives us the various peaks which are then compared to known molecular data

6) **Steps of DNA microarray analysis** include mRNA isolation, preparation of cDNA ,and further preparation of cRNA using fluorescent nucleotides. This is made to hybridize to a array having appropriate fixed oligo probes which may be customized or may be whole genome probes

The Teqniques learnt in the DHR_ - HRD fellowship 17th feb 2015 to 16th March 2015 at UCF , Orlando,Florida

1. mRNA expression Analysis from
    a) epithelial cell lines
    b) Aorta
2. Flow-cytometry ,
3. DNA microarrays basic protocol and insight into statistical evaluations of its data
4. Maintenance of cell culture lines
5. GC-MS
6. Calcium staining for aorta

1. a) mRNA expression of IL-6 , ALP, APOA1, MCP-1 and PON 1 in intestinal cells

Instestinal epithelial cell lines were used to study the techniques. These were being done as part of a project in Dr Parthasarathy's Lab . Cell lines are procurred from ABCC cell lines.They are supplied in DMSO. They can be adherent cell types like epithelial cells or in suspension like mmocytes. Addherant cell lines require treatment with trypsin before utilization . Cells are centrifuged to extract pellets and remove media. For storage , they are are re-suspended in DMSO and cryopreserved in liquid Nitrogen at -80.For working, they are put in the appropriate media for specific cell line in TC-75 tubes and sub-cultured on 6 well plates

ISOLATION OF TOTAL mRNA USING TRIZOL REAGENT

1) **Phase separation** : O.2 ml of chloroform was added per 1 ml of Trizol reagent. The tube was shaken by hand for 15 sec and further incubated at room temperature for 3 minutes.The sample was further centrifuged at 12,000xg for 15 min at 4ºC to obtain a colorless upper aqueous phase containing the RNA .The aqueous phase was pipetted out into a new tube.
2) **RNA isolation** :

   A) RNA precipitation : 5µg of RNA ase free glycogen was added as a carrier to the aqueous phase 0.5 ml of 100% isopropanol was added to the aqueous phase per iml of Trizol reagent used for homogenization. This was incubated for 10minutes at room temperature and further centrifugd at 12,000xg for 10minutes at 4ºC.The RNA was visible as a gel like pellet in the bottom of the tube

   B) RNA wash : The supernatant was discarded from the above tube leaving only the pellet which was further washed by 1ml of 75% ethanol per ml of initial Trizol reagent used for homogenization .Sample was briefly vortexed and centrifuged at 7500xg for 5minutes at 4ºC.The wash was discarded

   C) RNA re-suspension RNA was now dissolved in 50 µl of RNA'ase free water (Invitrogen) The sample was incubated in heat block at 55-60 ºC for 15min .
3) **Purity of RNA** isolated was determined by Nanodrop spectrophotometer at 260/280. Ratio between 1.7-2.0 was taken as acceptable

SYNTHESIS OF FIRST STRAND cDNA FORM THE mRNA ISOLATED

Super script 111 first strand synthesis super-mix for qRT-PCR was sourced from Invitrogen (cat.no 11752-050) .First a master-mix without RNA was prepared . This included 10 µl of 2xRT reaction mix (oligodT 2.5 µM, random hexamers:2.5ng/ µL, 10mM Mgcl2 and dNTPs) plus 2 µl of RT enzyme mix plus RNA upto 1 µl plus DEPC (diethylpyrocarbonate) treated water upto 20 µl. The tube contents were incubated as per protocol in PCR machine(genepro).The protocol was pre-set as incubation at 25 ºC for 10 min , 50 ºC for 30 mnand 85 ºC for 5min. After termination it was chilled on ice .Tubes were

taken out and 1 µl of Ecoli RNAase H was added and tubes further incubated at 37 ºC for 20 min.

REAL TIME QUANTITATIVE PCR for IL-6 , ALP, APOA1, MCP-1 and PON 1

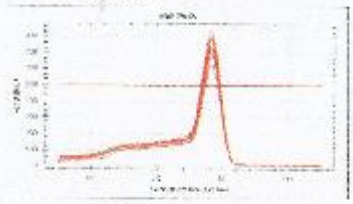
SYBR greenER qPCR supermix for icycler (cat.no 11761-100 : Invitrogen) was used as a ready to use cocktail containing all components except primers (F and R primers for IL-6 , ALP, APOA1, MCP-1 and PON 1 respectively) and template (cDNA) . 10 µL of SYBR mastermix was pipetted in appropriate tubes with forward and reverse primer 1 µL each for IL-6 , ALP, APOA1, MCP-1 and PON 1 (Invitrogen) . RNAase free water was added to make upto 19 µL with further addition of appropriate cDNA template in each tube. GAPDH (Glyceraldehyde 3 phosphate dehydrogenase) housekeeping reference gene was used as control with appropriate GAPDH primers to cross-check experimental conditions . The machine used was BIORAD CFX 96 Real Time System C1000 touch thermal cycler .

INTESTINAL EPITHELIAL CELLS (CANCER) - CELL LINES,

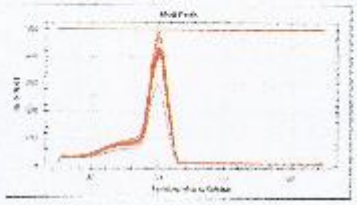
Target	Sample	Cq	Cq Mean	Cq Std. Dev
GAPDH	Control	17.5	17.62	0.172
GAPDH	Control	17.74	17.62	0.172
MCP-1	Control	30.81	30.83	0.029
MCP 1	Control	30.85	30.83	0.029
IL-6	Contro	32.13	32.41	0.391
IL 6	Contro	32.68	32.41	0.391
ALP	Control	24.54	24.59	0.072
ALP	Control	24.64	24.59	0.072
APOA1	Control	17.47	17.54	0.1
APOA1	Control	17.61	17.54	0.1
PON1	Control	27.8	27.87	0.1
PON1	Control	27.95	27.87	0.1
GAPDH	5uM cH2O2	17.52	17.47	0.065
GAPDH	5uM cH2O2	17.43	17.47	0.065
MCP-1	5uM cH2O2	30.99	30.79	0.28
MCP 1	5uM cH2O2	30.6	30.79	0.28
IL-6	5uM cH2O2	32.13	32.28	0.208
IL-6	5uM cH2O2	32.42	32.28	0.208
ALP	5uM cH2O2	24.65	24.57	0.115
ALP	5uM cH2O2	24.49	24.57	0.115
APOA1	5uM cH2O2	17.63	17.59	0.067
APOA1	5uM cH2O2	17.54	17.59	0.067
PON1	5uM cH2O2	27.81	27.79	0.021
PON1	5uM cH2O2	27.78	27.79	0.021
GAPDH	12.5uM	17.11	17.17	0.087
GAPDH	12.5uM	17.23	17.17	0.087
MCP-1	12.5uM	30.16	30.44	0.402
MCP-1	12.5uM	30.73	30.44	0.402
IL 6	12.5uM	33.17	33.61	0.618
IL-6	12.5uM	34.05	33.61	0.618
ALP	12.5uM	24.36	24.44	0.122
ALP	12.5uM	24.53	24.44	0.122
APOA1	12.5uM	17.22	17.24	0.029
APOA1	12.5uM	17.27	17.24	0.029
PON1	12.5uM	27.51	27.49	0.019
PON1	12.5uM	27.48	27.49	0.019
GAPDH	25uM	17.01	16.93	0.103
GAPDH	25uM	16.86	16.93	0.103
MCP-1	25uM	30.11	30.22	0.157
MCP-1	25uM	30.33	30.22	0.157
IL-6	25uM	32.9	32.61	0.398
IL 6	25uM	32.33	32.61	0.398
ALP	25uM	24.17	24.15	0.029
ALP	25uM	24.13	24.15	0.029
APOA1	25uM	17.04	16.96	0.114
APOA1	25uM	16.88	16.96	0.114

17days Caco2

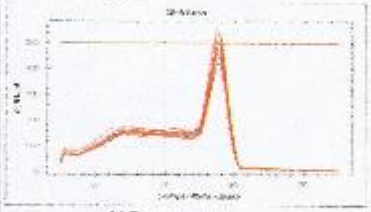
GAPDH



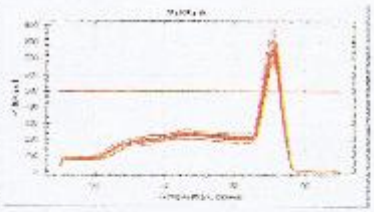
MCP 1



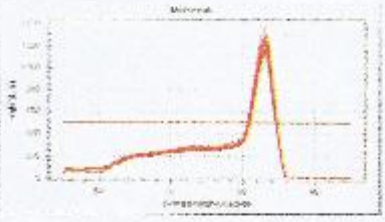
IL-6



ALP



APOA1



1. b) MMP-9 and MCP-1 mRNA expression in Paroxonase 1 and ApoE gene double Knock-out mice

_: Paroxonase and ApoE double knock out mice (age 13-14 months) on normal chow diet were sourced from the animal facility of Burnetts school of Biomedical Sciences where they were co-breeded and maintained by Dr Sampath Parthsarathy's Lab. The mice were initially sourced from http://jaxmice.jax.org. Three Apoe-Paroxonase double knock-out male mice were sacrificed as per standardized protocol using Iso-flurane for anesthesia and further exposure to Carbon di-oxide .The mice were dissected and aorta exposed . The aortic arch was dissected out and frozen in liquid nitrogen till further studies .

On day of experiment , aorta was further cut into small pieces to aid homogenization with the addition of 800 µl of Trizol reagent (Ambion-www.lifetechnologies.com) and centrifugation .The homogenized tissue was kept in ice and centrifuged in a cold centrifuge .(4ºC at 12,000 g).The homogenized sample was incubated at room temperature for 5 minutes

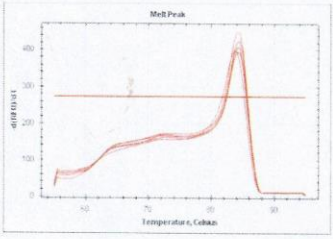
Further protocol of isolation of total mRNA using Trizol reagent and synthesis offirst strand cDNA from the mRNA isolated was followed as done for Intestinal cells . However during RNA precipitation , glycogen was not added.

REAL TIME QUANTITATIVE PCR for MMP-9 and MCP-1SYBR greenER qPCR supermix for icycler (cat.no 11761-100 : Invitrogen) was used as a ready to use cocktail containing all components except primers (F and R primers for MMP-9 and MCP-1 respectively) and template (cDNA) . 10 µL of SYBR mastermix was pipetted in appropriate tubes with forward and reverse primer 1 µL each for both MCP-1 and MMP-9 (Invitrogen) . RNAase free water was added to make upto 19 µL with further addition of appropriate cDNA template in each tube. GAPDH (Glyceraldehyde 3 phosphate dehydrogenase) housekeeping reference gene was used as control with appropriate GAPDH primers to cross-check experimental conditions . The machine used was BIORAD CFX 96 Real Time System C1000 touch thermal cycler .

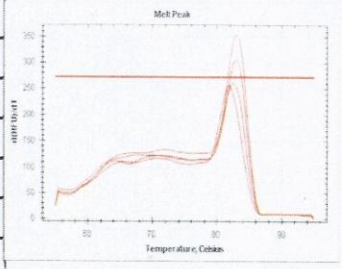
MICE AORTIC TISSUE PON-1 /APOE DOUBLE GENE KNOCKOUT

Target	Sample	Cq	Cq Mean	Cq Std. Dev
GAPDH	AP-10	21.57	21.62	0.07
GAPDH	AP-10	21.67	21.62	0.07
MMP9	AP-10	29	29.11	0.16
MMP9	AP-10	29.22	29.11	0.16
MCP1	AP-10	31.45	31.52	0.101
MCP1	AP-10	31.6	31.52	0.101
GAPDH	AP-11	22.07	22.06	0.019
GAPDH	AP-11	22.04	22.06	0.019
MMP9	AP-11	30.29	30.29	0
MMP9	AP-11	30.29	30.29	0
MCP1	AP-11	32.15	32.22	0.096
MCP1	AP-11	32.29	32.22	0.096
GAPDH	AP-12	23.05	23.07	0.018
GAPDH	AP-12	23.08	23.07	0.018
MMP9	AP-12	32.01	31.79	0.32
MMP9	AP-12	31.56	31.79	0.32
MCP1	AP-12	32.78	32.72	0.085
MCP1	AP-12	32.66	32.72	0.085

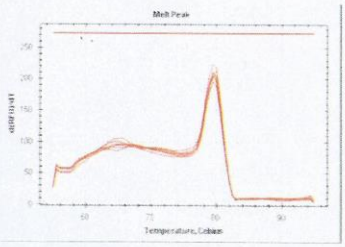
GAPDH

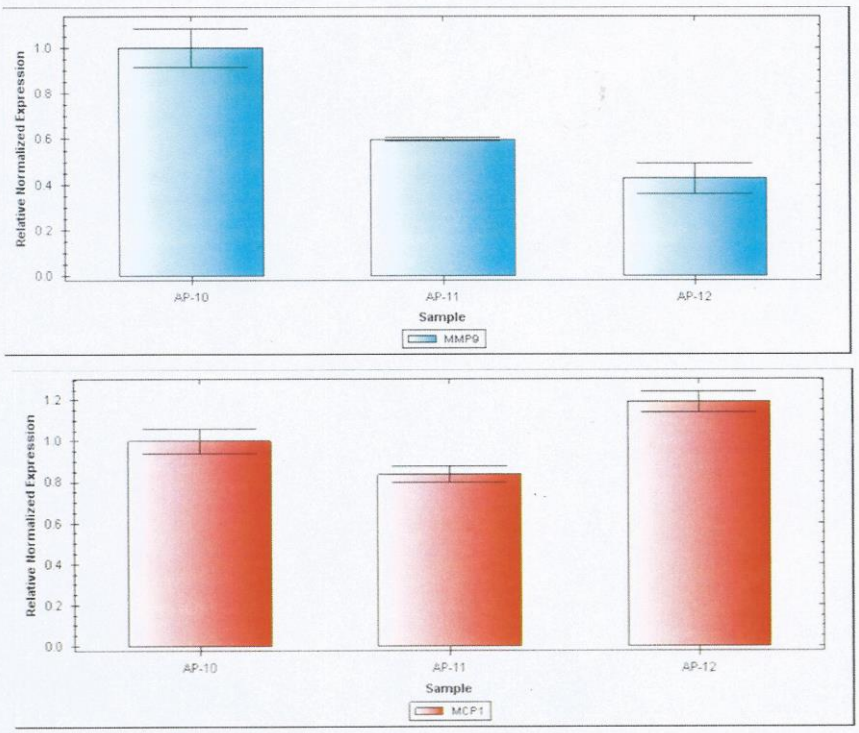


MMP9



MCP-1





Target	Sample	Expression	pression SEM
MCP1	AP-10	1	0.06021
MCP1	AP-11	0.83741	0.04028
MCP1	AP-12	1.19168	0.05086
		1.009697	0.05045
MMP9	AP-10	1	0.0856
MMP9	AP-11	0.5982	0.00782
MMP9	AP-12	0.42652	0.06691
		0.674907	

2. Flow Cytometry : The BD LSRFortessa™ X-20 cell analyzer used here for analysis of T cells ,B cells and NK cells

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.
A flow cytometer is made up of three main systems: fluidics, optics, and electronics.
• The fluidics system transports particles in a stream to the laser beam for interrogation.
• The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.
• The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

Blood Analysis by was done by flowcytometry for NK cells , T cells and B cells using reference beads

A. Purpose: to prepare and analyze components of blood collected from mice by flow cytometry.

B. Expected Results: samples should be relatively free of red blood cells and cells that are analyzed should be viable.

C. Reagents and Materials:
1. Blood collection tubes containing EDTA (lavender/pink top tubes)
2. Red blood cell lysis buffer: 150 mM Ammonia Chloride + 0.1 mM EDTA +10mM Potassium Bicarbonate, pH 7.3
3. 5mL BD tubes
4. Staining Buffer: DPBS + 2mM EDTA + 0.5% BSA
5. Cold DPBS
6. Desired antibody cocktails (See Section D)

D. Preparing Antibody Cocktails
1. Determined the amount of each antibody to add by using formula
   _____ µL/sample x _____ samples x 1.1 = _____volume of each ab
2. Determined the amount of staining buffer (C.4)
   _____ samples x 1.1 x 50µL - [the sum of the volumes of all ab's added] = total volume of staining buffer to add.
3. Added the volume of staining buffer determined in D.2 to a tube, and then added the volume of each antibody determined in D.1 until all antibodies for that cocktail have been added.
4. Kept cocktails cold and protected from light.
5. Example: Lymphocyte cocktail
   Per 1 Sample:
   2.5µL CD56-PE
   2.5µL CD3-APC
   2.5µL CD19-PC7
   2.5µL CD45-eFluor450
   Total of 5 samples
   5 samples x 1.1 x 2.5µL/sample = 13.75µL of each antibody.
   5 samples x 1.1 x 50µL = 275µL – 55µL (sum of all antibody volumes)=220µL staining buffer

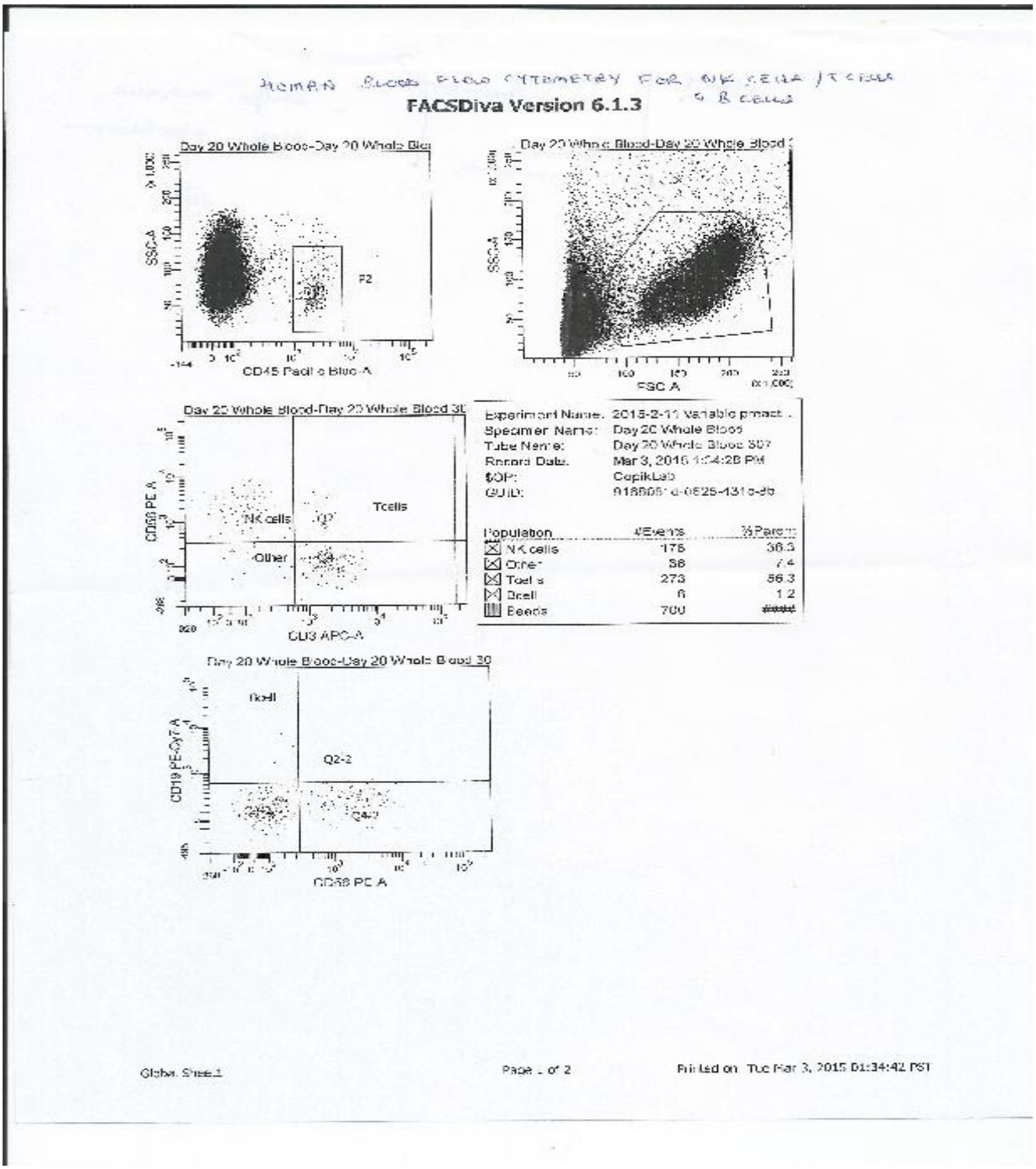
E. RBC Lysis Procedure
1. Transferred 50µL of whole blood to a 5mL BD tube (C.3) and added 500µL of RBC Lysis buffer (C.2) to it. Repeat in separate tubes for all samples.

2. Incubated samples for 15 minutes at room temperature.
3. Added 1 mL of cold DPBS to each tube and centrifuge at 200 x g for 4 minutes.
4. Aspirated supernatant and resuspend cells in 50μL of desired antibody cocktail (see D. for instructions on how to make cocktails).
5. Incubated for 30 minutes on ice protected from light.
6. Added 200μL of cold DPBS to each sample. Cells are ready to analyze on a flow cytometer

7. Important Notes:

1. Keep your antibodies and antibody cocktails cold and protected from light.
2. If you have multiple cocktails for each sample, repeat step 1 in separate tubes for each sample using 50μL of blood for each.
3. The amount of blood used for analysis can be increased. If so, make sure to maintain a 1:10 ratio of whole blood to RBC Lysis buffer and add twice as much cold DPBS as RBC lysis buffer after the 15 minute incubation.
4. If you notice a significant amount of red blood cells in the pellet at step E.3, aspirate the supernatant and add the same volume of RBC lysis buffer to the sample as was added in step E.1, incubating for only 7 minutes. Continue to follow the RBC lysis procedure starting at step E.3.

Graph of one blood sample for Tcells, B cells and NK cell :



3, DNA MICROARRAYS

A DNA microarray is a collection of microscopic DNA spots(oligos) attached to a solid surface. They can be used to measure the expression of levels of large numbers of genes simultaneously or to genotyoe multiple regions of a genomeor a whole genome . Each DNA spot contains a specific DNA sequence, which functions as a These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many gene expression analysis in parallel. Therefore arrays have dramatically accelerated many types of investigation. In standard microarrays, the probes are synthesized and then attached via surface engineering a solid surface by a covalent bond to a chemical matrix .The solid surface can be glass or a silicon chip, in which case they are colloquially known as an *Affy chip* when an Affymetrix chip is used. Other microarray platforms, such as Illumina , use microscopic beads, instead of the large solid support. Alternatively, microarrays can be constructed by the direct synthesis of oligonucleotide probes on solid surfaces. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.

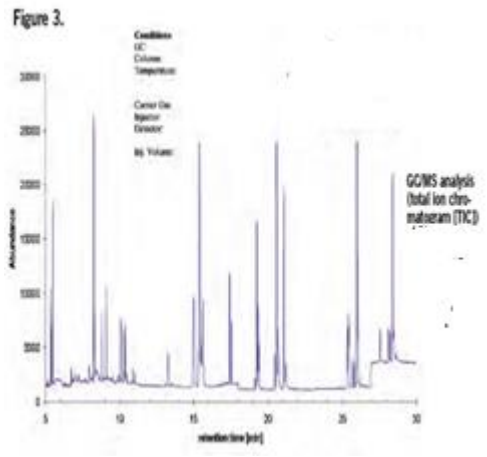
DNA microarrays can be used to measure changes in expression levels, to detect single cell nucleotide polymorpisms (SNPs), or for genotyping . Microarrays also differ in fabrication, workings, accuracy, efficiency, and cost.Additional factors for microarray experiments are the experimental design and the methods of analyzing the data

Steps of a basic DNA microarray

1: Isolation of the mRNA
2: Make cDNA by reverse transcription using fluorescent labeled nucleotides.
3: Apply the cDNA mixture to a microarray with a different gene in each spot. The microarray could be a whole genome microarray or a customized microarray
4: Rinse off excess DNA; scan for fluorescence.
5..A computer program will help analyse the data and check for hybridization areas. It will now give information of the mRNA expression of various genes in the particular sample

4.Gas chromatography–mass spectrometry (GC-MS) by Perkin Elmer : Used for fatty acids

Perkin Elmer instrument



Representation of data received after GCMS

is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio.

We used GC_MS by Perkin Elmer . Sebaic acid was used as internal standard. HpODE (hydroperoxy-9Z,11E-octadecadienoic acid) is produced by the oxidation of linoleic acid by lipoxygenase. 3 ml of ether was added .Nitrogen was used for drying the solvent methanol. Fatty acids were left as dry powder on the sides of the tube. These fattyacids were further dissolved in methanol which also donates the methy grp . PF3was used as catalyst .It was heated for 2 min at 90 ºC to make fatty acid volatile so that they can enter the gas phase .Cooled and hexane was added. Volatile fatty acids passed the GC and MS to appear as differentiated peaks which can be compared to known data programmed in GCMS for matched molecules

5.

Staining of Aorta to visualize calcium deposits

Representative stained slide

Von Kossa stain

Solutions and Reagents:

1% Aqueous Silver Nitrate Solution:
Silver nitrate ---------- 1 g
Distilled water ---------- 100 ml

5% Sodium Thiosulfate:
Sodium thiosulfate ---------- 5 g
Distilled water ---------- 100 ml

0.1% Nuclear Fast Red Solution:
Nuclear fast red ---------- 0.1 g
Aluminum sulfate---------- 5 g
Distilled water ----------100 ml
Dissolve aluminum sulfate in water. Add nuclear fast red and slowly heat to boil and cool. Filter and add a grain of thymol as a preservative.

Procedure:
1. **Deparaffinize paraffin sections and hydrate to water.**
2. **Rinse in several changes of distilled water.**
3. **Incubate sections with 1% silver nitrate solution in a clear glass coplin jar placed under ultraviolet light for 20 minutes (or in front of a 60-100 watt light bulb for 1 hour or longer). Note: If stain was weak or rinsed off in washing steps, it indicated the UV light was not strong enough. Longer staining is required for up to several hours.**
4. **Rinse in several changes of distilled water.**
5. **Remove un-reacted silver with 5% sodium thiosulfate for 5 minutes.**
6. **Rinse in distilled water.**
7. **Counterstain with nuclear fast red for 5 minutes.**
8. **Rinse in distilled water.**

9. Dehydrate through graded alcohol and clear in xylene.
10. Coverslip using permanent mounting medium.
Results:
Calcium salts ---------- black or brown-black
Nuclei ---------- red
Cytoplasm ---------- pink

Alizarin Red S staining

Fixation: Neutral buffered formalin or alcoholic formalin fixed, paraffin embedded tissue sections.
Positive Control: mouse embryo or a know calcium containing tissue sections
Solution and Reagents:
Alizarin Red Solution:
Alizarin Red S (C.I. 58005) ---------- 2 g
Distilled water ---------- 100 ml
Mix well. Adjust the pH to 4.1~4.3 with 10% ammonium hydroxide. The pH is critical, so make fresh or check pH if the solution is more than one month old.
Acetone (100%)
Acetone-Xylene:
Acetone (100%) ---------- 50 ml
Xylene ---------- 50 ml

Procedure:
1. De-paraffinize slides to distilled water.
2. Stain slides with the Alizarin Red Solution for 30 seconds to 5 minutes, and observe the reaction microscopically. Usually 2 minutes will produce nice red-orange staining of calcium.
3. Shake off excess dye and blot sections.
4. Dehydrate in acetone, 20 dips. Then in Acetone-Xylene (1:1) solution, 20 dips.
5. Clear in xylene and mount in a synthetic mounting medium.

Results:

Calcium deposits (except oxalate) -------- orange-red
This precipitate is birefringent.

ii) **Research results, including any papers,prepared/submitted for publication (Give in about 300 words)**

Proposed Paper (Rough draft)

Study of the MMP-9 and MCP-1 mRNA expression in Paroxonase 1 and ApoE double Knock-out mice as compared to healthy controls – A pilot study

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Introduction : . Atherosclerosis is a multifactorial and epidemic disease in which the lipids have been implicated as the main molecules involved in etiopathogenesis. Oxidative stress and inflammation are proposed to play supportive roles. We wished to study oxidative stress model of Paroxonase1 and Apo E gene knock-out mice to evaluate whether they would be prone to atherosclerosis even in the absence of high cholesterol chow or high cholesterol levels in the blood of these mice . After gross visualization of aorta , we wish to further study the mRNA

Expression of MMP-9 and MCP-1 in theses mice .Chemokines cause direct migration of inflammatory cells .MCP-1 (monocyte chemoattractant protein -1) promotes atherosclerosis by recruiting monocytes to the sub-endothelial layers . Matrix metalloproteinases (MMPs) form a family of zinc-dependent enzymes with proteolytic activity against connective tissue proteins such as collagen, proteoglycans, and elastin..MMP-9 also known as gelatinaseB or 92-kDa type IV collagenase is one of the MMPs found to be highly expressed in the vulnerable regions of atherosclerotic plaque and region of foam cell accumulation and may contribute to the remodeling processes associated with atherogenesis and plaque instability.

Aim : To study the mRNA expression of MMP-9 and MCP-1 in Paroxonase 1 and ApoE double Knock-out mice as compared to healthy controls

Material and methods : Paroxonase and ApoE double knock out mice (age 13-14 months) on normal chow diet were sourced from the animal facility of Burnetts school of Biomedical Sciences where they were co-breeded and maintained by Dr Sampath Parthsarathy's Lab. The mice were initially sourced from http://jaxmice.jax.org. Three ApoE-Paroxonase double knock-out male mice were sacrificed as per standardized protocol using Iso-flurane for anesthesia and further exposure to Carbon di-oxide .The mice were dissected and aorta exposed . The aortic arch was dissected out and frozen in liquid nitrogen till further studies . Before assay , aorta was homogenized, total mRNA extracted using trizol reagent from ambion .cDNA was synthesized using super-mix by invitrogen and further quantified by qPCR super-mix for icycler .Appropriate primers for MMP_9 and MCP-1 were used and quantification done on BIORAD CFX 96 Real Time System C1000 touch thermal cycler using appropriate protocol .

Results and Discussion

The aorta which was dissected out of these mice was grossly atherosclerotic as compared to normal controls

The relative expression of MCP-1 was 1+/- 0.06 SEM, 0.83+/-0.04SEM, 1.19+/- 0.05 SEM respectively in mice labeled AP 10,11 and 12. The values for MMP-9 was 1+/- 0.08 SEM , 0.59 SEM and 0.67 +/- 0.06 SEM respectively .

iii) **Proposed utilization of the experience in the Parent Institute. (Please specify the project developed whether originally proposed/ new project)** :

Project continues to be the one origionally proposed with few modifications which is as follows

' **1.Title** Oxidative stress and inflammatory biomarkers for evaluation of coronary atherosclerosis.

2.Background Current projections suggest that India will have the largest cardiovascular disease burden in the world[1,2,3,4]by 2020 .One fifth of the deaths in India are from coronary heart disease.By the year 2020, it will account for one third of all deaths[4,5] in IndiaAtherosclerosis is a multifactorial disease with genetic and environmental influences.Inspite of success in therapeutic lowering of LDL cholesterol , the epidemic of CAD continues to surge. The current atherogenic indices available are also not sensitive or predictive enough to be translated into patient care . Large-scale Studies all over the world have confirmed that low levels of HDL cholesterol is still the single best predictor of atherosclerosis risk. However, it still cannot evaluate or predict the extent of Atherosclerosis or plaque unstability . Coronary Angiography is a invasive procedure which is routinely done for this assessment

5.Aims and Objectives

To evaluate blood oxidative stress markers (Paroxonase , glutathione peroxidase, super-oxide dismutase) inflammatory markers (hsCRP ,TNFα , NFκB,IL-6) cell adhesion molecules (MMP-9) ,and markers of Insulin resistance (HOMA-IR) in atleast 1000 patients who have had a recent (within 2 days) Acute Coronary event and compare them to age and sex matched healthy controls

To do a extended lipid profile in all study population

To statistically evaluate whether any or a group of markers could have predicted or been associated with the acute coronary event

To do complete genome analysis (or a customized microarray)by DNA microarray of at least 10 patients with acute coronary event but without any/very few of the above biomarkers deranged. Same number of age and sex matched healthy controls will also be subject to DNA microarray analysis
To formulate a blood atherogenic indice involving these novel and relevant bio-markers which can conclusively highlight the atherogenic state in the body plus have the potential of serving as a better predictor of cardio-vascular risk .

Material and Methods
Patients will be enrolled from G.B.Pant hospital and L.H.M.C medical units
1000 patients suffering from a acute coronary in the past 2 days will be enrolled in the study after informed consent
Age and sex matched individuals with no history of a acute coronary event will be taken as controls
Detailed history and examination will be done for each patient with particular reference to diet , family history, BMI, and current medical illness including drug treatments
6 ml of blood will be taken from each of the study population
Extended lipid profile (Total cholesterol, HDL-C,LDL-C,VLDL-C,ApoA1,ApoE) levels will be done by automated methods in fully automatic analyser
Oxidative stress markers (Paroxonase, super-oxide dismutase, Glutathione peroxidase)will be done by ELISA/automated methods
Inflammatory markers (hsCRP ,TNFα , NFκB,IL-6) evaluated by ELISA
Cell adhesion molecules (MMP-9) ,and
Insulin levels will be done by chemiluminescence and markers of Insulin resistance (HOMA-IR) will be calculated
Adiponectin and leptin levels will be measured by ELISA
Phospholipase A2 and MCP1 levels will also be measured in peripheral blood
Measurement of platelets and their surface proteins by Flow cytometry

10 patients with family history of Acute Coronary Events and very few predisposing factors will be subject to whole genome/customized DNA microarray analysis and compared to age and sex matched healthy controls

Appropriate statistical methods will be applied to formulate a atherogenic indice which could give a association or prediction of a acute coronary event
.Expected Impact
Formulation of a atherogenic/thrombotic index inclusive of platelets ,oxidative and inflammatory biomarkers besides lipid levels . This has the potential of being used in patient care

Signature of Fellow