



Characterisation of circulating extracellular vesicles in human obesity

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Contents

List of contents.....	ii
Executive summary.....	ix
Acknowledgements.....	x
Publications and presentations.....	xi
List of Abbreviations.....	xii
List of Figures.....	xvi
List of Tables.....	xxi

Table of Contents

Chapter 1.....	1
Introduction	1
1.Extracellular vesicles.....	2
1.1 History.....	2
1.2 EVs nomenclature	2
1.3 EV biogenesis	5
1.3.1 Exosome secretion -the classical pathway.....	5
1.3.2 Microvesicle secretion- the direct pathway	6
1.4 EV content.....	8
1.4.1 EV proteins.....	8
1.4.2 Genetic material in EVs.....	11
1.4.2.1.RNA in EVs.....	11
1.4.2.2. DNA content of EVs.....	11
1.4.3 Lipids in EVs.....	12
1.5 EV uptake mechanisms and interaction with membrane receptors	12
1.6 EV isolation.....	13
1.6.1 EV isolation from blood	13
1.6.2 EV isolation - cell culture medium	14
1.6.3 EV isolation techniques.....	14
1.7 EV sample storage.....	16
1.8 EV characterisation methodology.....	17
1.8.1 EV morphology.....	17
1.8.2 EV concentration and size.....	17
1.8.3 EV content.....	18
1.8.3.1 ISEV recommendations.....	18
1.9 EV function.....	20
1.10 Potential application of EVs	23
1.10.1 EV role as biomarkers	24
1.10.2 EVs’ role in pharmacotherapy.....	24
1.10.2.1 Modulation of EV secretion	24
1.10.2.2. EVs used as pharmacotherapy vectors	25
2. Adipose tissue	27
2.1 Overview	27
2.2 AT classification.....	27
2.3 Adipose tissue turnover	29

2.4. AT function.....	30
2.4.1 Energy storage	30
2.4.2 Adipose tissue as a secretory organ	31
2.5 AT dysfunction and obesity- overview.....	39
2.6 Obesity	41
2.6.1 Epidemiology.....	41
2.6.2 Pathophysiology.....	41
2.6.3 Obesity and inflammation	42
2.6.4. Adiposity and atherosclerosis	44
2.6.5 Metabolic syndrome and cardiovascular disease in obesity	44
2.6.6. Obesity and Cancer	45
2.7 Obesity management.....	46
2.7.1 Lifestyle modification.....	46
2.7.2 Pharmacotherapy for obesity	46
2.7.3 Bariatric surgery.....	49
2.7.4 Faecal microbiota transplantation.....	50
2.8 Study hypothesis and aims	50
Chapter 2.....	52
General Methods	52
2.1 Study approvals.....	53
2.2 Study support.....	53
2.3 Study participants	53
2.4 Anthropometric measurements	53
2.5 Isolation and characterisation of plasma-derived extracellular vesicles (EVs).....	54
2.5.1 Differential ultracentrifugation.....	54
2.5.1.1 Background	54
2.5.1.2. Experimental methodology	54
2.5.2 Size exclusion chromatography columns.....	55
2.5.2.1 Background	55
2.5.2.2 Experimental methodology	56
2.5.3 Storage of extracellular vesicles	60
2.5.4 Size and concentration analysis of extracellular vesicles	60
2.5.4.1 Background	60
2.5.4.2 Experimental methodology	62
2.5.5.Immunophenotyping of extracellular vesicles.....	63
2.5.5.1 Background	63

2.5.5.2 Experimental methodology	64
2.5.6 Electron microscopy.....	68
2.6 Plasma measurements.....	70
2.6.1 Measurement of plasma adipocytokines.....	70
2.6.1.1 Human plasma FABP4.....	70
2.6.1.2 Human Plasma PPAR γ	70
2.6.1.3 Plasma IL6, interferon γ , TNF α and adiponectin.....	71
2.6.3 Plasma insulin measurement.....	74
2.6.4 Plasma glucose measurement	74
2.6.5 HOMA-IR	74
2.7 Protein concentration	74
2.8 The isolation and characterisation of adipocyte-derived EVs (ADEVs) from primary adipocyte culture.....	75
2.8.1. Sample preparation	75
2.8.2 Commercial preadipocyte culture	79
2.8.3 Adipocyte-derived EV isolation.....	81
2.8.3.1 Differential centrifugation	81
2.8.3.2 Size exclusion chromatography columns.....	82
2.8.4 ADEV concentration and size	82
2.8.5 Proteome Profiling.....	82
2.8.6 Oil Red O staining.....	84
2.9 Statistical analysis	85
Chapter 3.....	86
Characterisation of circulating extracellular vesicles and plasma adipocytokines in healthy lean and obese subjects.....	86
3.1 Introduction	88
3.2 Aims.....	89
3.3 Scientific question/hypothesis.....	89
3.4 Methods.....	90
3.4.1 Recruitment/ Ethics	90
3.4.2 Anthropometric measurements	91
3.4.2.1. BMI (Body Mass Index)	91
3.4.2.2 Waist: Hip Ratio	91
3.4.3 Blood Pressure Measurement	91
3.4.4 Venepuncture	91
3.4.5 Plasma analysis	91

3.4.5.1 Fasting glucose.....	91
3.4.5.2 Fasting insulin	92
3.4.5.3 HOMA-IR	92
3.4.5.4 Plasma adipokines.....	92
3.4.6 EV isolation and analysis.....	92
3.4.6.1 EV isolation	92
3.4.6.2 EV concentration and size.....	92
3.4.6.3 EV cellular origin and content.....	92
3.4.7 Statistical analysis	93
3.5 Results.....	93
3.5.1 Baseline anthropometric and metabolic characteristics of the healthy cohort.....	93
3.5.2 Glucose homeostasis	95
3.5.3 Circulating plasma cytokine and adipokine profile.....	95
3.5.3.1 Interleukin 6.....	96
3.5.3.2 TNF α	96
3.5.3.3 Interferon γ	96
3.5.3.4 Adiponectin.....	96
3.5.3.5 Fatty Acid Binding Protein 4.....	98
3.5.3.6 PPAR γ	99
3.5.4 Characterisation of circulating plasma EVs in the healthy population.....	100
3.5.4.1 EV concentration and size distribution.....	100
3.5.4.2. EV cellular origin	102
3.4.3.3 EV adipocytokine expression	103
3.5.4.4 Expression of the exosomal marker CD9	108
3.6 Discussion.....	110
3.6.1 Anthropometric measurements and glucose metabolism	110
3.6.2 Plasma adipocytokines.....	111
3.6.2.1 Plasma IL6	111
3.6.2.2. Plasma TNF α	112
3.6.2.3. Plasma Adiponectin	112
3.6.3 Circulating plasma EVs in healthy volunteers.....	115
3.6.3.1 Plasma EVs and obesity.....	116
3.6.3.2 Plasma EVs and gender.....	117
3.6.4 Exosomes as carriers of adipokines	118
3.7 Summary	119

3.7.1 Study limitations	119
3.7.2 Study strengths	119
3.7.3 Conclusions	119
Chapter 4.....	121
Characterisation of cell-derived extracellular vesicles in morbidly obese individuals undergoing bariatric surgery.....	121
4.1 Introduction	123
4.2 Aims.....	124
4.3 Methods.....	124
4.3.1 Study population.....	124
4.3.2 Types of intervention	126
4.3.3. Study design.....	127
4.3.4 Ethical approval.....	128
4.3.5 Clinical measurements.....	128
4.3.6 Sample collection	128
4.3.7 EV isolation and characterisation	128
4.3.8 Plasma measurements.....	128
4.3.9 Statistical analysis	129
4.3.9.1 Sample size.....	129
4.3.9.2 Statistical tests	129
4.3.10 My involvement	129
4.4 Results.....	130
4.4.1 Population characteristics.....	130
4.4.2 EV concentration, cellular origin and content	132
4.4.3 Plasma FABP4.....	139
4.4.5 Additional analysis	141
4.5 Discussion.....	142
4.6 Study limitations	144
4.7 Conclusions	145
Chapter 5.....	146
The effects of a multidisciplinary weight loss programme on circulating extracellular vesicles.....	146
5.1 Introduction	148
5.2 Aims.....	149
5.3 Methods.....	150
5.3.1 Study design.....	150
5.3.2 Study population.....	151

5.3.3 Ethical approval.....	152
5.3.4 Anthropometric measurements	152
5.3.4 Sample collection, processing and storage.....	152
5.3.5 EV isolation, measurement and analysis.	152
5.3.5.1 Size exclusion chromatography columns.....	152
5.3.6 Plasma adipokine and cytokine concentration.....	155
5.3.7. Other plasma measurements	155
5.3.7 Evaluation of overall wellbeing/health.....	155
5.3.8 Statistical analysis	155
5.3.9. Sample size and power calculations	155
5.4 Results.....	157
5.4.1 Baseline and follow-up anthropometric characteristics, glucose, HbA1c and lipids.....	157
5.4.2 Measurement of psychological well being	158
5.4.3 Plasma adipocytokines.....	158
5.4.3.1. Plasma IL-6	158
5.4.3.2 Plasma adiponectin.....	159
5.4.3.3. Plasma TNF α	160
5.4.3.4 Plasma FABP4.....	161
5.4.4 The effects of lifestyle intervention on circulating plasma EVs concentration, cellular origin and profile.....	163
5.4.5. Differences in EV profile between morbidly obese subjects and healthy volunteers.....	170
5.4.6 Changes in pre- and post-lifestyle intervention in plasma EV profile isolated by size exclusion chromatography	173
5.5 Discussion.....	178
5.5.1 Lifestyle intervention and circulating EVs.....	178
5.5.2 Differences in EV profile between healthy volunteers and morbidly obese subjects.....	182
5.5.3. Effects of methodology on EV analysis – quality vs quantity?.....	183
5.6 Conclusions	186
Chapter 6.....	187
Evaluation of <i>ex vivo/in vitro</i> human adipocyte cultures for adipocyte-derived EV characterisation	187
6.1 Introduction	189
6.2 Aims.....	189
6.3 Methods.....	190
6.3.1 Adipose tissue harvesting	190
6.3.2 Ethical approval.....	190
6.3.3. General methods	190

6.3.3.1 Cell culture	190
6.3.3.2 Adipocyte-derived EV isolation.....	190
6.3.3.3 Characterisation of ADEVs	190
6.4 Results	191
6.4.1 Subcutaneous and visceral preadipocyte culture: methodological observations.....	191
6.4.2 The culture of commercial visceral preadipocytes – methodological observations	195
6.4.3 ADEVs concentration and size	197
6.4.4 The use of SEC columns	201
6.4.5 Evaluation of ADEV content.....	205
6.5 Discussion.....	211
6.5.1. Characteristics and function of ADEVs.....	211
6.5.2 Methodological pitfalls, limitations and future considerations.....	216
6.6 Conclusions	218
Chapter 7.....	219
Conclusions and future directions	219
7.1 Relevance of research undertaken	220
7.2 Contribution of my findings to the field of EV research	220
7.3 Suggestions for further research	222
7.3.1 Developments in plasma EV isolation and characterisation in obese cohorts	222
7.3.2 Development of Adipocyte-derived EV isolation and characterisation.....	223
7.3.3. Functional studies: why do these matter?	223
7.4 Conclusions	226
References	227
Appendix	265

Executive summary

Introduction

Extracellular vesicles (EVs) are submicron vesicles released by most cells. They contain protein, enzymes and microRNA of the donor cells and are believed to play a role in paracrine communication. Circulating EVs might reflect heightened inflammatory status in obese individuals and play a role in chronic low grade inflammation associated with obesity.

Aims

To compare circulating plasma EVs between healthy volunteers across a range of BMI and morbidly obese individuals attending a multidisciplinary weight loss clinic, and to assess the effects of lifestyle changes and bariatric surgery on the circulating EV profile. Finally, to characterise EVs derived from subcutaneous and visceral fat depots.

Methods

Plasma EVs were isolated by differential centrifugation. EV cellular origin (platelets CD41, monocytes/macrophages CD11b, erythrocytes CD235a, endothelial cells CD144) and adipocytokine expression (IL6, TNF α , interferon γ , adiponectin, FABP4, PPAR γ) were evaluated by TRF immunoassay.

Results

Circulating EV profile and concentration in metabolically healthy volunteers was unaffected by BMI (all $p=ns$). The EV profile in healthy men was more pro-inflammatory compared to women, with higher EV-expressed CD41, CD144, IL-6, interferon γ and FABP4 (all $p<0.05$). This was accompanied by lower plasma adiponectin concentration in males (128 $\mu\text{g}/\text{mL}$ vs. 272.3 $\mu\text{g}/\text{mL}$, $p<0.005$). Plasma FABP4 correlated strongly with BMI ($r=0.91$, $p<0.005$) and was lower in healthy lean versus obese individuals (13.5(6.4) vs 23.8(6.4) ng/mL , respectively ($p<0.05$)).

Dietary and lifestyle management affected the EV profile, with lower signals observed from platelet-derived EVs ($p<0.05$) as well as FABP4-, TNF α - and Interferon γ -expressing EVs at 6 months' follow-up ($p<0.05$, $p=0.05$, $p=0.06$, respectively). Bariatric surgery led to transient increase in EV and plasma FABP4 at 1 month ($p<0.05$) which is likely secondary to increased lipolysis during that period. The exosomal marker CD9 correlated with FABP4, interferon γ , adiponectin and TNF α ($r=0.49$, $r=0.41$, $r=0.59$, $r=0.53$, all $p<0.05$), suggesting that exosomes are the main carriers of these adipokines.

Adipocyte-derived EVs from both visceral and subcutaneous fat depots were found to express numerous adipokines involved in various biological processes and molecular pathways.

Conclusion

EVs can be regarded as diverse biological vectors playing an important role in regulation of adipose tissue homeostasis and inflammatory processes. Their concentration, cellular origin and content do not directly correlate with BMI but are affected by gender and the presence of obesity-driven comorbidities.

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Presentations

Posters:

- **Gender-related differences in circulating microparticle characteristics: implications for cardiovascular risk?** (40 highest scoring posters authored by Early Career Endocrinologists) at British Endocrine Society 2015 Meeting, Edinburgh, UK
- **Differences in circulating extracellular vesicles between healthy volunteers and patients with established erectile dysfunction: do endothelial microvesicles play an ambivalent role?**, UK Extracellular Vesicles Interest Group Meeting (UKEV) 2015, Cardiff UK
- **Fatty Acid Binding Protein (FABP4) concentration in plasma is an important early biomarker of metabolic dysfunction**, Diabetes UK, 08-10.03.17, Manchester, UK
- **Extracellular Vesicles (EVs)-associated and plasma FABP4 fluctuations following bariatric surgery**, International Society of Extracellular Vesicles Conference, Toronto, Canada, 18-20.05.17
- **Alterations in concentration and characteristics of circulating extracellular vesicles in morbid obesity**. British Endocrine Society Meeting, Harrogate, November 2017
- **Effects of a multidisciplinary lifestyle management programme on circulating extracellular vesicles: implications for cardiovascular risk**. Diabetes UK, 14-16.03, London, UK
- **Cell-derived extracellular vesicles as important intercellular messengers in obesity**. ESE, 19-22.05.18, Barcelona, Spain (selected for guided poster tour)

Oral presentations:

- **Changes in adipocyte-derived extracellular vesicles following bariatric surgery** (abstract nominated for Young Diabetologist and Endocrinologist Travel Award), Diabetes UK, 08.03.17, Manchester, UK
- **Differences in Circulating Extracellular Vesicles Between Morbidly Overweight Individuals and Healthy Volunteers**. 5th European Young Endocrine Scientists Meeting, 08-10.09.17, Porto, Portugal
- **Cell-derived extracellular vesicles as important intercellular messengers in obesity**. Welsh Endocrine and Diabetes Society Meeting, 05.10.17, Miskin, UK

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Abbreviations

A

ADEV	Adipocyte derived extracellular vesicles
ALIX	ALG-2 interacting protein X
AMPK	Adenosine monophosphate-activated protein kinase
AT	Adipose tissue
ATP	Adenosine triphosphate
a.TRF	Arbitrary TRF units

B

BAT	Brown adipose tissue
BMI	Body mass index
BP	Blood pressure
BPD	Biliopancreatic diversion
BSA	Bovine serum albumin

C

Ca ²⁺	Calcium
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CRP	C-reactive protein
CVD	Cardiovascular disease

D

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DUBASCO	Dudley Bariatric Surgery Comorbidity Score

E

EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport

EV	Extracellular vesicle
F	
FABP	Fatty acid binding protein
FC	Flow cytometry
FCS	Foetal calf serum
FDA	Federal Drug Agency
FFA	Free fatty acid
FGF	Fibroblast Growth Factor
G	
GB	Gastric banding
GH	Growth Hormone
GLUT	Glucose transporter
H	
HDL	High density lipoprotein
HIF	Hypoxia-inducible factor
HOMA	Homeostatic model assessment
HUVEC	Human umbilical vein endothelial cells
HV	Healthy volunteers
I	
IGT	Impaired glucose tolerance
IL	Interleukin
ILV	Intraluminal vesicle
IR	Insulin resistance
ISEV	International Society of Extracellular Vesicles
K	
kDa	kilodaltons
L	
LDL	Low density lipoprotein
M	
MCP	Monocyte chemoattractant protein

MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
mL	millilitre(s)
μL	microlitre(s)
mm	millimetre(s)
μm	micrometre(s)
mmHg	millimetres of mercury
MMP	Matrix metalloproteinase
MO	Morbidly obese
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MVB	Multivesicular body
N	
NICE	National Institute for Health and Care Excellence
NK	Natural killer
nm	nanometre(s)
NTA	Nanoparticle tracking analysis
O	
O ₂	Oxygen
OD	Optical density
OSA	Obstructive sleep apnoea
P	
PAI	Plasminogen activator inhibitor
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PFP	Platelet-free plasma
PPAR	Peroxisome proliferator-activated receptor
PPP	Platelet-poor plasma
PREF	Preadipocyte factor
PS	Phosphatidylserine

R

RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT	Room temperature

S

S-AT	Subcutaneous adipose tissue
SEC	Size exclusion chromatography
SG	Sleeve gastrectomy
SGBS	Simpson-Golabi-Behmel Syndrome

T

TEM	Transmission electron microscopy
TF	Tissue factor
TG	Triglycerides
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRF	Time resolved fluorescence
TSG	Tumor susceptibility gene
TSH	Thyroid Stimulating Hormone
TWL	Total weight loss
T2D	Type 2 diabetes

U

UCP	Uncoupling protein
-----	--------------------

V

V-AT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor

W

WAT	White adipose tissue
WHR	Waist:Hip Ratio
WHO	World Health Organisation
WIMOS	Welsh Institute of Metabolic and Obesity Surgery

List of figures

Fig.1.1: Summary of EV nomenclature	4
Fig.1.2: Extracellular vesicle generation pathways.....	8
Fig.1.3: Development of metabolic complications of obesity.	40
Fig.2.1: Isolation of EVs by differential centrifugation.....	55
Fig.2.2: Schematic principle of SEC columns.....	57
Fig.2.3: Validation experiment of plasma EV isolation from healthy donors:	59
Fig.2.4: NTA operation	60
Fig.2.5: The modified Stokes-Einstein equation.	61
Fig.2.6: The principle of TRF using a long half-life fluorophore.....	63
Fig. 2.7: TRF Immunoassay:.....	67
Fig.2.8: TEM images of plasma-derived EVs isolated (fresh) by differential centrifugation from 3 healthy donors (a-c)	69
Fig.2.9: Schematic illustration(a) and photograph (b) of separation process during preadipocyte isolation following adipose tissue digestion and 1500rpm centrifugation.....	76
Fig.2.10: Timeline of primary adipocyte culture experiments from subcutaneous and visceral fat depots	80
Fig.2.11: Differential centrifugation steps used to isolate adipocyte derived EVS (ADEVs).....	81
Fig.2.12: Example of a result obtained following incubation of visceral adipose tissue derived EVs obtained at day 21 of differentiation using the adipokine proteome profiler assay.	84
Fig.3.1: The BMI distribution: a) percentage BMI distribution in the healthy female cohort b) percentage BMI distribution in the male cohort	95
Fig. 3.2 Spearman correlation analysis of plasma adiponectin and EV concentration/mL of plasma (a) and EV expressed cellular markers (b,c) and adipokines (d-g).....	97

Fig.3.3. a) Plasma FABP4 concentration (mean (SD)) in healthy lean, overweight and obese volunteers. b) Spearman analysis of correlation between plasma FABP4 and BMI.	99
Fig.3.4 a-b Differences in size distribution of plasma EVs between male and female healthy volunteers. Male subjects had significantly higher EV concentration within the exosomal range (100-200nm in diameter).	101
Fig.3.5 a) Expression of cell of origin markers (CD41 platelets, CD11b monocyte/macrophages, CD235a erythrocytes, CD144 endothelial cells) in plasma EVs isolated from healthy lean, overweight or obese individuals. b) EVs adipocytokine expression in lean, overweight and obese individuals ..	105
Fig.3.6 a) Effects of gender on expression of cell-of-origin markers (CD41 platelets, CD11b monocyte/macrophages, CD235a erythrocytes, CD144 endothelial cells). b) Effects of gender on EV adipokine expression.	107
Fig.3.7: Spearman correlation analysis of the exosomal marker CD9 with EV-expressed interferon γ , adiponectin and FABP4 (a-c).....	109
Fig.4.1: EV concentration/mL plasma and size distribution at 3 timepoints	133
Fig.4.2: Bariatric surgery did not alter EV cellular origin	134
Fig.4.3: Median EV expression of CD9, adiponectin, TNF α , interferon- γ , IL-6 and PPAR γ ,	135
Fig. 4.4: a) Changes in EV FABP4 (median with IQR) reveal significantly lower TRF signal at 6 months' follow-up compared to 1-month (p<0.05) b) Individual pattern of changes in EV FABP4 TRF signal for all study participants at 3 timepoints c) Median EV FABP4 signal was significantly lower in the cohort that underwent biliopancreatic bypass diversion surgery at 6 months' follow-up . Spearman's correlation analysis revealed significant correlation between the exosomal marker CD9 and EV-derived: FABP4 (d), adiponectin (e), TNF α (f) and interferon γ (g).....	138
Fig.5.1: Isolation of EV fractions using Exo-Spin Midi Columns (Cell Guidance Systems) a) Loading of 1mL of plasma b) Collection of early fractions c) Collection of EV-rich fractions.....	154

Fig.5.2: Differences in plasma IL-6 concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (HV)(n=40) and morbidly obese (MO) patients (n=21),.....	159
Fig.5.3: Differences in plasma adiponectin concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (n=40) and morbidly obese patients (n=21).....	160
Fig.5.4: Differences in plasma TNF α concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (n=40) and morbidly obese patients (n=21).....	161
Fig.5.5: Differences in plasma FABP4 concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (n=40) and morbidly obese patients (n=21),.....	162
Fig.5.6: a) Mean EV concentration/mL of plasma pre-and post-lifestyle intervention did not differ (p=0.6); b) and c) The size distribution analysis by two-way ANOVA did not reveal any differences in EV concentration/mL between the timepoints when analysed 'bin by bin' (b) or grouped in 100nm intervals (c).....	164
Fig.5.7: Expression of EV cellular origin markers (CD41:platelets, CD11b:monocytes/macrophages, CD235a: erythrocytes, CD144: endothelial cells) and exosomal markers (CD9 and CD63) at baseline and 6 months after lifestyle intervention.....	166
Fig.5.8: Changes in EV-expressed FABP4 at baseline and 6 months following lifestyle intervention	167
Fig. 5.9. EV expressed TNF α , interferon γ , adiponectin and PPAR γ at baseline and 6 months following lifestyle intervention.....	168
Fig.5.10: Differences in EV concentration/mL of plasma between healthy volunteers (n=49) and morbidly obese individuals (n=21).....	170
Fig. 5.11: Comparison of median (ranges) expression of markers of EV cellular origin between healthy volunteers (HV, n=49) and morbidly obese subjects (MO, n=21)	171

Fig.5.12: Differences (median(range)) in EV-expressed adipocytokines (TNF α , interferon- γ , FABP4 and PPAR γ) between healthy volunteers (n=49) and morbidly obese subjects (n=15)	172
Fig.5.13: Distribution of TRF signal for main EV cellular origin markers (CD41-platelets, CD11b-monocytes/macrophages, CD235a-erythrocytes, Cd144-endothelial cells) in baseline and 6 months follow up EV samples isolated by differential ultracentrifugation (UC) (a) and size exclusion chromatography columns (SEC) (b)	174
Fig.5.14: Differences in EV cell of origin and exosomal markers pre-and post- lifestyle intervention using Exo Spin columns for EV isolation (n=8).....	175
Fig.5.15: Differences in EVs expression of adipocytokines pre-and post-lifestyle intervention using Exo Spin columns for EV isolation (n=8).	177
Fig.6.1 Visceral preadipocyte cultures at day 0 (a,b) and day 21 (c,d) of differentiation.....	192
Fig. 6.2: Subcutaneous preadipocyte cultures at day 0 (a,b) and day 21 of differentiation (c,d)	193
Fig.6.3: Oil Red O absorbance of subcutaneous (a) and visceral (b) adipocyte cultures measured at day 0 and day 21.	194
Fig.6.4: Visceral preadipocytes culture from a healthy (control) donor at day 0 (a-b) and day 21 (c-d); from the diabetic donor: day 0 (e) and day 21 (f) and from the donor with diabetes and heart disease: day 0 (g), day 21 (h)	196
Fig.6.5: Oil Red O absorbance signals for healthy (control) adipocytes at day 0 and day 21.....	197
Fig.6.6: Size distribution general (a-b) and in 100nm intervals (c-d) of EVs obtained from visceral and subcutaneous adipose tissue explants at day 0 and day 21 of differentiation (n=5).	199
Fig.6.7: Overall size distribution (a,c, f) and in 100nm intervals (b,d,f) at day 0 and day 21 of EVs obtained from culture of commercial visceral preadipocytes from various donors: healthy(control) a-b, diabetic c-d and diabetic with heart disease e-f.....	200
Fig.6.8. Analysis of protein concentration (mg/mL) by Nanodrop and nanoparticle concentration/mL by NTA of 6 fractions obtained from EXOspin columns revealed a peak of EVs in fraction 2 and 3 in	

(a) EVs isolated from visceral preadipocyte medium at day 0, and (b) EVs isolated from visceral adipocyte medium at day 21, (n=3).....	202
Fig. 6.9. Differences in mean EV concentration pre- and post-SEC column at day 0 and day 21 of differentiation for 3 different visceral ADEVs samples. 1-3 represent paired samples from 3 different donors.....	204
Fig.6.10: Heatmap of adipokines expression presented as mean pixel density for subcutaneous and visceral ADEVs at day 0 and d21 (n=4).....	206
Fig.6.11: Spearman correlation analysis of 58 ADEV expressed adipokines at day 0 and day 21: a) subcutaneous ADEVs day 0 vs day 2; b) visceral ADEVs day 0 vs d21; c) subcutaneous vs visceral EVs at day 0; d) subcutaneous versus visceral EVs day 21.	207
Fig.6.12: Functional enrichment analysis by FunRich of visceral ADEV-expressed proteins' involvement in various biological processes (a-b) and molecular pathways (c-d); a,c-day 0 , b,d- d21 of differentiation (n=5).	208
Fig.6.13: Functional enrichment analysis by FunRich of subcutaneous ADEV-expressed proteins' involvement in various biological processes (a-b) and molecular pathways (c-d); a,c-day 0 , b,d- d21 of differentiation(n=4).....	215
Fig.6.14: Functional enrichment analysis by FunRich of Lonza control non-diabetic ADEV-expressed proteins' involvement in various biological processes (a-b) and molecular pathways (c-d).....	216

List of Tables

Tab.1.1 Common markers used in EV analysis of biological fluids	10
Tab.1.2: AT secreted molecules and their biological relevance	32
Tab.2.1: Pre- and post- analytical settings used for EV sample analysis by NTA.....	62
Tab. 2.2: Primary antibodies used in the EV immunophenotyping by TFR.....	66
Tab.2.3: The specifications of commercially available Abcam kits used for estimation of plasma concentration of IL6, TNF α , interferon γ and adiponectin.	72
Tab.2.4: The concentration of standards used in the Abcam assay kits used for measurement of plasma IL6, TNF α , interferon γ and adiponectin.....	73
Tab.2.5: Ingredients and their respective volumes required to prepare 200mLs of complete medium with 10%FCS and 100mLs of x2 differentiation medium mix.	78
Tab.2.6: Alphabetical list of adipokines detected using the Proteome Profiler Assay.	83
Tab.3.1: Anthropometric and metabolic characteristics of the healthy volunteer cohort.	94
Tab.3.2: Spearman correlation analysis between EV concentration/mL of plasma and other evaluated parameters.....	102
Tab.4.1: The Swansea modified DUBASCO score.	125
Tab.4.2: Anthropometric and metabolic characteristics of patients undergoing bariatric surgery at baseline, 1- and 6-months' follow-up.....	131
Tab. 5.1: Summary of anthropometric and metabolic variables pre-and post-intervention of the sub-cohort that completed the study.....	158
Tab.5.2: Comparison of EV concentration, cellular origin and adipokine content at baseline between participants with T2DM and those with no diabetes.	169
Tab.6.1: Summary of manuscripts on ADEVs in murine and rodent models to date in chronological order	215

Chapter 1

Introduction

1. Extracellular vesicles

1.1 History

Extracellular vesicles (EVs) are a heterogeneous group of submicron vesicles released by most cells during the normal cell cycle as well as during cell stress. Although EVs were first reported over 70 years ago, they have only gained more attention over the last 3 decades as we learnt more about their mechanisms of secretion and multiple biological roles.

The first report of EVs dates back to 1946 when Chargraff and West [Chargraff E, 1946] reported platelet-derived particles in normal plasma, later described by Wolf as 'platelet dust' [Wolf P, 1967]. However, the significance of these findings was not clear at the time. In 1987, an exosomal secretion pathway from multivesicular bodies was first described from reticulocytes [Johnstone RM, 1987]. A decade later, evidence emerged that these vesicles are also released from antigen presenting cells (B lymphocytes) which triggers antigen-specific MHC class II- restricted T cell responses [Raposo G, 1996]. This implicated exosomes in playing a role in immune responses. EVs have since been isolated from most biological fluids including blood, urine, cerebrospinal fluid, bile, saliva, synovial fluid, semen and amniotic fluid [Yanez-Mo, M 2015]. They have been found to contain membrane and cytoplasmic components of their parental cells, including proteins, enzymes and cytokines. [Yanez-Mo, M, 2015] When functional RNA was detected within EVs (evRNA) just over a decade ago [Ratajczak J, 2006], the interest in EVs rose exponentially, and they have since been recognised as biological vectors that can influence gene expression in recipient cells [Valadi H, 2007].

The International Society of Extracellular Vesicles (ISEV) was founded in 2011 and is now established as a scientific reference body which evaluates and promotes EV research. Currently, the role of EVs as biological messengers involved in numerous physiological and pathological processes is widely acknowledged and the main focus of current research in this area is on improvement and standardisation of EV isolation and separation methodology, and this will point the way to potential clinical applications.

1.2 EVs nomenclature

EVs are a heterogeneous group but can be divided broadly into 3 major subgroups based on their size and mode of secretion: exosomes (<150nm in diameter) originating from multivesicular bodies, microvesicles (150nm-1µm in diameter) released by outwards budding of the plasma membrane, (Fig.1.1) and oncosomes and apoptotic bodies originating from cells undergoing apoptosis (1-5µm in

diameter). The latter are not evaluated by the research in this thesis hence will not be described in detail. The division of EV subtypes based on size is arbitrary as they do overlap. Moreover, the commonly reported exosomal markers such as CD9 and CD63 are also present in larger EVs [They C, 2017]. Additionally, it must be noted that in reality biological fluids are a mixture of various EVs secreted by cells and at the time of undertaking the research in this thesis no reliable method of isolation of only one EV subtype exists.

The mechanisms controlling the sorting of EV protein and RNA content are not yet fully understood. They exert their effects on target cells via receptor-ligand interaction but EVs can also be internalised via phagocytosis and micropinocytosis. The circulating EV concentration is believed to depend on the balance between generation and clearance; the latter is believed to mainly occur via uptake into target cells. Therefore, EV biodistribution and half-life varies depending on the cell of origin and the proximity of the target tissue [Yanez-Mo M, 2015].

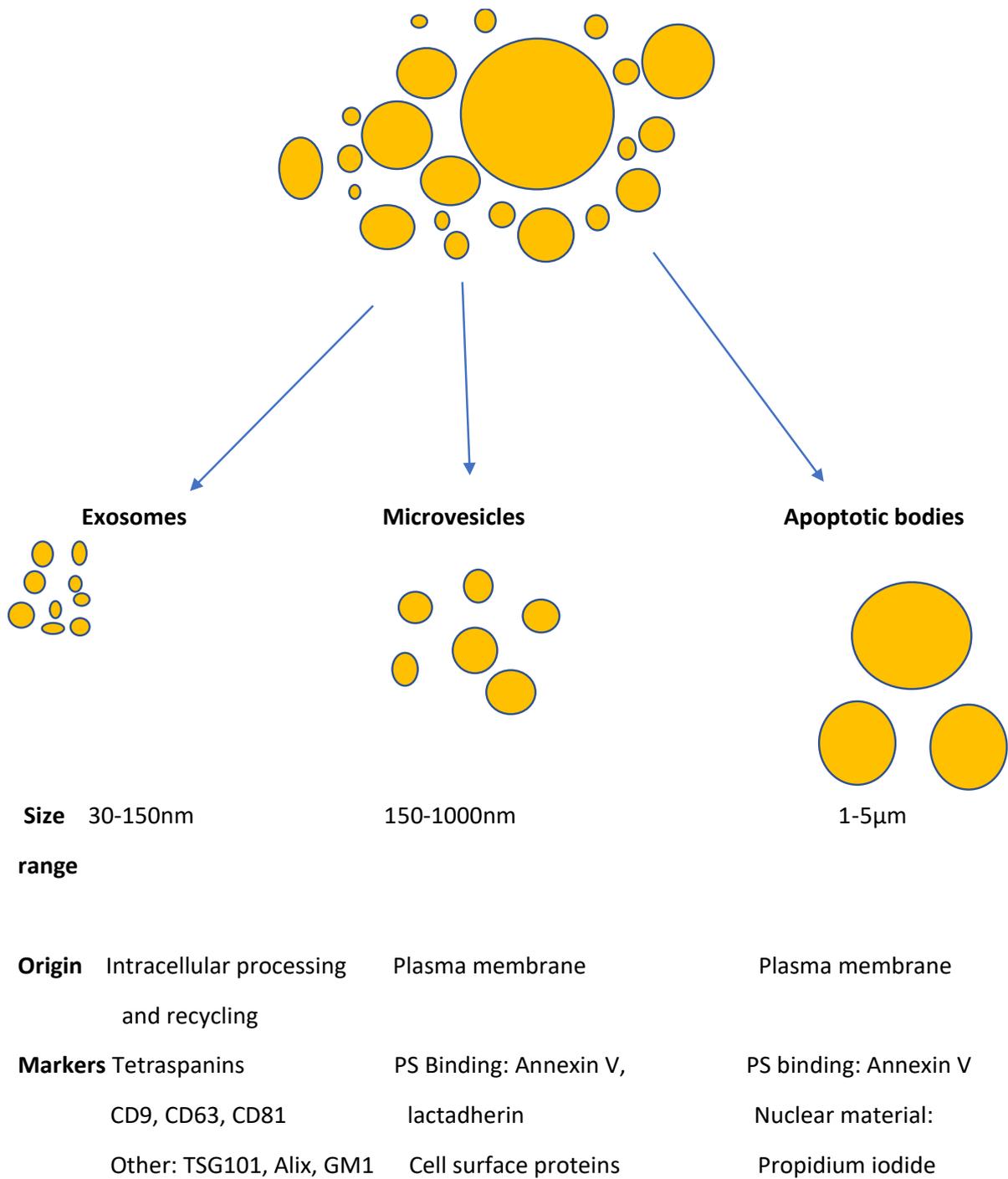


Fig.1.1: Summary of EV nomenclature.

1.3 EV biogenesis

EVs differ not only in size but also in the mode of generation. There are 2 major pathways involved in EV formation: the classical pathway for exosome generation and the direct pathway where microvesicle budding occurs.

1.3.1 Exosome secretion -the classical pathway

This pathway forms part of the endocytic pathway which regulates the expression of cell surface receptors which are internalised and degraded or recycled depending on the biological circumstances [Vanlandingham PA, 2009]. Briefly, in this process an intracellular vesicle is formed firstly by invagination of the plasma membrane and endocytosis of the membrane proteins and surrounding material. Next, intraluminal vesicles (ILVs) are generated by an inward budding of the intracellular vesicle membrane. This process is regulated by the Endosomal Sorting Complex Required for Transport (ESCRT) [Colombo M, 2014]. There are 4 different ESCRT complexes which regulate different functions: ESCRT complexes 0, I and II are involved in recognition and ubiquitination of the endocytosed proteins, whilst ESCRT III complex (Alix, TSG101 (tumour susceptibility gene 101)) regulates the inward budding and scission of the ILVs, which leads to formation of multivesicular bodies (MVBs) [Colombo M, 2014, Gruenberg J, 2004]. The Alix protein plays an important role by simultaneously binding to the TSG101 component of the ESCRT-I complex and CHMP4 (charged multivesicular body protein 4) [McCollough J, 2008], a component of ESCRT-III. MVBs can then fuse with the plasma membrane leading to exosome release or they can be degraded by lysosomes. The exact pathways behind these processes are still being researched as it is not clear what mechanisms determine the sorting of MVBs into those that are degraded versus those that are released from the cells.

Interestingly, there are some reports suggesting that ubiquitination is not necessary for sorting the proteins into ILVs. Baietti *et al* described involvement of syndecan protein in ESCRT III-driven exosome generation [Baietti MF, 2012]. A cytosolic protein called syntenin was shown to connect syndecan to Alix protein and clustering of syndecan by heparanase drives syntenin-ALIX-ESCRT-mediated sorting and exosome generation [Roucourt B, 2015]. Intriguingly, this pathway mainly stimulates selective incorporation of CD63 into exosomes, rather than CD9 or CD81 and is regulated by the small GTPase ADP ribosylation factor 6 (ARF6) and phospholipase D2 (PLD2) [Ghossoub, 2014]. Moreover, Trajkovic *et al* recently reported that ceramide (released from sphingomyelin by neutral sphingomyelinase 2 (nSMase2)) is involved in ILVs budding into MVBs by inducing negative membrane curvature [Trajkovic K, 2008].

These various generation pathways may target different exosome subpopulations and regulate different exosomal cargos which can define their biological function. Therefore, further insight into mechanisms regulating these pathways could have a significant effect into the potential use of exosomes in transitional medicine.

As mentioned above, the MVBs can either fuse with the plasma membrane and release the exosomes into the extracellular fluid or fuse with lysosomes and undergo degradation. It is not currently understood what determines the fate of MVBs, but recent studies have provided some insight into potential regulatory mechanisms of this process. Fader *et al* reported that a change in the cellular conditions such as starvation can lead to decreased exosome release and increased fusion of MVBs with autophagosomes [Fader C, 2008]. Post-translational modifications were also shown to play a role in exosome secretion. Villaroya-Beltri *et al* demonstrated in *in vivo* and *in vitro* mice studies that conjugation of ISG15, an interferon (IFN)- α/β -induced ubiquitin like protein(UBL), to target proteins called “ISGylation” (ubiquitin-like modification) leads to decreased MVB number due to their co-localisation with lysosomes. This effect was shown on the TSG101 protein which led to MVB aggregation and degradation, and subsequently reduced exosome release from cells [Villaroya-Beltri C, 2016]. Additionally, the sumoylation of heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) was shown to play a role in the sorting of microRNAs into exosomes [Villaroya-Beltri C, 2013].

The transport of ‘secretory’ MVBs to the plasma membrane is controlled along microtubules by various kinesin isoforms which are regulated by RAB7- and Arl8-dependent protein complexes [Raiborg C, 2015, Pu J, 2015]. Once ‘docked’ to the plasma membrane, MVBs bind to the SNARE complex (soluble N-ethylmaleimide-sensitive component attachment protein receptor), with small GTPases such as RAL-1 believed to play a role in this process [Hyenne V, 2015].

In recent years a variety of different SNARE proteins were described such as Synaptobrevin homolog YKT6, Syntaxin-1a, Syntaxin-4 and Syntaxin-5, SNAP23 (synaptosomal-associated protein 23), and VAMP7 (vesicle associated membrane protein 7) [Bebelman M, 2018]. The formation of SNARE complexes is controlled by various mechanisms such as their phosphorylation status and calcium-sensing SNARE interacting protein synaptotagmin-7 [Bebelman, M, 2018].

1.3.2 Microvesicle secretion- the direct pathway

This heterogeneous subtype of EVs is released directly in the process of vesicles budding from the plasma membrane which is the result of dynamic interaction between contractions of cytoskeletal proteins and phospholipid redistribution [Hugel B, 2005]. In a resting state the plasma membrane phospholipid bilayer consists of phosphatidylcholine and sphingomyelin primarily located in the

outer leaflet, and phosphatidylserine and phosphatidylethanolamine located in the inner leaflet [Seigneuret M, 1984]. The process of microvesicle secretion is controlled by enzymes: flippase (a translocase that moves the phospholipids from the outer leaflet to the inner leaflet of the plasma membrane), floppase (a translocase which moves the phospholipids from the inner to the outer layer) and scramblase (which can transfer the negatively charged phospholipids in both directions and does not require ATP). When the cell is activated, cytosolic calcium increases leading to increased activity of floppase and scramblase, and inactivation of flippase [Daleke D, 2003]. The subsequent reorganisation of the phospholipids in the plasma membrane by movement of phosphatidylserine to the outer leaflet leads to microvesicle secretion/ “budding” which is supported by cytoskeletal contractions [Hugel B, 2005, Akers C, 2017]. The GTP-binding protein ADP-ribosylation factor 6 (ARF6), a member of the rho family, is involved in the process of microvesicle secretion by inducing the signalling cascade from the activation of phospholipase D, recruitment of the extracellular-signal regulated kinase (ERK) up to phosphorylation and activation of the myosin light chain by myosin light chain kinase (MLCK) [Muralidharan-Chari V, 2009]. Other proteins such as caspase and calpain are believed to play a role in this EV secretion pathway but the exact mechanisms behind it are not fully understood yet.

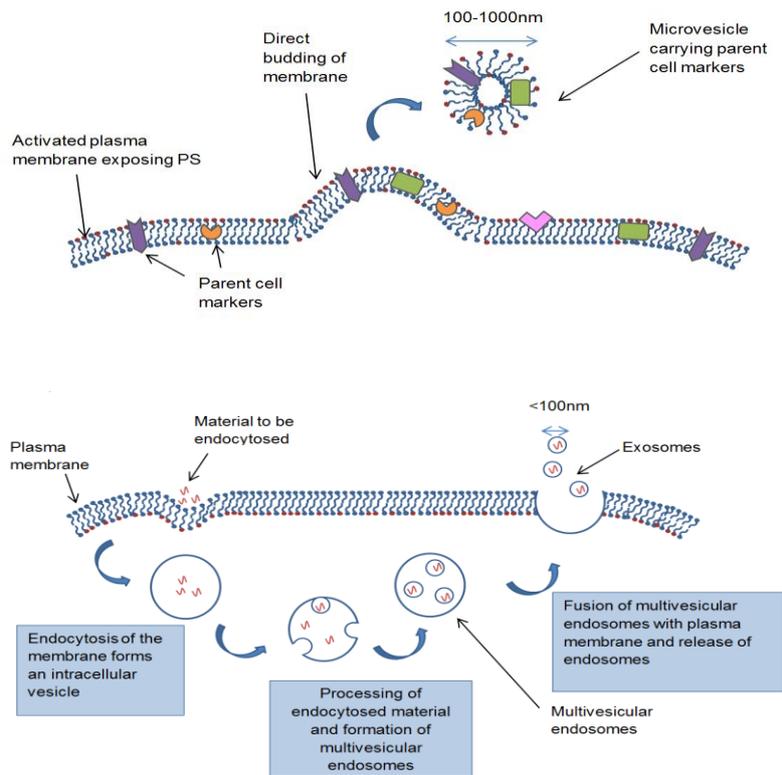


Fig.1.2: Extracellular vesicle generation pathways.

Microvesicles are released directly from plasma membrane budding; exosome formation occurs within multivesicular bodies which subsequently fuse with the cell membrane. (Images courtesy of Dr K. Connolly, Cardiff Metropolitan University)

1.4 EV content

1.4.1 EV proteins

EVs have been shown to contain a vast amount of various proteins with online databases available for reference which are continuously updated such as Vesiclepedia [Kalra H, 2012], EVpedia [Kim DK, 2013] and ExoCarta [Simpson RJ, 2012]. Generally, EV-expressed proteins can be divided into those that are considered as EV markers and those that reflect the EV cellular origin, localisation and mechanisms of secretion. Cytosolic, cytoskeletal, plasma membrane and heat shock proteins have been found to be the most prevalent groups of EV proteins, although the data on EV proteomic

profile varies depending on the isolation and proteomic analysis techniques [Yanez-Mo M, 2015], which as discussed above are currently not unified and still being developed.

Unfortunately, the EV proteins which are commonly used as markers often lack specificity but have been used in the literature due to the fact they are enriched in EV populations. These include tetraspanins (CD9, CD63, CD81 and CD82), 14-3-3 proteins, major histocompatibility complex (MHC) molecules and cytosolic proteins such as Alix, Tsg101 and heat shock proteins [Witwer KW, 2013]. Up until recently, it was believed that CD9, CD63 and CD81 could be used as exosome-specific markers but recent studies have shown that these tetraspanins are also present in apoptotic bodies and microvesicles [Tauro B, 2013, Crescitelli R, 2013].

EVs have also been found to contain an array of various cytokines such as Interleukin 1 β (IL-1 β), Interleukin 1 α (IL-1 α), Interleukin 6 (IL-6), Interleukin 8 (CXCL8), Transforming growth factor β (TGF β), Macrophage migration inhibitory factor (MIF) and vascular endothelial growth factor (VEGF) [Yanez-Mo M, 2015].

Table 1.1 demonstrates cell-specific markers used for EV analysis based on current experience from the literature. [Gustafson CM, 2015, Yuana Y, 2013]

Cell type	Surface marker/antigen
Erythrocytes	CD235a, CD236
Retikulocytes	CD71
Platelets	CD41, CD42a, CD42b, CD61
Leukocytes	CD15, CD45
Monocytes	CD11b, CD14
B cells	B cell receptor, CD19
T cells	CD2, CD3, CD86
Endothelial cells	CD31, CD62E, CD144, CD146, CD105

Tab.1.1 Common markers used in EV analysis of biological fluids.

1.4.2 Genetic material in EVs

1.4.2.1. RNA in EVs

The presence of functional RNA was first described in EVs just over a decade ago [Ratajczak J, 2006, Lotvall J, 2007]. It typically consists of less than 700 nucleotides [Chen TS, 2010, Batagov AO, 2013] and can be in the form of intact mRNA and RNA fragments, miRNA, long-non-coding RNA, ribosomal RNA, piwi-interacting RNA and lastly, fragments of tRNA-, vault- and Y-RNA [Yanez- Mo M, 2015]. Batagov *et al* reported that exosomes secreted by human cells contain mostly mRNA fragments enriched in the 3'-untranslated regions rather than intact mRNA. These regions contain sites for regulatory miRNA binding which suggest that RNA delivered to the recipient cells by EVs may compete with cellular RNA for miRNA binding and play an important role in translation [Batagov AO, 2013]. Secretion of RNA into EVs appears to be controlled by an active sorting mechanism as studies have shown that certain nucleotide motifs have been enriched in the RNA isolated from EVs [Batagov AO, 2011]. The heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1 appears to play an important role in this process as it recognises the EXOmotif (GGAG tetranucleotide) in miRNAs and controls the loading of these miRNAs into EVs [Villarroya-Beltri C , 2013].

EV-contained RNA has been shown to affect the recipient cells in a number of studies starting from the landmark discoveries by Ratajczak *et al* whereby murine bone marrow mononuclear cells treated with EVs derived from embryonic stem cells and enriched in Oct4 mRNA showed an increase in Oct4 protein expression [Ratajczak J , 2006]; and by Lotvall *et al* showing that mouse mast cell-derived EVs incubated with human mast cells transfer their murine RNA into the recipient cells leading to translation into murine proteins [Lotvall J, 2007]. Since then, numerous studies have shown a role for EV-contained RNA in biological processes such as angiogenesis, tissue repair, cell differentiation and proliferation, and immune regulation [Bruno S, 2009]. It also appears that the mRNA EV content is determined by the parental cell functional and physiological status as it was reported that EV RNA content differed in mast cell-derived EVs grown under normal conditions and oxidative stress [Eldh M, 2010].

1.4.2.2. DNA content of EVs

The physiological role of DNA present in EVs is currently not well understood. Nevertheless, various forms of DNA have been described in EVs so far: mitochondrial DNA (mtDNA), single-stranded DNA, double-stranded DNA (dsDNA) and oncogene amplifications (i.e. c-Myc) [Balaj J, 2011, Gruescini M, 2010, Thakur BK, 2014, Waldenstrom A, 2012, Lee TH, 2014]. The analysis of DNA content of EVs

reflects the mutations in the parental tumour cells [Thakur BK, 2014] which may have potential use in development of novel biomarkers in oncological conditions.

1.4.3 Lipids in EVs

Lipids are believed to play an important role in EV physiology and there have been numerous studies in the last 2 decades aiming to describe EV lipid composition and the differences between various cells/ clinical conditions. Generally, compared to the cells of origin, EVs are enriched in sphingomyelin, cholesterol, PS and glycosphingolipids [Record M, 2014]. Lipids play a role in EV generation and release; these include cholesterol, ceramide, lysobisphosphatidic acid (LBPA) and phosphatidic acid [Yanez-Mo, 2015]. Moreover, EVs also act as cargos for bioactive lipids between cells such as eicosanoids, fatty acids and cholesterol [Record M 2014]. These EV-contained lipids were reported to play a role in angiogenesis [Kim CW, 2002], reproduction, [Palmerini CA, 2006] and inducing cell death in cancer cells [Beloribi S, 2012]. Interestingly, the composition and concentration of EV fatty acids alters during the different stages of their cell of origin's growth and differentiation, with some fatty acid composition changes mimicking those occurring in the cells and some being unique to the EVs as previously shown by our research group [Connolly K, 2015].

1.5 EV uptake mechanisms and interaction with membrane receptors

EVs are involved in intercellular cell signalling in a number of ways: by being internalised by the target cell, by surface receptor/ligand interaction and finally by transfer of active receptors such as CCR5 (C-C Chemokine Receptor type 5) [Mack M, 2000], EGFRvIII (the epidermal growth factor receptor variant iii) [Al-Nedawi K, 2008] or tyrosine-protein kinase Met [Peinado H, 2012]. EV internalisation can occur via phagocytosis [Morelli AE, 2004,], micropinocytosis or direct fusion, but the latter is limited to the low pH conditions common within the tumour microenvironment (as the fluidity of EVs and plasma membranes differs at neutral pH) [Parolini I, 2009].

Intriguingly, some molecules delivered by EVs are thought to be more biologically active than their free soluble forms. This was shown for Fas ligands and metalloproteinases [Tanaka M, 1998, Shimoda M, 2013].

The receptor-ligand reactions between EVs and target cells are regulated by EV-expressed proteins such as MHC I and II [Simons M, 2009, Fevrier B, 2004], tetraspanins [Nazarenko I, 2010] and transferrin receptors [Calzolari A, 2006], which trigger various communication pathways in the recipient cells such as mitogen-activated protein kinase (MAPK) [Calzolari A, 2006], integrins and calcium signalling or natural killer group 2D (NKG2D) signalling [Clayton A, 2008, Macario AJ, 2010].

For instance, human natural killer (NK) cells and tumour cells have been shown to release EVs which express ligands triggering cell death in their target cells such as FasL, TNF-related apoptosis-inducing ligand (TRAIL) or perforin [Lugini L, 2012, Cai Z 2012, Andreola G, 2002]. Moreover, some of those tumour-derived EVs are capable of inducing lysis of lymphocytes that were programmed to kill the tumour cells [Andreola G, 2002, Huber V, 2006] which has significant clinical implications in the context of malignant disease progression.

Another example of important receptor-ligand interaction EVs participate in, is the role of platelet-derived EVs (containing platelet-endothelium attachment receptors (CD41, CD61, and CD62), cytokine receptors (TNF-RI, TNF-RII, and CD05), and ligands such as CD40L and PF-4) in facilitating platelet adhesion and coagulation pathways [Baj-Krzyworzeka M, 2002]. Hence, it is not surprising that patients with established cardiovascular disease, acute strokes or vascular dementia were found to have increased levels of circulating platelet-derived EVs in blood [Del-Conde I, 2005].

1.6 EV isolation

As mentioned above, EV research is a novel area and at present, there are lots of variations in approach to EV isolation which makes it challenging for direct comparison of work from various research groups. Therefore, the International Society of Extracellular Vesicles (ISEV) has focused efforts in the last few years on creating recommendations for standardisation of EV research methodology [Witwer K, 2013].

It has been stressed that for research questions aimed at characterisation of EV size, concentration and content, particular attention must be paid to the reproducibility of assays used and optimisation of their sensitivity.

1.6.1 EV isolation from blood

For studies on blood-derived EVs, basic anthropometric and clinical information should be available such as age, gender, ethnicity, smoking status, use of anticoagulants and current medication list. It is also important to standardise the sample collection protocols by using the same needle type and same type of collecting tube/vacutainer, appropriate sample handling and use of the same type of centrifuge, rotor and speed [Witwer K , 2013]. The effects of the circadian rhythm on EV concentration are not fully known but it is recommended to standardise the collection time for the purpose of the study. A similar rationale is applied to time intervals from the last meal, hence fasting samples are most likely to minimise unnecessary sample-to-sample variations [Witwer K, 2013].

It is also believed that it is plasma rather than serum which is the medium for EVs in blood under physiological conditions. Hence, unless specific questions relating to platelet-derived EVs are being

addressed, plasma is the biological fluid of choice for blood-derived EV characterisation [Witwer K, 2013].

The optimal anticoagulant is still debated. Whilst the use of heparin-based additives is generally contraindicated due to their potential effect on PCR- readings or potential heparin binding to EVs and blockage of their uptake [Yokata M, 1999, Beutler E, 1990], the choice between alternative anticoagulants such as EDTA [2,2',2'',2'''-(Ethane-1,2-diylidinitrilo)tetraacetic acid], sodium fluoride/potassium oxalate or sodium citrate is still not clear. Macey M *et al* suggested that the use of calcium chelators may lead to *in vitro* associations between platelets and EVs, and lower the overall EV count in blood collected into EDTA or citrate tubes, but further research is required to validate these findings [Macey M, 2002].

For sample collection, a 21-gauge needle or larger is recommended; the first few millilitres of blood should be discarded due to contamination with fibroblasts and the platelet activation effect from the tourniquet pressure. Vacutainer tubes should be handled gently and processed promptly with the maximum time from venepuncture to centrifugation of 30 minutes [Jayachandran M, 2012].

1.6.2 EV isolation - cell culture medium

For the analysis of EVs released spontaneously from a certain cell type, collection of medium following a 24-48-hour incubation period is recommended. Shorter incubation periods may be required if an effect of a certain stimulus on cell-derived EV release is being analysed. As most tissue culture media are supplemented by sera (such as foetal calf serum (FCS) or human serum) contamination with those sera EVs and RNA is likely to occur. In order to eliminate this risk an overnight 100000g ultracentrifugation of the media should be performed, which should not contain more than 20% serum [Witwer K, 2013]. A second solution is to culture cells in serum-free media for a set period of time. The disadvantage of the first method is the need for additional experiments to ensure a lack of bovine proteins, whilst the second approach may stimulate stress response in cells by rapid nutrient depletion, thus the profile of EVs may not fully reflect the physiological milieu. The percentage of dead cells in the culture should also be established as well as ensuring no microbial contamination [Witwer K, 2013].

1.6.3 EV isolation techniques

There have been many approaches to EV isolation reported to date such as differential ultracentrifugation, filtration or immunoaffinity isolation, and the field continues to rapidly expand. The main disadvantage of current methods is the fact that they are usually time consuming which limits their adoption in clinical laboratories for rapid patient sample analysis.

Differential centrifugation is the most commonly used method of EV isolation. The aim of this method is to deplete the starting sample from platelets, cells and apoptotic bodies by at least one low speed centrifugation step, which is then followed by high speed centrifugation to pellet EVs (100000g-200000g). Some approaches suggest an interim speed centrifugation at 10000-20000g in order to pellet larger EVs [Witwer K, 2103]. The main limitation of this method is the inability to perform accurate EV separation based on size; during the procedure aggregation of different EV types may occur. Secondly, the pellet may contain contamination from protein complexes or lipoproteins. [Webber J, 2013] Some advocate pellet resuspension in PBS and repeat ultracentrifugation. This may increase sample purity, but such an approach is likely to result in loss of the EV yield. Additionally, various inconsistencies in reproducibility of this method arise from differences in centrifugation speed, time, temperature and rotor type [Livshits MA, 2015]. However, when my study was commenced, differential ultracentrifugation was the most common EV isolation method and it was used in the majority of my experimental work.

In order to address the issue of sample purity, various density gradient separating techniques were developed recently originating from sucrose density gradient studies [Thery C, 2006], which allow separation of protein and RNA aggregates from vesicles. The approaches to this method differ, with many variations in sample loading time and sucrose tube length which may affect the characteristics of the EV pellets obtained. Furthermore, the risk of contamination with protein aggregates is not fully overcome [Thery C, 2006, Witwer K, 2013].

Size exclusion for EV isolation is based on the assumption that by forcing sample passage through physical barriers such as filters or chromatography, separation of EVs based on their size will occur. This method allows exclusion of larger particles from the analysed sample. The downside of this method is the potential effect of forcing particles through filters leading to EV deformation and break up of large EVs causing inaccuracies in final results [Witwer K, 2013]. Moreover, this method as I describe later in my results, leads to significant sample dilution/loss. Therefore, it is recommended that size exclusion is performed by gravity [Gyorgy B, 2011].

Immunoaffinity isolation is a method that uses the presence of certain characteristic surface proteins on EVs to select the desired EV population (immunoenrichment) or, in contrast, to deplete the sample of EV populations that are unwanted (immunodepletion) [Witwer K, 2013]. Antibodies are usually associated with beads (typically against tetraspanins (CD9, CD63, CD81) or immune-regulator molecules (MHC I and II) present on the EV surface, which enables physical separation by magnetic techniques or low speed centrifugation [Oliveira-Rodríguez M, 2016, Lynch S, 2009]. The obvious advantage of this approach is its high specificity when analysing a particular EV

subpopulation, but this is compromised by lower EV yields as well as high cost and importantly, the potential loss of EV functionality [Thery C, 2006].

Polymeric precipitation is a method where biological samples are incubated with commercially available polymeric precipitation mixtures; subsequent centrifugation generates an EV precipitate, particularly exosomes. The potential disadvantage of this method relates to possible sample impurity, since the precipitating liquid may bind to non-EV particles such as lipoproteins [Witwer K, 2016].

New methods on the horizon

An affinity-based technique was recently described by Nakai *et al* which is based on Tim4 protein binding to phosphatidylserine (PS) which is present on the EV surface as described above. The authors used K562 cells and peritoneal macrophages to show enrichment in CD63, Flotillin2, CD9 and CD81 markers in the small EVs (sEVs) in comparison to older isolation methods such as ultracentrifugation, with good recovery of the EV protein profile [Nakai W, 2016]. However, this method does not allow separation of exosomes from microvesicles and apoptotic bodies, since all these EV subpopulations can express PS.

Microfluidic devices are another emerging alternative which have the advantage of being quick and requiring only a small amount of sample. They can be divided into sub-categories based on the methodology applied within them: sieving nanoporous membranes trapping EVs on porous structures, and immunoaffinity EV trapping [Liga A, 2015]. However, these devices also have numerous limitations at present and further improvements are required before they can be applied more widely.

As all EV isolation methods available currently have some disadvantages, the combination of different isolation techniques is often performed in practice, such as ultracentrifugation and size filtration. However, this may again affect the final EV yield. With the rapidly developing improvements in EV methodology, a main focus over the next few years will be to develop a gold standard approach which can be reproduced easily and reliably across various laboratories.

1.7 EV sample storage

Should the biological fluid require a period of storage prior to EV isolation (as is the case with complex clinical studies requiring multiple samples), it is recommended that it should be rendered platelet- and cell-free prior to storage. There is no consensus, however, on the effects of different temperatures on EV recovery and characteristics. The work conducted by my colleague Dr K Connolly on the methods (4°C, -20°C, -80°C, liquid nitrogen) and length (1 day, 1 week, 1 month, 3

months) of storage of EVs revealed that EVs kept short term in the fridge (4°C) retained the closest concentration to that of fresh EVs and that long term storage irrespective of method increases EV concentration. [Connolly K, 2015] Therefore, the experimental work presented in this thesis evaluating EV characteristics is performed on samples which were stored for a maximum of 5 days at 4°C following isolation from plasma/medium.

The most widely used long-term storage temperature is -80°C and PBS is the diluent of choice [Thery C, 2006]. Moreover, siliconised storing vessels should be used to prevent EV adherence to surfaces and their subsequent loss [Witwer K, 2013]. Whilst data on the effect of repeat freeze/thaw cycles on EV recovery are limited, this should be avoided nevertheless. If required, samples should be frozen and thawed rapidly for the best preservation of their function and morphology [Witwer K, 2013].

1.8 EV characterisation methodology

1.8.1 EV morphology

Electron microscopy (EM) is a useful tool for visualisation of EV morphology and size measurement but cannot be used to assess EV concentration. It can also be used to evaluate the presence of membrane markers when nanogold particles coupled with immunoglobulins are used [Witwer K, 2013]. Atomic force microscopy is an alternative method to evaluate EV morphology [Gyorgy B, 2011].

1.8.2 EV concentration and size

Nanoparticle tracking analysis (NTA) is a technique where EV size and distribution can be established using commercial equipment, in which a laser beam scatters through a particle suspension and their Brownian motion are recorded by CCD camera. The Stokes-Einstein equation is then applied to calculate the particles' mean velocity. The limitation of this method is the fact that it cannot distinguish EVs from large protein complexes, hence the concentration calculated by the software may be skewed by the presence of such complexes.

Resistive pulse sensing (qNano) is an alternative technique to NTA for measurement of size and concentration. This method is based on the assumption that transport of a vesicle through a nanoporous membrane will lead to a transient decrease of the ionic current. However, for characterisation of heterogeneous EV populations, various nanopore membranes may need to be used as each of those membranes only detects particles of a certain size [Witwer K, 2013].

Flow cytometry (FC) enables quantitative and qualitative measurements of small particles but its use in EV research was until recently limited by the fact that most flow cytometers have detection

thresholds around 300-5000nm and are thus unable to detect small EVs such as exosomes. However, with the advent of new generation flow cytometers such as Gallios (Beckham Coulter) or DD-Influx (Becton Dickinson), smaller particles down to 100-200nm in diameter can now be detected which will likely result in more widespread adoption of this method for EV characterisation [Witwer K, 2013].

1.8.3 EV content

Western Blot has been widely used to demonstrate the presence of proteins regarded as EV markers (such as CD9, CD63, CD81) or cytosolic proteins such as Alix or Tsg 101. The limitation of this method is that it does not allow measurement of EV quantity or distinguish between various EV subpopulations. In addition, it may reflect the presence of contaminating proteins.

Immunoaffinity assays can be used to detect the presence of an antigen of interest within the EV population. It has been adapted by our group based on the work of Webber J *et al* [Webber J, 2014] and is described in more detail in chapter 2.

For functional EV studies, dose-response experiments should be included and density gradients for isolation are recommended, in order to ensure that it is the intrinsic content of EVs that is responsible for any observed effect. Labelling studies are also encouraged [Witwer K, 2013].

1.8.3.1 ISEV recommendations

Given current limitations in EV isolation and processing as described above, ISEV created a set of minimal experimental criteria to define EVs and their function, in an attempt to standardise the wide variations reported in EV methodology [Lotvall J, 2014].

Firstly, as mentioned above, EVs should be isolated from extracellular fluids such as cell culture medium, plasma or other bodily fluids and samples should be handled gently to prevent cell disruption which could contaminate the samples with vesicles from intracellular compartments.

For EV characterisation and protein content/expression, at least a semi-quantitative method should be used and at least 3 proteins should be reported in an EV preparation.

This should include various groups of protein: a) proteins that are expected to be present within the EV population such as tetraspanins or certain cytosolic proteins, and b) proteins that are not expected to be seen, and therefore their presence or absence in the analysed sample will give an indication of sample purity [Lotvall J, 2014].

The use of EV protein databases such as Vesiclepedia is encouraged to compare the protein profiles of various samples. It is encouraged that negative controls are used to compare EV samples against EV-depleted fluids when various commercially available antibodies are used first [Lotvall J, 2014].

For the characterisation of a single vesicle, two main methods should be applied: size distribution such as NTA or dynamic light scattering, and electron or atomic force microscopy [Lotvall J, 2014].

The four main groups of proteins expected to be analysed in EV samples are summarised below: [Lotvall J, 2014]

1. Transmembrane or lipid-bound extracellular proteins such as tetraspanins (CD9, CD63, CD81), heterotrimer G proteins, adhesion molecules/integrins, GH (Growth Hormone) factor or lactadherins are proteins which are usually present/enriched in EVs/exosomes which prove the presence of a membrane in the isolated sample.
 2. Cytosolic proteins with membrane or receptor binding capacity such as ALix, TSg101 or syntenins are also present or enriched in EVs/exosomes.
 3. Intracellular proteins which are associated with other than membrane/endosomes cellular compartments and should be absent or depleted in exosomes but can be present in other EV types. Examples include: Endoplasmic reticulum (Grp94=HSP90B1, calnexin=CANX), Golgi (GM130), Mitochondria (cytochrome C=CYC1), Nucleus (histones=HIST*H*) or Argonaute/RISC complex (AGO*).
 4. Extracellular proteins which have variable associations with EVs as they can bind specifically or non-specifically to membranes and co-isolate with EVs. Examples include: cytokines, metalloproteinases, collagens, serum albumin, growth factors or acetylcholinesterase.
- At least one protein of groups 1-3 should be quantified in the EV preparation.

The EV-TRACK knowledge base has recently been launched [van Deun J, 2017] which enables scientists to use various tools for effective EV research and to obtain an EV-METRIC for their manuscript on the methodology used. This facilitates transparency.

ISEV recognised, however, that our knowledge on what the EV and non-EV proteins are, is still developing which may affect the 'minimal criteria' of EV characterisation. The Society also acknowledges that it sometimes may be difficult to perform all the protein expression experiments due to limited amount of EV material [Witwer K, 2017].

1.9 EV function

EVs have been reported to be involved in numerous biological processes to date. EV biological roles can be broadly divided into 3 categories: remodelling and/or removal of cellular components; vectors of intercellular communication by direct EV content uptake by neighbouring and distal cells, transfer of cell surface proteins or receptor-ligand reaction, and finally regulation of the local or distal cell microenvironment by modification of immune reactions, cell migration and angiogenesis [Gonda D, 2013].

Interestingly, the proposed function of EVs in the context of certain pathologies can differ which may be due to variability in isolation and characterisation methodology between various research laboratories. However, it could also indicate biological plasticity of EVs and the fact that different intracellular compartments of origin subsequently lead to different EV function, reflected in a different EV composition. Therefore, tools for better separation of EV populations are essential in order to advance the potential use of EVs in clinical practice.

Plasma-derived EVs comprise a heterogeneous group of EVs originating from cellular blood components as well as endothelial cells. In pathological conditions, however, other EV sources may also appear in the circulation, such as tumour-derived EVs in malignancy. Platelet-derived EVs are believed to be the most abundant circulating EV population, comprising around 25% of total blood EVs [Aatonen MT, 2014]. They are mainly involved in haemostasis and possess procoagulant properties, which may hold particular relevance in conditions such as cardiovascular disease, diabetes mellitus, sepsis or cancer [Aatonen MT, 2013]. Leukocyte-derived EVs seem to play an important role as paracrine messengers in both innate and acquired immunity. Along with platelet-derived EVs, they are also involved in angiogenesis. Reticulocyte –derived EVs are involved in the erythrocyte maturation pathway. There is also a growing body of evidence for EV involvement in tissue repair and organogenesis, bone calcification, liver homeostasis and neuronal communication [Aatonen MT, 2013]. It is, however, important to state that at present the proportions of circulating plasma EV subpopulations described in various published manuscripts depend to a certain degree on the isolation and characterisation techniques used. As discussed previously these have various limitations and there is currently no method with 100% detection/capture rates that would ensure a true reflection of physiological plasma EV distribution. Moreover, circulating plasma EVs profiles are not static and change in response to various stages of acute illness. For instance, Chiva-Blanch *et al* compared the circulating EV profile from patients at various timepoints following an acute ischaemic stroke (within 48 hours of onset, day 7 and day 90) to EVs from a high cardiovascular risk control group. They reported increased EVs shedding from blood and vascular compartment cells and neural progenitor cells at all those timepoints in the stroke arm and also changes in the EVs proportion at

90-day follow-up in those with the largest post-stroke cerebral lesions – with decrease in neural precursor cell derived EVs and increase in smooth muscle cell-derived EVs [Chiva-Blanch G, 2016].

EVs also play a role in cellular waste management as they may contain redundant intracellular material which is then secreted within EVs by the cells and removed from the circulation through phagocytosis by the other cells. The spleen is likely to be involved in clearing these EVs from the circulation [Davilla M, 2008].

Inflammation/immune system

EVs have been found to modulate the immune response in a number of ways, exhibiting both pro- and anti-inflammatory properties on the local microenvironment. With regard to anti-inflammatory responses, EVs were described to affect the expansion of T regulatory cells and stimulate their function which leads to suppression of the host immune reaction [Szajnik M, 2010]. They contain a variety of different cytokines such as interleukin 10, cytotoxic T-lymphocyte antigen-4, transforming growth factor- β 1 and also Fas ligand, which initiates Fas-dependent apoptosis in lymphocytes [Kim JW, 2005]. In melanoma patients, tumour-derived EVs were shown to hinder the differentiation of monocytes into dendritic cells (DCs) and stimulate the differentiation of myeloid cells with Transforming Growth Factor- β -Mediated Suppressive Activity on T Lymphocytes (CD14+HLA-DR-/low) [Valenti R, 2006]. They were also found to contain annexin-1 which has anti-inflammatory properties [Dalli J, 2008].

Interestingly, not only leukocyte- but also platelet-derived EVs have been found to be involved in modulation of immune responses by delivering CD40 ligands (also known as CD154) to promote adaptive immune responses via CD4+ T cells [Sprague DL, 2008].

Additionally, EVs are enriched in molecules involved in antigen presentation, such as major histocompatibility complex class I and II molecules [Androela G, 2002]. It has been shown that cells infected by various pathogens release EVs presenting the pathogen-derived antigens which leads to stimulation of T cells [Walker JD, 2009]. Berckmans *et al* showed that leukocyte-derived EVs from synovial fluid from patients with rheumatoid arthritis stimulate synoviocytes to secrete inflammatory molecules such as monocyte chemoattractant protein-1 (MCP-1), IL-8, IL-6, RANTES (regulated on activation, normal T cell expressed and secreted), ICAM-1 (Intercellular Adhesion Molecule-1) and VEGF (Vascular Endothelial Growth Factor) [Berckmans RJ, 2005].

Angiogenesis

EVs have been shown to have both a pro- and anti-angiogenic role in studies evaluating endothelial- and platelet-derived EVs. Endothelial cell-derived EVs contain metalloproteinases such as MMP-2

and MMP-9 which play a role in matrix degradation and thus help to initiate formation of new blood vessels [Tarabolletti G, 2002]. EVs derived from platelets have also been shown to promote formation of capillary-like structures in both *in vitro* [Kim KH, 2004] and *in vivo* mice models [Brill A 2005]. This may be particularly important from the tumour biology point of view since platelet-derived EVs play a role in tumour metastasis and angiogenesis by stimulating the expression of growth factors such as VEGF and HGF (hepatic growth factor), matrix metalloproteinases and interleukin-8 [Janowska-Wieczorek A, 2008]. Interestingly, tumour cell-derived EVs can also promote angiogenesis themselves as they are enriched in IL6, IL8 and angiogenin [Skog J, 2008].

Moreover, adipocyte-derived EVs (from 3T3-L1 cells and rat adipocytes) were recently shown to contain various angiogenic factors and exhibit angiogenic activity *in vivo* in obese mice. They also stimulated cell migration and tube formation of endothelial cells derived from human umbilical veins and promoted endothelial cell invasion through extracellular matrix [Aoki N, 2010].

On the other hand, endothelial cell-derived EVs as well as lymphocyte-derived EVs have the ability to hinder angiogenesis by increasing the production of reactive oxygen species (ROS) and reducing nitrite oxide (NO) [Yang C, 2008, Burger JA, 2006].

Coagulation

The role of EVs in coagulation is not yet fully characterised though they have been widely reported to initiate and augment platelet aggregation. In 1999, Giesen *et al* reported that cell-derived vesicles obtained from plasma of healthy individuals expressed tissue factor (TF) which initiates coagulation. [Giesen PI, 1999]. As EVs also express phosphatidylserine (PS), they provide the catalytic surface for formation of the tenase (factors VIIIa, IXa, and X) and prothrombinase (factors Va, Xa, and II) coagulation cascade complexes [Tripisciano C, 2017]. Moreover, PS might play a role in changing TF into its biologically active form [Spronk HM, 2014]. Interestingly, not only blood but also urine and saliva contain EVs expressing coagulant TF under physiological conditions [Berckmans RJ, 2011]. Numerous studies have reported the presence of procoagulant TF in EVs derived from patients suffering from malignancy-associated venous thromboembolism [Tesselaar ME, 2007], sepsis [Nieuwland R, 2000], coronary artery disease [Nieuwland R, 1997] or sickle cell disease [Shet As, 2003]. On the other hand, in bleeding disorders such as Scott syndrome defective microvesicle formation was described secondary to impaired scramblase activity resulting from a calcium-gated ion channel mutation (TMEM16F) [Malvezzi M, 2013]. The decreased PS exposure and decreased procoagulant EV secretion is believed to lead to low prothrombinase activity in this condition [Toti F, 1996].

1.10 Potential application of EVs

Circulating EVs are elevated in numerous conditions including diabetes mellitus, cardiovascular disease, malignancies, liver disease and autoimmune disorders [Van der Pol E, 2012]. Given that they contain protein and miRNA derived from their parental cells, they may represent novel biomarkers for early disease detection and assessment of treatment response, particularly in cancer management [Kalluri R, 2016].

However, although specific EV subtypes are increased in various pathological conditions, their biological role under such circumstances appears to be ambivalent; they might promote the pathological process but might also promote protective and tissue repair pathways. Endothelial EVs are a good example of this plasticity and diversity; on the one hand, they have been found to impair endothelial function and dilatation, whilst on the other they can stimulate endothelial proliferation and repair [Digant-George F, 2011].

Of additional interest, EVs appear to be promising therapeutic agents within the field of nanomedicine. This potential is being explored in the context of cancer and infectious disease vaccinations, immunosuppressive and regenerative therapies and as drug-delivering nanovectors, with numerous trials already in progress [Fais S, 2016].

1.10.1 EV role as biomarkers

EVs have a potential use as biomarkers in establishing the presence of disease, predicting the risk of developing it or evaluating response to therapy. For instance, the presence of leukocyte-, platelet- and endothelial cell- derived EVs was shown to affect the Framingham risk score (a risk assessment tool to estimate a patient's 10-year risk of developing CVD) in recent studies [Chironi G, 2006, Ueba T, 2010, Nozaki T, 2009]. In oncology, EV content seems to reflect tumour cell plasticity and molecular changes; samples are also easier /quicker to access than traditionally used methods such as repeated biopsies. This may be particularly important in the management of rapidly progressing tumours such as glioblastoma multiforme (GBM) as it may help to predict the effectiveness of planned therapy and/or development of resistance to therapy used. For instance, the microfluidic chip device developed recently showed that EVs isolated by this method from GBM patients expressed increase EGFR, EGFRvIII, podoplanin, and IDH1 R132H proteins compared to healthy volunteers. Higher levels of these proteins were associated with failure to respond to standard treatment with temozolomide and radiotherapy [Wykosky J, 2011].

Gene expression analysis of EV-contained DNA, mRNA, miRNA, methylation status and proteins by microarray as well as sequencing of EV nucleic acids in a search for tumour-specific mutations offers another potential tool for clinical application but is currently hindered by methodological limitations.

1.10.2 EVs' role in pharmacotherapy

1.10.2.1 Modulation of EV secretion

Given the multiple biological functions of EVs as described above, there are various possibilities to use them in modern pharmacotherapy. These can be broadly divided into blocking of EV secretion and uptake, and the use of EVs as cargos for immunotherapies or targeted gene/drug delivery.

As discussed, EVs have been shown to be raised in numerous pathological conditions and affect various processes such as angiogenesis, immune responses, and tumour invasion and metastasis [Gonda D, 2013]. Therefore, development of mechanisms that stop their release from cells and their uptake could in theory help to stop the pathological processes mediated by EVs. Interestingly, some commonly used old generation drugs such as amiloride, imipramine and proton pump inhibitors (PPI) were shown to reduce EV secretion by various mechanisms: amiloride, a diuretic, decreases calcium influx into the cells [Bianco F, 2009]; the antidepressant imipramine inhibits sphingomyelinase [Bianco F 2009] and PPIs affect EV release by interaction with the tumoral acidic extracellular environment [Luciani F, 2004]. Iero *et al* showed that patients with cancer treated with amiloride had reduced EV production and tumour-mediated immunosuppression [Iero M, 2008]

whilst mice studies on melanoma showed that pre-treatment with PPIs leads to increased sensitivity to chemotherapy with cisplatin by reducing the efflux of the drug via EVs [Chalmin F, 2010]. Al Nedawi *et al* hindered EV uptake into surrounding cells by inhibiting their fusion to plasma membranes by reducing the horizontal propagation of the EGFRvIII oncogene using annexin V, a scaffolding protein that blocks phosphatidylserine exposure [Al Nedawi K, 2008].

Other potential EV specific strategies include interference with the ESCRT pathway by silencing genes encoding proteins such as HRS, Rab27a, STAM, or TSG101 which are required for EV production [Savina A, 2003]. Bobrie *et al* also showed in their *in vivo* study on mammary carcinoma cells that blockade of Rab27a resulted in decreased exosome secretion and subsequent decreased primary tumour growth and lung dissemination of a metastatic carcinoma [Bobrie A, 2012]. Moreover, my colleague, Dr N Burnley-Hall showed that release of EVs from hypoxia-exposed human endothelial cells can be reduced by treating the cells with sodium nitrite (NaNO₂) which increases HIF1 α degradation. This effect is further attenuated by the addition of allopurinol which inhibits xanthine oxidoreductase, thus stopping the conversion of nitrite to nitric oxide [Burnley-Hall N, 2017].

1.10.2.2. EVs used as pharmacotherapy vectors

EVs are promising vectors for novel therapies for a number of reasons and they appear advantageous over artificial nanoparticles that have been developed for drug delivery. Firstly, EVs have the right biophysical properties in the form of a gel-like cytoplasm-derived core and deformable cytoskeleton [Hood JL, 2012], and near neutral, slightly negative zeta potential which enables them to exist longer the circulation [Zhao W, 2011]. Moreover, due to their size, EVs have the ability to cross the gap between endothelial cells in the tumour vasculature (which is typically around 400nm) and transfer into the tumour microenvironment [Hood JK, 2012]. Lastly, EVs are well tolerated by the immune system compared to synthetic nanoparticles and contain various ligands and co-receptors which enable intercellular signalling processes, as discussed previously. EV modification for the purpose of pharmacotherapy can be undertaken endogenously at the cellular level or exogenously following EV isolation. The latter can be modified by alteration of their surface molecules, loading of hydrophobic therapeutics into the EV membrane or loading of hydrophilic molecules into the EV core [Gonda D, 2013].

As discussed above, EVs are involved in modulation of various immune processes. The role of immunotherapies in oncology has recently gained considerable interest. The potential ability of EVs to stimulate cytotoxic T lymphocytes against tumour cells has made them a promising novel therapeutic vector which is already reflected by some recent preliminary studies both on animal

models and in phase I trials. For instance, Escudier *et al* reported that metastatic melanoma patients who received a 1-month weekly cycle of subcutaneous and intradermal autologous dendritic cell-derived EVs pulsed with MAGE 3 (Melanoma associated antigen 3) showed promising results in terms of disease progression control with no major toxicity reported [Escudier B, 2005]. Similarly, Morse *et al* applied autologous dendritic cell-derived EVs loaded with MAGE-A3, -A4, -A10, and MAGE-3DPO4 peptides used as 4 treatment cycles for patients with advanced non-small cell lung cancer (n=9). They also demonstrated good response, no major side-effects and achieved long term stability of disease in some patients [Morse M, 2005]. Moreover, Dai *et al* showed that in patients with advanced colorectal cancer (n=40) subcutaneous injection of autologous exosomes isolated from ascitic fluid, in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF), induced a tumour-specific anti-tumor cytotoxic T lymphocyte (CTL) response [Dai S, 2008].

Finally, EVs can potentially be used as vectors for targeted delivery of therapeutic agents which is particularly important in the management of malignancies since systemic chemotherapy is associated with significant side-effects and damage to healthy cells. A proof-of-concept study by Dai *et al* on murine models showed that si-RNA was successfully delivered through the blood-brain-barrier to neurons by EVs derived from dendritic cells which were engineered to express rabies viral glycoprotein (RVG) and Lamp2b protein, a constituent of exosomes [Dai S, 2008]. Apart from transferring small RNAs, transfer of active signalling molecules is also being explored in order to affect cells' response to therapy [Lachenal G, 2011].

Once the mechanisms of EV protein and miRNA sorting are fully elucidated, targeted drug delivery and potential modulation of the recipient cell's gene expression could ultimately revolutionise future pharmacotherapy.

2. Adipose tissue

2.1 Overview

Adipose tissue (AT) is now considered as an active endocrine organ secreting a variety of hormones and cytokines and playing an important role in modulation of various metabolic processes. This is in addition to the traditionally well-known role of AT in 'energy storage' by accumulation of neutral triglycerides (TG) via the lipogenic pathway. AT consists of adipocytes and preadipocytes, fibroblasts, macrophages, endothelial cells and leukocytes. The storage of TGs in the adipocytes leads to increased size of lipid droplets and subsequent adipose expansion and development of obesity [Tan CY, 2008]. On the other hand, AT-stored TGs can undergo lipolysis into free fatty acid (FFA) and glycerol when energy expenditure is increased under conditions of starvation [Lafontan M, 2009]. Under such circumstances, FFAs and glycerol are transported via blood to muscles, liver and other organs which modulates the energy homeostasis in the body [Frayn KN, 2002].

2.2 AT classification

In general, AT can be divided into 2 types: white adipose tissue (WAT) and brown adipose tissue (BAT). The latter is mainly involved in the dissipation of surplus energy into heat via uncoupling protein 1 (UCP1). Interestingly, over the last few years it has emerged that WAT contains an adipocyte population described as beige or brite ("brown-like-in-white") in which UCP1 expression can be stimulated by cold stress or β 3-adrenoceptor agonists [Barbatelli G, 2010, Bostrom P, 2012]. Given the thermogenic properties of beige and brown adipose tissue, there has been interest in recent years in their potential application in the management of obesity [Ishibashi J, 2010]. Mice studies have shown that an increase in circulating factors such as natriuretic peptides, Fibroblast Growth Factor 21 (FGF-21) and irisin can enhance the function of brown and beige AT depots [Harms M, 2013]. Therefore, therapies which enhance BAT activity or augment its content, or lead to trans-differentiation of non-BAT progenitors into BAT-preadipocytes, could potentially open a new chapter in targeted therapies for obesity [Reddy NL, 2014].

White, brown and beige adipocytes differ in morphology, origin, mitochondrial content and thermogenic abilities. WAT mainly consists of white adipocytes varying from 25-200 μ m in size and containing a unilocular lipid droplet and very few mitochondria. They therefore have a low oxidative rate [Jeanson Y, 2015] but, as discussed above, have the ability to store energy in the form of TGs. White adipocytes originate from resident mesenchymal cells within WAT. Generally, there are 2 main types of WAT based on their anatomical location: visceral adipose tissue (V-AT) such as mesenteric, retroperitoneal and perigonadal fat, and subcutaneous adipose tissue (S-AT). These two depots, however, differ by much more than location. Other differences include adipocyte

development, cell morphology, biological function and molecular signature [Berry DC, 2013]. S-AT appears to be morphologically more heterogeneous as it contains both mature unilocular and small multilocular adipocytes and increased interstitial tissue component. In contrast, V-AT mainly consists of large unilocular cells [Tchernof A, 2006]. S-AT also displays an increased rate of cell turnover, leading to an increase in new adipocyte formation. It is believed that 'younger' adipocytes are not associated with the risk of developing metabolic dysfunction [Salans LB, 1973]. There are also differences in lipolytic rates between these two depots in response to external stimuli such as steroid hormones. Shi *et al* demonstrated in human and mice studies that the accumulation of AT within the viscera or neck tissue is more responsive to glucocorticoids whereas the accumulation of adipose tissue in a subcutaneous location appears to be stimulated by oestrogens [Shi H, 2009].

Finally, there are differences in transcription factors such as members of the homeobox (HOX) and forkhead box (FOX) family [Gesta S, 2006], and in gene expression. For example, expression of leptin, angiotensinogen and glycogen synthase is increased in S-AT compared to omental fat, whereas the latter shows elevated expression of the insulin receptor, 11 β hydroxysteroid dehydrogenase (11 β HSD) and interleukin 6 (IL6) [Gesta S, 2007].

It is therefore believed that these two AT depots have distinct biological functions. Simplistically, S-AT is thought to be the more protective "good fat" whilst V-AT may be considered as "bad fat" associated with the metabolic complications of obesity [Berry DC, 2013].

In recent years, ectopic fat accumulation around the heart (pericardial and epicardial fat depots) as well as the PVAT (perivascular adipose tissue) adjacent to adventitia of most arteries has gained lots of interest due to its effects on cardiovascular function [Iozzo P, 2011]. The morphology and physiology of PVAT depends on its location; for instance, adipose tissue surrounding thoracic aorta was found to resemble BAT [Fitzgibbons TP, 2011] whilst PVAT in mesenteric arteries resembles WAT [Guzik B, 2013]. These fat depots apart from providing structural support secrete a variety of adipokines such as TNF α , MCP-1, PAI-1, IL-6 which affects the inflammatory responses [Salgado-Somoza A, 2010], vascular tone [Maenhaut N, 2011], migration and proliferation of smooth muscle cells [Miao C, 2012] and neointimal hyperplasia and formation [Takaoka M, 2010]. Obesity leads to an increase in PVAT mass [Greenstein AS, 2009] and to alterations in its function. Increased production of pro-inflammatory and vasoconstrictive adipokines affects the vascular relaxation response [Gao YJ, 2005, Xia N, 2017] and contributes to the development of cardiovascular disease in obese subjects. In the clinical context, measurement of pericardial/epicardial fat and perivascular fat by echocardiography or computed tomography (CT) was shown to correlate with visceral adipose

tissue mass, BMI/waist circumference, LDL cholesterol, insulin sensitivity and low plasma adiponectin [Iacobellis G, 2003, Gorter PM, 2008].

Brown adipose tissue (BAT) was initially believed to play a role predominantly in temperature regulation in the neonatal period [Spiegelman B, 2001] where the adipocytes are mainly located in the dorsal region between the scapulae [Cannon B, 2004]. However, studies using ^{18}F -fluorodeoxyglucose (FDG) positron emission tomography-computed tomography (PET-CT) have since shown that BAT is also present in some adults in the supraclavicular and lower neck areas [Nedergaard J, 2007]. Brown adipocytes originate from the dermomyotome, a tripotent engrailed 1 (EN1)-positive cell lineage from which brown fat fibres, myocytes and the dermis develop [Atit R, 2006]. Interestingly, a subset of brown as well as white adipocytes descends from a myogenic factor 5 (MYF5)-positive source [Sanchez-Gurmaches J, 2012]. Brown adipocytes are enriched in mitochondria, which gives them their characteristic colour, and contain specialised proteins such as UCP1 which collapses the electron gradient causing heat rather than ATP generation.

Beige adipocytes are a distinctive cell line mainly arising from Myf5- progenitor cells (similar to WAT) but they have a multilocular morphology and positive UCP1 expression [Wu J, 2012]. Beige adipocytes can develop via a number of different pathways, but the exact mechanisms are still poorly understood. Firstly, they can arise from Myf5- progenitor cells *de novo*. PDGFR α + adipocyte precursor cells have also been shown to differentiate into beige adipocytes when stimulated by interleukin 4 receptor α (IL4R α) signalling [Lee YH, 2012], and Sca1+ progenitor cells can develop into beige AT when induced with bone morphogenetic protein 7 (BMP7) [Schulz TJ, 2011]. 'Browning' of WAT has also been induced by cold stress or its mimics via β 3-adrenoceptor agonists [Young P, 1984].

2.3 Adipose tissue turnover

Our knowledge on the formation of adipose tissue is predominantly based on *in vitro* cell culture models. In fact, the term "adipogenesis" was first used to describe the transition of confluent 3T3-L1 fibroblasts into lipid-laden cells following incubation with artificial inducers [Green H, 1975]. It has subsequently been shown that adipogenesis is a complex process of differentiation of committed preadipocytes into mature adipocytes, with peroxisome proliferator-activated receptor γ (PPAR γ) believed to play a key role in its regulation [Rosen ED, 2000] and multiple other signalling molecules involved such as insulin, thyroid hormone, glucocorticoids and TGF β superfamily members (BMP2/BMP4/GDF3) [Berry D, 2013].

In response to positive energy balance, AT can expand from 2-3% up to 60-70% of body weight [Hossain P, 2007]. There are two main types of adipose tissue expansion: hypertrophy and

hyperplasia, with the relative contribution of each component depending on a variety of factors such as genetic background, hormonal milieu, diet and fat depot site [Berry D, 2013]. Adipocytes are able to increase their storage of TGs leading to a 2-3-fold increase in their volume [Hirsch J, 1976]. This has a range of biological consequences, ranging from increased rate of adipocyte apoptosis and a shorter lifespan [Strissel K, 2007], to reduced sensitivity to insulin [Hossain P, 2007] and increased local inflammation and secretion of cytokines [Osborn O, 2012].

In contrast, adipose tissue hyperplasia may lead to formation of small adipocytes which may play a protective role [Strissel K, 2007]. Studies have estimated that some 8% of adipocytes in humans turn over each year [Spalding K, 2008]. This suggests that adipose stem cell proliferation replenishes the adipocyte pool but the exact mechanisms initiating and controlling this process are not clear, although it appears this may be AT depot-specific [Berry D, 2013, Joe A, 2009].

2.4. AT function

2.4.1 Energy storage

AT is a primary organ for the storage of excess energy in the form of fat which is determined by the balance between lipogenesis and lipolysis. Lipogenesis is the process of fatty acid synthesis which then serves as energy storage. It is a dynamic process which is responsive to dietary intake with glucose being the main substrate, hence fasting leads to inhibition of lipogenesis whilst an increase in plasma FFAs and a high carbohydrate diet lead to its stimulation and a subsequent increase in plasma triglycerides [Kersten S, 2001]. Glucose is taken up by adipocytes via the insulin-dependent glucose transporter 4 (GLUT-4) and undergoes glycolysis to provide the substrate required for lipogenesis, glycerol-3-phosphate (glycerol-3-P). Lipid droplets of triacylglycerols are synthesized by esterification of glycerol-3-P from FFAs delivered to AT from the liver within VLDLs (very low-density lipoproteins) and chylomicrons from the intestine [Coehlo M, 2012]. AT-secreted hormones are involved in the regulation of lipogenesis, playing either stimulatory (angiotensin, acylation stimulating protein (ASP)) or inhibitory (leptin) roles. The opposite process, termed lipolysis, describes the hydrolytic breakdown of fat stores in the form of triglycerides into FFAs and glycerol to create energy. FFAs can be used as an energy source by other tissues by β -oxidation which converts them into acetyl coenzyme A molecules [Bemhlor DA, 2002, Coehlo M, 2012]. The main enzymes involved in lipolysis are hormone-sensitive lipase (HS-L) which is inhibited by insulin and monoacylglycerol lipase. The HS-L breaks the ester bonds in position 1 and 3 of the triacylglycerol molecules, with 2-monoacylglycerol lipase catalysing the hydrolysis of the remaining ester, leading to release of the FFA molecules and glycerol. FFAs are then bound to plasma albumin and

transported to peripheral tissues (muscles, liver) where they undergo oxidation whereas glycerol is moved back to the liver where it can be utilised for gluconeogenesis or oxidation [Coehlo M, 2012].

2.4.2 Adipose tissue as a secretory organ

As mentioned above, AT is now considered as a potent endocrine tissue which secretes a variety of molecules named adipokines. These play important roles in the regulation of metabolism and inflammatory responses. They can be broadly divided into those secreted mainly by adipocytes such as leptin, adiponectin, resistin and chemerin and those secreted by AT resident immune and endothelial cells such as TNF α , IL6, MCP1, PAI1 or visfatin [Coehlo M, 2012]. A detailed analysis of all of these secreted factors is beyond the scope of this chapter hence only selected adipokines are described below. Table 1.2 summarises the main biological functions of the principal adipokines .

Adipokine	Biological role
Leptin	Food intake, energy expenditure, reproduction, immune response, β -cells function, angiogenesis, haematopoiesis
Adiponectin	Insulin sensitivity, energy expenditure, inflammation suppression; protective role against T2DM and CVD
Resistin	Possible role in insulin resistance; inflammation
IL6	Pro-inflammatory; lipid and glucose metabolism; regulation of body weight
TNF α	Affects insulin receptor signalling, insulin resistance, inflammation, lipolysis, cell survival, adipokine action
Adipsin	Links complement pathway with AT metabolism
IGF-1	Stimulation of proliferation of various cells; mediates effects of growth hormone
PAI-1	Inhibits fibrinolysis by blocking activation of plasminogen; vessel wall remodelling; adipokine action
Angiotensinogen	Regulation of electrolytes and blood pressure
ASP	Affects the rate of triacylglycerol synthesis in AT
VEGF	Angiogenesis stimulation
FGF21	Browning of WAT, thermogenesis
TGF β	Regulation of AT differentiation, fibrosis and endocrine and metabolic functions
Chemerin, apelin, visfatin, MCP-1	Inflammation

TNF α – tumour necrosis factor α , IL-6 – interleukin-6, PAI-1 – plasminogen activator inhibitor 1, ASP – acylation stimulating protein, VEGF – vascular endothelial growth factor, IGF-1 – insulin-like growth factor 1; TGF β - Transforming Growth Factor β ; MCP-1- monocyte chemoattractant protein

Tab.1.2: AT secreted molecules and their biological relevance.

Adiponectin

Adiponectin has been found to have a number of beneficial metabolic effects such as cardiovascular protection, improvement of insulin sensitivity, anti-inflammatory potential and stimulation of fatty acid oxidation and glucose uptake by peripheral tissues [Tomas, 2002, Haluzik, 2004]. Adiponectin is related to the complement 1q family and also shares some sequence homology with collagen VIII and X [Weisberg SP, 2006]. It is found in significant concentrations in human plasma compared to other adipokines (between 10-30 µg/mL) with no significant fluctuation; this suggests that its release is not regulated acutely but rather is determined by long-term metabolic status [Kadowaki T, 2005]. It circulates in the form of trimers (approx. 67kDa), hexamers (approx. 120kDa) and multimers (>300kDa) with the latter believed to have the strongest insulin-sensitising potential [Tsao T, 2002, Waki H, 2003]. Each monomer of adiponectin consists of 3 domains: an α-helical stalk of multiple G-X-X repeats, an approximately 140 amino acid distinctive globular C-terminal, and a variable N-terminal [Galic S, 2010]. The interchange between various adiponectin isoforms does not occur once in the plasma as the isoform is determined at the stage of secretion from adipocytes [Schraw T, 2008]. Interestingly, a globular form of adiponectin has also been described (gAd) which is involved in activation of the AMPK pathway in skeletal muscles, leading to increased fatty acid oxidation and glucose uptake [Yamauchi T, 2002].

Adiponectin exerts its action on various tissues via adiponectin receptor 1 and receptor 2 [Yamauchi T, 2003, Yamauchi T, 2007] which each contain seven transmembrane domains, but which differ in structure and function and in binding to T-cadherin [Denzel MS, 2010]. Various metabolic pathways are activated following receptor binding which contribute to the insulin-sensitising effect: these include AMPK, calcium, PPARα, ceramide and S1P (sphingosine-1 phosphate) [Yamauchi T, 2007, Zhou L, 2009, Holland W 2011]. The liver is the crucial organ for adiponectin's involvement in energy homeostasis and insulin sensitivity where it has been shown to work in a number of ways: by reducing the expression of gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxylase [Nawrocki AR, 2006]; by enhancing ceramidase activity thus reducing hepatic ceramide content; and by affecting the biology of growth factors such as heparin-binding epidermal growth factor (HB-EGF), fibroblast growth factor (FGF), and platelet-derived growth factor [Wang Y, 2005]. Adiponectin concentration decreases in obesity and correlates inversely with BMI, triglycerides, glucose and insulin as well as visceral fat accumulation [De Rosa A, 2013]. It plays an important role in preserving endothelial function and vascular remodelling, thus exhibiting antiatherogenic properties [Iwashima Y, 2004, Di Chiara T, 2014].

Leptin

Leptin was the first adipokine to be described [Zhang Y, 1994]. It is known as the “satiety hormone” as it has the ability to cross the blood-brain barrier to work on the hypothalamus and regulate food intake [Zhang Y, 1994, Morton GJ, 2011]. Following leptin’s binding with its receptors, orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons are inhibited [Schwartz MW, 1996]. Feeding is also controlled by regulation of other orexigenic neuropeptides, such as melanin-concentrating-hormone (MCH), galanin, orexin and galanin-like peptide [Schwartz MW, 1996], and anorexigenic ones such as POMC, cocaine and amphetamine-regulated transcript (CART), neurotensin, corticotropin-releasing hormone and brain-derived neurotrophic factor (BDNF) [Liao GY, 2012]. Mice studies have shown that ob/ob mice (with deficiency of leptin) or db/db (with leptin receptor dysfunction) suffer from decreased energy expenditure and early onset obesity [Zhang Y, 1994]. Plasma and AT leptin levels are dependent on the overall energy balance, hence obese individuals have higher concentration of plasma leptin and similarly fasting leptin levels are lower than in the post-prandial state [Coehlo M, 2012]. Females have been found to have higher levels of plasma leptin which can be explained by the inhibitory effect of androgens and stimulating effect of oestrogens but also by AT depot-related differences in leptin synthesis, which is greater in subcutaneous fat (higher in females) [Coehlo M, 2012].

Interestingly, apart from involvement in the regulation of energy expenditure and appetite, leptin receptors are expressed in various peripheral tissues suggesting its role is much more complex. For instance, leptin also has the ability to affect pancreatic β cells modulating insulin secretion [Marroqui L, 2012], to regulate immune responses [Naylor, 2016] and to act on reproductive organs [Perez-Perez A, 2015].

Despite its huge potential use in the management of obesity, the results from the use of recombinant leptin have thus far been disappointing [Farooqi IS, 1999] whilst clinical trials of the leptin sensitizer pramlintide demonstrated an unacceptable side-effect profile [Bluher M, 2014].

TNF α

Adipocytes are capable of synthesising and secreting TNF α , but it is the stromal-vascular fraction containing macrophages that is the main source of this cytokine within AT [Coehlo M, 2012]. Interestingly, visceral AT contains more macrophages than subcutaneous AT [Trzeciak-Rydzek A, 2011] which is one of the reasons visceral AT is believed to play a more important role in obesity pathogenesis. TNF α was the first described link between obesity, chronic inflammation and diabetes as this cytokine has been shown to impair insulin signalling in hepatocytes and adipocytes [Cai D,

2005], and decrease insulin-stimulated glucose uptake in skeletal muscles [Galic S, 2010]. Rodent studies revealed that neutralisation of TNF α improves insulin sensitivity in individuals without established T2DM, but it does not exhibit this action once T2DM is established [Galic S, 2010]. TNF α activates serine kinases such as the c-Jun-N-terminal kinase (JNK) or inhibitor of NF- κ B kinase (IKK) and increases expression of suppressor of cytokine signalling 3 (SOCS3) which leads to inhibition of the signalling capability of insulin receptor substrate (IRS) [Coehlo M, 2012]. TNF α also reduces fatty acid oxidation in the liver which leads to accumulation of bioactive lipids such as diacylglycerols, in turn activating protein kinase C and inhibiting IRS [Galic S, 2010].

TNF α has been shown to have various effects on adipose tissue biology through activation of numerous signalling pathways: it induces insulin resistance in AT through suppression of PPAR γ and GLUT4 mRNA expression, affects expression of genes encoding proteins involved in responses to oxidative stress, decreases production of insulin-sensitising adipokines and promotes secretion of those with proinflammatory properties. It also inhibits FFA uptake and lipogenesis, stimulates lipolysis and suppresses the recruitment and differentiation of new adipocytes, thus diminishing AT lipid storage capacity [Cawthorn WP, 2008].

Interleukin 6 (IL-6)

AT contributes to approximately 30% of circulating plasma IL-6, with V-AT producing more IL6 than S-AT [Coehlo M, 2012]. IL-6 secretion stimulated by IL-1 and TNF α is increased in obesity, correlating with body weight and plasma free fatty acid concentration [Coehlo M, 2012]. Similarly to TNF α , the majority of AT-derived IL6 is secreted by the stromal-vascular fraction, with only around 1/3 produced by adipocytes. IL6 impairs insulin signalling by upregulation of SOCS3 and it also induces lipolysis, inhibits lipoprotein lipase and increases glucose uptake [Galic S, 2010].

Fatty Acid Binding Protein 4 (FABP4)

Fatty Acid Binding Protein 4 (FABP4), also known as aP2, belongs to a family of intracellular lipid chaperons that are involved in intracellular lipid trafficking and responses [Furuhasi M, 2008]. The expression of this protein is induced during adipogenesis and controlled by PPAR γ , FFAs, insulin and dexamethasone [Amri E, 1991, Distel RJ, 1992, Kletzien RF, 1992, Cook JS, 1998, Melki S, 1993]. Within AT, FABP4 accounts for about 1% of all soluble proteins [Baxa C, 1989]. FABP4 has also been found in macrophages and dendritic cells [Makowski L, 2001], and also in endothelial cells within small vessels in the heart and kidneys [Elmasri H, 2009].

Interestingly, it has been shown that FABP4 can be secreted from adipocytes via ER-Golgi independent pathways: through activation of lipolysis by phosphorylation of HSL and the NPR-A-

mediated GC-PKG (natriuretic peptide receptor A guanyl cyclase protein kinase G) pathway [Furuhasi M, 2014], and also through secretion by EVs [Nickel W, 2009]. Both of these mechanisms are upregulated by ionomycin which increases intracellular calcium and is downregulated by insulin [Kralisch S, 2014].

FABP4 has been shown to exert multiple extracellular roles such as enhancing hepatic glucose production [Cao H, 2013] and glucose-mediated insulin secretion from pancreatic β cells [Wu LE, 2014] or suppressing cardiomyocyte contractions *in vitro* [Lamounier-Zepter V, 2009]. In recent years plasma FABP4 has also emerged as a potent biomarker of metabolic dysfunction. It correlates positively with insulin resistance, waist circumference and blood pressure [Xu A, 2006], thus subjects with established T2DM, hypertension, cardiac dysfunction and atherosclerosis have higher circulating plasma levels than healthy controls [Yeung DC, 2007, Xu A, 2006]. FABP4 also appears to be a promising marker to predict future risk of developing cardio-metabolic dysfunction in apparently healthy individuals, as shown by recent 5- and 10-year prospective studies by Xu *et al* and Tsao *et al* [Tsa A, 2007, Xu A, 2007]. Therefore, pharmacological modifications of FABP4 could lead to development of a new class of drugs targeting obesity and its complications. Indeed, trials are currently being conducted to evaluate various strategies to inhibit or neutralise FABP4 [Furuhasi M, 2007, Sulsky R, 2007].

Fibroblast growth factor 21 (FGF21)

FGF21 has gained lots of attention in recent years due to its diverse biological functions as a hepato-adipo- and myokine [Luo L, 2016]. However, its exact mechanisms of action are not yet fully understood. As discussed above, cold exposure induces FGF21 which, via its receptor 1c and coreceptor β -klotho [Itoh N, 2010], affects the expression of thermogenic genes in the BAT and inguinal WAT [Fisher FM, 2012, Adams AC, 2013]. Though FGF21 'browning' and thermogenic effects are likely to have anti-obesogenic potential, circulating levels of this adipokine in obese subjects are elevated, suggesting possible resistance to its action in the presence of obesity [Chen W, 2008].

Resistin

As its name implies, resistin is believed to be implicated in the development of insulin resistance. It is secreted by adipocytes [Savage D, 2001] as well as monocytes and macrophages [Patel L, 2003] and circulates as low molecular weight complexes and hexamers, with the latter being its active form [Patel S, 2004]. Resistin secretion is stimulated by inflammation, LPS (lipopolysaccharide), hyperglycaemia or IL6. The effects of resistin on glucose metabolism is through its negative modulation of insulin signalling on glucose uptake and by increasing hepatic gluconeogenesis

[Coehlo M, 2012]. It is likely to play a role in adipogenesis as its expression is higher in preadipocytes than in mature adipocytes [Steppan C, 2007].

In rodents, resistin levels are significantly elevated in obesity and T2DM [Steppan C, 2001] but the results from human studies have been somewhat confusing, with some studies reporting similar associations [Oliver P, 2003, Gerber M, 2005] and others showing no significant correlation [Lee JH, 2003, Iqbal N, 2005]. Nonetheless, resistin has been shown to stimulate the production of pro-inflammatory cytokines and adhesion molecules such as IL6, TNF α , ICAM1, VCAM1 [Verma S, 2003], and to downregulate adiponectin signalling [Benomar Y, 2016].

Visfatin

Visfatin, also known as pre-B cell colony-enhancing factor (PBEF), is mainly produced by visceral adipocytes and visceral AT resident macrophages, with only small quantities being made in subcutaneous AT. Its expression is increased in mature adipocytes compared to preadipocytes [Coehlo M, 2012]. Visfatin is considered a pro-inflammatory cytokine which has the ability to activate leukocytes and increase secretion of IL6 and TNF α [Coehlo M, 2012]. On the cellular level, visfatin is involved in regulating energy metabolism by affecting NAD⁺ biosynthesis and the activity of NAD⁺/NADH dependent enzymes [Manolescu B, 2008]. Interestingly, visfatin can act as an insulin mimetic: in *in vitro* studies as well as mice studies it was shown to lower glucose levels after binding to the insulin receptor [Wang P, 2009].

Other adipokines

Amongst other AT secreted molecules, angiotensin, acylation stimulation protein (ASP) and plasminogen activator inhibitor 1 (PAI-1) are also worth mentioning given their potent biological roles. Angiotensin II was found to stimulate adipocyte differentiation, lipogenesis and prostacyclin synthesis, hence is thought to regulate adipocyte growth and differentiation [Coehlo M, 2012]. Interestingly, all components of the RAAS system are expressed by AT, including renin, angiotensinogen, angiotensin I-converting enzyme and the angiotensin II type 1 receptor [Ahima RS, 2000]. This suggests that in obese individuals these AT-derived peptides play a role in blood pressure regulation [Carey A, 2006].

PAI-1 is a serpin predominantly secreted by endothelial and vascular smooth muscle cells but other sites including AT, stromal cells, monocytes and macrophages have been shown to produce it as well. Visceral AT is more involved in PAI-1 production and the contribution of AT -derived PAI-1 into the total plasma circulating pool is determined by the total fat mass [Coehlo M, 2012]. As PAI-1 is

mainly involved in fibrinolysis and its levels correlate with visceral fat it is often considered as an important link between CVD and obesity [Coehlo M, 2012].

ASP (acylation stimulating protein) stimulates triacylglycerol synthesis in AT and augments glucose transport by affecting the translocation of glucose transporter type 4 (GLUT4). It also inhibits the actions of hormone specific lipase [Cianflone K, 2003]. Circulating plasma ASP levels are therefore increased in the postprandial state, obesity T2DM and CVD, whilst weight loss, exercise and starvation decrease circulating concentrations. Post-prandially, it is the subcutaneous AT depot that is involved in increased ASP production [Coehlo M, 2012].

2.5 AT dysfunction and obesity- overview

AT dysfunction leads to development of obesity and numerous related comorbidities such as cardiovascular disease (CVD), diabetes mellitus type 2 (T2DM) and malignancies. Obesity is considered as a state of chronic systemic low-grade inflammation [Itoh M, 2011]. The expansion of adipocytes leads to AT hypertrophy. Circulating monocytes are attracted to AT by a variety of cytokines secreted by adipocytes such as monocyte chemoattractant protein (MCP-1), macrophage migration inhibition factor (MIF-1), macrophage inflammatory proteins (MIP-1 α), chemokine CCL5 (RANTES) and macrophage colony stimulating factor (M-CS F) [Ouchi N, 2011]. The proinflammatory cytokines are secreted from M1 macrophages infiltrating the AT originating from circulating monocytes, with the number of macrophages infiltrating the AT correlating with fat mass [Coelho M, 2012]. This leads to dysregulated adipokine secretion, with an increase in the pro-inflammatory cytokines IL-6 and TNF α , as well as leptin, resistin and free fatty acids, and a decrease in adiponectin. These secreted adipokines work not only in a paracrine and autocrine manner but also affect systemic metabolic homeostasis by decreasing insulin sensitivity in liver and muscles. This results in increased gluconeogenesis and glycogenolysis in the liver, and low glucose uptake and fatty acid oxidation in the muscles [Coelho M, 2012]. Repeated cycles of these processes eventually lead to an increase in plasma glucose, insulin resistance and its clinical sequelae. (Fig.1.3)

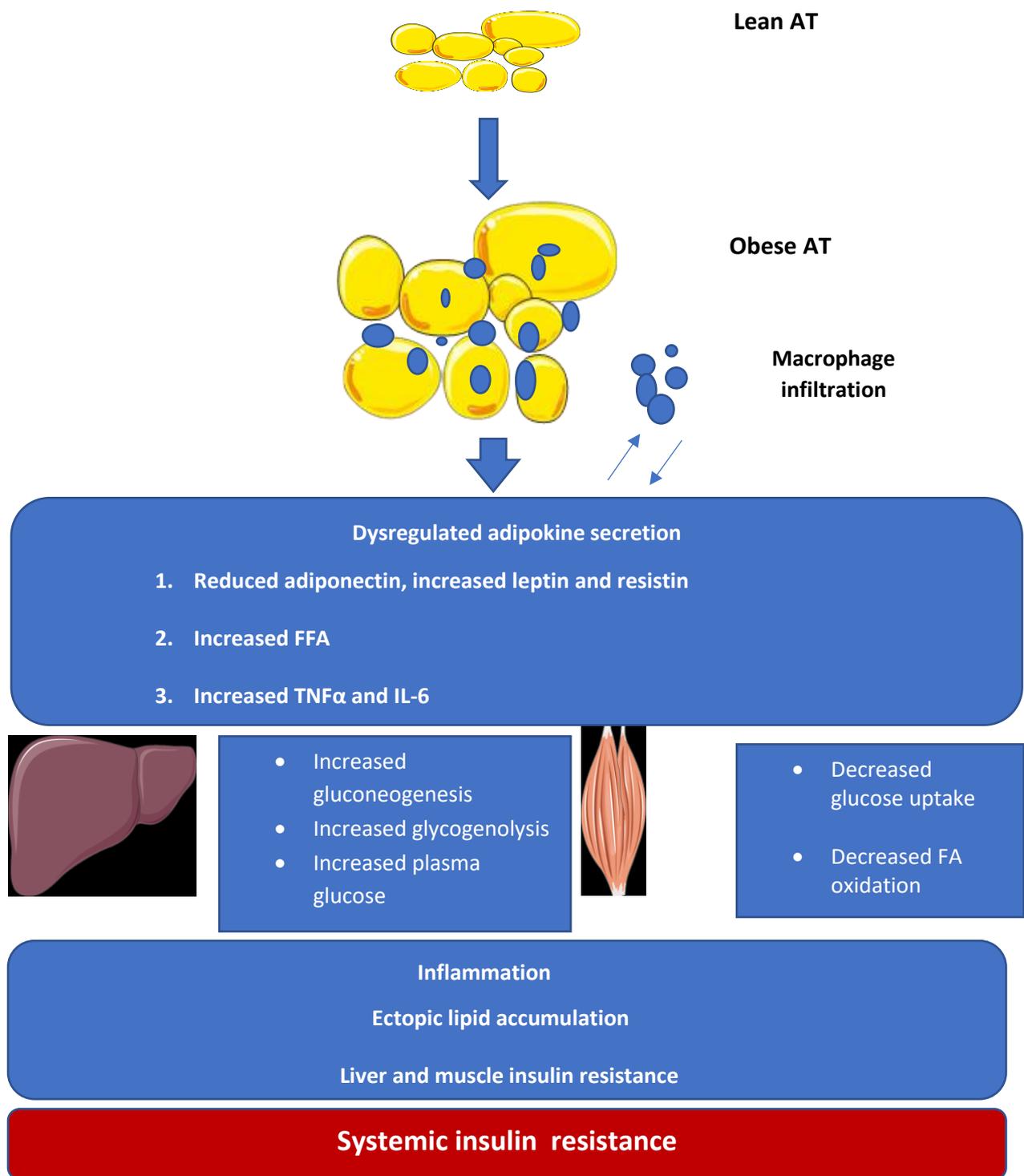


Fig.1.3: Development of metabolic complications of obesity.

Expansion of AT results in adipocyte hypertrophy and release of chemokines that lead to macrophage infiltration of AT, which further enhances local inflammation due to increased release of cytokines. This is accompanied by increased release of FFA and dysregulated secretion of leptin, adiponectin and resistin. These local AT changes lead at

the systemic level to increased ectopic lipid deposition and inflammation, which results in decreased muscle and liver insulin sensitivity and increased hepatic gluconeogenesis and glycogenolysis. This is accompanied by reduced glucose uptake and low FFA oxidation in the muscles leading to increased availability of glycerol as a substrate for liver.

Subsequently, plasma glucose rises, and insulin resistance increases. (Adapted from: Galic *S et al.*, 2010)

2.6 Obesity

2.6.1 Epidemiology

The prevalence of obesity is rising worldwide, placing a significant burden on health care systems. It is estimated that by 2030 there will be 11 million more obese adults in the UK which will be associated with additional health care costs of £1.9-2billion/year [Wang C, 2011]. This is due to the fact that obesity leads to numerous chronic debilitating health conditions such as T2DM, CVD, malignancies, mobility problems and decreased quality of life.

It is estimated that 26% of the adult population in the UK is obese (Body Mass Index (BMI) $>30\text{kg/m}^2$), with the most prevalent regions being the North of England and the Midlands, and most prevalent age groups being 45-74 yrs for men and 45-84 yrs for women [Niblett P, 2018]. Wales has a reported prevalence of obesity of 23%, with a further 36% of the population being reported as overweight. Being obese or overweight is more prevalent amongst men in Wales (65%vs 53%) with obesity rates highest in the most deprived areas and in 54-64 years olds (67%) [Baker C, 2018].

According to the National Health Service (NHS) statistics, in England in 2016/17 there were 10,705 hospital admissions with a primary diagnosis of obesity and 617,000 admissions where obesity was a factor, representing an increase of 8% and 18% respectively compared to the previous year [Niblett P, 2018]. Interestingly, in both cases there was a clear predominance of female patients (72% and 66% respectively).

2.6.2 Pathophysiology

Obesity is a complex condition resulting from numerous metabolic disturbances driven by excess energy provision from the diet and inadequate energy expenditure, which leads to disturbed glucose and lipid metabolism. In evolutionary terms, food storage in the form of fat ensured substrate provision at times of starvation. The change in food accessibility in many societies and change in

behaviours and attitudes to food, including binge eating and food addiction disorders as well as sedentary lifestyle, have led to excessive fat storage resulting in obesity.

Excessive fat storage in the form of triacylglycerol leads to increased lipolysis (enhanced by increased sympathetic drive) which releases FFAs from the fat depots and causes oxidative stress to the mitochondria and endoplasmic reticulum (ER) of various tissues such as AT, pancreas and liver; this contributes to development of the metabolic syndrome [Redinger R, 2007, Hutley L, 2005]. Moreover, circulating FFAs then inhibit lipogenesis with subsequent development of hypertriglyceridaemia secondary to decreased clearance of serum triacylglycerol. FFAs are also released by endothelial lipases from circulating triglycerides which exacerbates the lipotoxicity further and eventually leads to dysfunction of insulin receptors, insulin resistance and subsequent hyperglycaemia [Redinger R, 2007]. This is further exacerbated by enhanced hepatic gluconeogenesis and decreased utilisation of glucose by muscles. Eventually the prolonged lipotoxicity from excess FFAs leads to gradual β cell exhaustion in the pancreas [Redinger R, 2007].

2.6.3 Obesity and inflammation

Various forms of immune dysfunction have been described in obesity ranging from higher infection rates and impaired wound healing through to changes in white blood cell counts, with higher leukocytes, neutrophils, monocyte and lymphocyte counts but diminished T and B cell mitogen-induced proliferation and decreased antigen production in vaccinated obese patients [Marti A, 2001].

As mentioned previously, adipose cells, particularly visceral adipocytes and resident AT immune cells, secrete a range of active adipokines. These play an important role in the development of the metabolic consequences of obesity due to paracrine as well as systemic actions, since they are transported via the systemic circulation to various peripheral sites and reach the liver via the portal vein. Elevated levels of cytokines such as CRP, IL6 and TNF α have been described in obese and overweight individuals. Plasma levels correlate with measures of visceral adiposity such as waist circumference or waist:hip ratio (WHR) [Festa A, 2001], including in lean individuals [Lapice E, 2009].

Interestingly, white adipocytes and immune cells share a common embryonic origin. Moreover, AT has been described as a site for formation and maturation of immune cell precursors [Poglio S, 2010]. Rodent studies have shown that adipose tissue in obese mice is more significantly infiltrated by macrophages compared to lean ones [Weisberg SP, 2003]. Furthermore, 2 types of AT resident macrophages were described : the proinflammatory M1 (classically activated) and the anti-inflammatory M2 (alternatively activated) with the shift from M2 to M1 phenotype observed in

obesity [Lumeng C, 2007]. The proinflammatory macrophages secrete monocyte chemoattractant protein 1 (MCP-1), macrophage migration inhibiting factor (MMIF) and resistin, which enhance insulin resistance. As immune stimulators, they enhance the mitogen activated protein kinase family and activate NF κ B, leading to dephosphorylation of IRS-1 and 2 (Insulin Receptor Substrate 1 and 2) docking proteins, inhibition of GLUT4 and consequent insulin resistance [Tham D, 2002, Redinger R, 2007]. Apart from macrophages, other resident immune cells within AT include: IL-10 producing invariant natural killer T (iNKT) cells and T regulatory cells (Tregs), IL-4 producing eosinophils and Ig-M producing B cells with loss of iNKT and Tregs described during adipose tissue expansion in obesity [Exley MA, 2014].

The exact mechanisms linking obesity with chronic systemic inflammation are not clear but are likely to involve various pathways. Firstly, as discussed earlier, in obesity there is an imbalance between the levels of the anti-inflammatory 'protective' adipokine adiponectin and pro-inflammatory leptin, which has a feedback effect on the production of other AT-secreted adipokines. Increased levels of FFAs can also induce inflammation through lipotoxic effects on other tissues. Furthermore, differences in FFA composition have been described between lean and obese individuals [Pietilainen KH, 2011]. FFAs can affect the production and secretion of adipokines and also act directly on cell receptors involved in inflammatory pathways, particularly through acting as ligands for PPAR γ . PPAR γ is a transcription factor with a number of metabolic effects including modulation of adipocyte differentiation and M2 to M1 macrophage transition, regulation of cell metabolism and transcription factor activity such as that of NF- κ B [Coll T, 2010]. Subsequently, a vicious cycle of metabolic processes develops whereby increased proinflammatory cytokines raise circulating FFAs with upregulated lipolysis and diminished lipogenesis within AT, and concomitantly increased lipogenesis in muscles and liver [Mei M, 2011, de Heredia FP, 2012].

ER stress has also been implicated in the development of the chronic inflammation of obesity. As the ER is the main site for protein and triacylglycerol droplet synthesis within cells, a state of excessive nutrient availability puts stress on this cellular structure which leads to activation of 'the unfolded protein response' (UPR) which is a mechanism leading to increased production of reactive oxygen species and thus increased oxidative stress. This then activates inflammatory pathways leading to secretion of proinflammatory cytokines such as TNF α , IL6 and IL8 or MCP-1 [de Heredia FP, 2012].

Adipose tissue hypoxia resulting from inadequate vascular supply to hypertrophied adipocytes also leads to production of proinflammatory molecules to stimulate local angiogenesis. These processes are regulated by HIF1 α (Hypoxia Inducible Factor 1) which, as indicated by its name, is a

transcription factor unstable in normoxia but stable in hypoxia that regulates a variety of genes involved in metabolism and inflammation [Trayhurn P, 2010].

Proinflammatory macrophages accumulate around hypoxic areas in AT, whilst increased lactate production from anaerobic glycolysis also stimulates inflammatory responses [de Heredia FP, 2011] and increases ER stress. As a consequence of these processes, AT becomes fibrosed which then further exacerbates AT dysfunction [Halberg N, 2009].

All of these mechanisms appear to act synergistically to stimulate chronic systemic inflammation, and feedback positively on each other.

2.6.4. Adiposity and atherosclerosis

Chronic low-grade inflammation is also involved in the development of atherosclerosis which subsequently leads to the development of CVD, the leading cause of death globally [Mentis S, 2014]. Adipokines, including perivascular fat depots such as PAI-1, VEGF, angiotensinogen, renin and angiotensin II, are implicated in the development of vasomotor dysfunction and endothelial injury. This subsequently leads to formation of foam cells resulting from uptake of oxidised low density lipoproteins, FFAs and other lipid metabolites into endothelium. Furthermore, a decrease in nitric oxide (NO) in obesity secondary to increased oxidative stress and the effects of proinflammatory cytokines, leads to vasoconstriction and increased vascular resistance [Poirier P 2005]. Moreover, proinflammatory cytokines such as IL6 inhibit the activity of endothelial and adipose lipoprotein lipase which results in increased plasma triglyceride levels [Redinger R, 2007]. Macrophages infiltrating smooth muscle cells secrete MCP-1, MMIF and endothelin-1 which further fuels the inflammation within the atheromatous plaque. This is further exacerbated by adipokines with prothrombotic properties such as TNF α , PAI-1, TGF β , and IL6 and matrix metalloproteinases, also secreted by WAT which take part in collagen remodelling. The cascade of events finally leads to thinning of the atheroma cap and plaque rupture, release of tissue factor and intravascular thrombosis which leads to clinical presentation of CVD in the form of acute coronary syndrome or stroke.

2.6.5 Metabolic syndrome and cardiovascular disease in obesity

The main complication of the chronic low-grade systemic inflammation triggered by obesity is the development of metabolic syndrome which consists of impaired glucose metabolism exacerbated by TNF α and other inflammatory adipokines, hypertension resulting from enhanced endothelial vasomotor tone affected by AT-secreted renin, angiotensinogen and angiotensin II, and dyslipidaemia (hypercholesterolaemia and hypertriglyceridemia) [Redinger R, 2007]. These conditions combined with ongoing lipotoxicity from FFAs finally lead to the development of

atherosclerosis and cardiovascular disease, as discussed previously. Moreover, the effects of adipose tissue mass on the cardiovascular system contribute further to the development of comorbidities since the total adipose tissue mass can constitute a significant proportion of total body weight in obese individuals, which has a significant effect on an increase in cardiac output, total metabolic demand and cardiac workload [Alpert MA, 2001]. The increase in left ventricle filling pressure and volume leads to chamber dilatation, increase in cardiac wall stress and muscle mass, and eventually eccentric hypertrophy [Meserli FH, 1986]. Left atrial dilatation is also commonly observed which is linked with a risk of atrial fibrillation in obesity [Wang TJ, 2004]. Obese subjects are therefore susceptible to develop both systolic and diastolic left ventricular dysfunction [Poirier P, 2000]. Furthermore, adipositas cordis (“fatty heart”) which develops secondary to excessive epicardial fat and fatty infiltration of the myocardium leads to cardiac conduction defects due to replacement of the sinus node musculature, atrioventricular node and right bundle branch by fat [Balsaver AM, 1967]. Additionally, fat infiltration also leads to development of a restrictive cardiomyopathy [Dervan JP, 1991]. Apart from increased risk of acute coronary syndrome and ischaemic stroke, obesity is also associated with increased risk of venous thromboembolism, pulmonary emboli, hypertension, sleep apnoea and pulmonary hypertension [Poirier P, 2005].

2.6.6. Obesity and Cancer

Obesity is an important risk factor for many types of cancers such as colon, breast, endometrium, prostate, hepatocellular, renal and oesophageal malignancies [Redinger R, 2007]. Various mechanisms appear to be involved in this process depending on the type of malignancy, including chronic low-grade inflammation, angiogenesis, perturbed cellular proliferation, dedifferentiation and apoptosis, with adipokines such as leptin and insulin growth factor-1 believed to play an important role [Kim S, 2006]. The first insight into the causative relationship between obesity and cancer was provided by Schoen *et al* who reported that hyperinsulinaemia in obese patients is a risk factor for bowel cancer [Schoen RT, 1999]. Non-alcoholic fatty liver disease (NAFLD) which is common in obesity and can progress to liver cirrhosis, is associated with the development of hepatocellular carcinoma, with high circulating levels of leptin in obesity believed to play a stimulating role for the growth of these malignant cells [Wang SN, 2006]. The chronic oesophageal inflammation resulting from acid reflux, again common in obesity and exacerbated by hiatus hernia, is a consequence of decreased oesophageal sphincter tone from pressure from visceral adiposity. This leads to Barrett’s oesophagus and subsequently oesophageal metaplasia [Chow W, 1998]. Chronic pancreatic inflammation resulting from gluco- and lipotoxicity leads to pancreatic dysplasia and development of pancreatic adenocarcinoma [Calle E, 2004]. Genetic and environmental factors are also likely to play

a role in the development of various malignancies in obese individuals. More information to explain the pathogenesis of these cancers is still needed.

2.7 Obesity management

2.7.1 Lifestyle modification

The aim of lifestyle intervention is to aid weight loss by decreased caloric intake in order to create a negative energy balance and increased energy expenditure through exercise. According to the American Heart Association, in order to maintain health, moderate exercise of 30 minutes 5 times a week is required [Haskell W, 2007]. Numerous studies to date have evaluated the effects of lifestyle intervention on various health markers/outcomes. Though dietary intervention alone is likely to lead to weight loss, the results are difficult to maintain without the addition of regular exercise [Tate D, 2007] and the long-term cardiovascular benefits are not clear. The recent LookAhead trial was designed to compare intensive lifestyle intervention (ILI) to Diabetes Support and Education (DSE) in obese T2DM patients with regard to CVD risk over time. It consisted of 175 minutes /week of unsupervised exercise and reduction in caloric intake to either 1200–1500 Kcal/day with 40–50 gm of fat for initial weights of <250 lbs or 1500–1800 kcal/day with 50–60 gm of fat for initial weights >250 lbs [Wadden T, 2006]. ILI led to 8.6% weight reduction in the first year which was maintained at 6% at the end of intervention (median 9.6 years). It was accompanied by improved glycaemic control and lipid profile, reduction in liver fat and severe kidney disease, improvement in OSA and sexual dysfunction, reduced need for diabetes medications, maintenance of physical mobility and overall care costs, as well as improved quality of life. Surprisingly, however, there was no effect on cardiovascular outcomes between the groups [Pi-Sunyer X, 2014].

Unfortunately, adherence to lifestyle modification in obese subjects is quite poor and often limited by various social and psychological barriers. The long-term maintenance of weight following weight loss is usually low, ranging from 2 to 20% [Blackburn G, 2005]. Therefore, alternative treatment modalities, as described below, have been intensively explored in recent years.

2.7.2 Pharmacotherapy for obesity

The development of a safe and effective pharmacotherapy approach to treat obesity has been quite challenging, partly because obesity as discussed above is a complex condition affecting various metabolic pathways, hence monotherapy with an agent targeting a single protein or mechanism will have limited efficacy. According to Federal Drug Agency (FDA) criteria a weight loss agent is believed to be effective if it induces >5% loss of body weight compared to placebo or if >35% of study participants lose >5% of their initial body weight [Narayanaswami V, 2017]. Generally, pharmaceutical agents in obesity management can be divided into 3 groups: a) medication that acts

centrally to decrease food intake b) medication that decreases fat absorption peripherally c) agents that increase energy expenditure [Narayanaswami V, 2017]. Unfortunately, many of the therapies that appeared promising in recent years were terminated due to unfavourable cardiovascular or psychiatric side-effects. Therefore, at present there are only 5 effective FDA-approved therapeutic agents used for the treatment of obesity: orlistat, liraglutide, phentermine/topiramate, lorcaserin and naltrexone/bupropion [Homayoun A, 2016].

Phentermine (2-methyl-1-phenylpropan-2-amine) is a sympathomimetic that has been available for over 50 years for short-term (less than 12 weeks) obesity management in addition to lifestyle modifications. The most common side-effects are insomnia, dry mouth, palpitations/tachycardia and hypertension but data from long safety and efficacy trials are not available. A recent placebo-controlled study by Kang *et al* on slow release 30mg phentermine used in obese individuals, showed improvement in their lipid profile (cholesterol and LDL) and mean body weight reduction by 8kg (compared to 2 kg in the placebo group) [Kang JG, 2010].

Since 2012, phentermine immediate release has been also available in combination therapy with topiramate (2, 3, 4, 5-bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate), a GABA agonist initially developed for the management of epilepsy but found to have appetite-suppressing properties aiding weight loss [Ben-Menachem E, 2003]. It is not clear whether the results observed are due to increased GABA-A receptor stimulation, and/or inhibition of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor)/kainite glutamate receptors or the effects on voltage gated ion channels [Richard D, 2000]. Topiramate has since been effectively used in treating binge eating disorder [McElory S, 2007]. The combination of 46mg of topiramate with 7.5mg of phentermine led to 7.8% weight loss compared to 1.2 % in the placebo arm after 1 year of treatment [Gadde K, 2011] and was also shown to improve glycaemic control in patients with T2DM as well as lipid profile and blood pressure control [Allison D, 2012].

Orlistat ((S)-((S)-1-((2S, 3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl) 2-formamido-4-methylpentanoate) is a lipase inhibitor that impairs absorption of dietary fat by inhibiting gastrointestinal lipases (gastric and pancreatic). Therefore, it is commonly associated with gastrointestinal side-effects such as flatulence, bloating, abdominal pain and diarrhoea. Treatment can also lead to deficiency of fat-soluble vitamins (A, D, E, K) hence supplementation is recommended [Padwal RS, 2007]. Cases of severe liver failure and acute kidney injury have also been rarely reported [Karamadoukis L, 2009].

Lorcaserin ((1R)-8-chloro-1-methyl-2, 3, 4, 5-tetrahydro-1H-3-benzazepine) is a selective 5-HT_{2c} agonist approved for use in conjunction with lifestyle modification at a dose of 10mg twice daily in

people with BMI of ≥ 30 , or ≥ 27 kg/m², with at least one weight-related comorbidity, such as hypertension, T2DM or dyslipidaemia [Narayanaswami V, 2017]. However, it should be discontinued if weight loss of $>5\%$ is not achieved after 12 weeks. Lorcaserin acts centrally on 5-HT_{2C} receptors in POMC (proopiomelanocortin) neurons of the arcuate nucleus and promotes hypophagia by increasing satiety [Lam D, 2008]. A 1-year, randomised, double-blind, placebo-controlled trial by O'Neill *et al* in obese patients with T2DM showed that 38% of the participants in the intervention arm achieved $>5\%$ body weight loss. After 52 weeks of treatment the intervention group lost 4.5% of initial body weight compared to 1.5% in the placebo arm [O'Neill, 2012].

Side-effects include back pain, headache, cough and hypoglycaemia, hence modification of diabetes treatment regimen is required in patients with diabetes. As it stimulates the serotonergic system, lorcaserin can also potentially lead to serotonin syndrome, especially in patients using other serotonergic drugs which are quite commonly prescribed, hence more safety studies are required. The lorcaserin side-effect profile is, however, significantly better than that of dexfenfluramine (a non-selective 5HT agonist) which led to hallucination, cardiac valvopathies and pulmonary hypertension, and was eventually withdrawn [Narayanaswami V, 2017].

Naltrexone (((4R,4aS,7aR,12bS)-3-(cyclopropylmethyl)-4a,9-dihydroxy-2,4,5,6,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7-one) and Bupropion (((±)-2-(tert-butylamino)-1-(3-chlorophenyl)propan-1-one) is another new combined therapy option for obesity management. Naltrexone is a non-selective opioid receptor antagonist whilst bupropion inhibits DA (dopaminergic) and NE (norepinephrine) transporters. Both agents were previously used in the therapy of addictions. It is not exactly understood how weight loss is achieved by using these agents, but the proposed mechanisms of action include modulation of homeostatic (hypothalamic melanocortin system) and non-homeostatic systems (mesolimbic DA reward system) [Narayanaswami V, 2017]. The first proposed mechanism suggests that bupropion stimulates release of α -MSH (melanotropin) which binds to MC4Rs (melanocortin 4 receptors) leading to decreased oral intake and increased energy expenditure. Simultaneously, β -endorphins are secreted which normally act by negative feedback to prevent further α -MSH secretion. However, as naltrexone blocks opioid receptors, the negative feedback mechanism is impaired and POMC activity is increased which causes subsequent weight loss [Billes S, 2014]. The DA reward system is also likely involved as shown by an fMRI study by Wang *et al* where obese subjects treated with bupropion/naltrexone for 4 weeks showed reduced activation in the hypothalamus and increased activation in regions involved in inhibitory control (anterior cingulate), internal awareness (superior frontal, insula, superior parietal) and memory (hippocampal) when exposed to food cues [Wang G, 2014].

Apovian *et al* showed in their double-blind, placebo-controlled randomised study that obese individuals with hypertension and/or dyslipidaemia following 56 weeks of therapy with naltrexone 32 mg/day and bupropion 360 mg/day achieved weight loss of 6.4% compared to 1.2% in the placebo group which was also associated with improvement in cardiometabolic risk factors and quality of life [Apovian C, 2013]. The side-effect profile consisted of nausea and vomiting, dizziness, constipation/diarrhoea and headache, but a black box warning has also been issued regarding increased risk of suicidal behaviour and neuropsychiatric symptoms [Narayanaswami V, 2017].

Liraglutide is a long-acting GLP-1 receptor analogue given subcutaneously, initially developed for the therapy of T2DM (dose 1.2 and 1.8mg) which has later been approved to be used at 3mg daily as a weight loss agent in individuals with at least one weight-related comorbidity [Narayanaswami V, 2017]. A 56-week randomised trial showed reduction of body weight of 8.1% compared to 2.7% in the placebo group with associated improvement in cardiometabolic risk factors, glycaemic variables, and β cell function and insulin sensitivity [Pi-Sunyer X, 2015]. The side-effect profile includes dizziness, nausea and vomiting, headache, constipation/diarrhoea, dyspepsia and fatigue with serious side-effects including acute pancreatitis, bronchitis and chest pain [Narayanaswami V, 2017]. Rodent studies indicate that this agent may lead to the development of medullary thyroid cancer though there are no data from human subjects on this to date [Narayanaswami V, 2017].

2.7.3 Bariatric surgery

Bariatric surgery is the only treatment modality for obesity at present which is shown to have established long-term efficacy. The most frequently performed procedures are: gastric banding (GB), Roux-en Y gastric bypass (RYGB) and laparoscopic sleeve gastrectomy (SG) [Samuel I, 2006]. Weight loss is achieved by a reduction in caloric intake through restrictive and malabsorptive consequences of the procedures, but the exact mechanisms involved are complex and include neurophysiological and neuroendocrine pathways [Zhang Y, 2014]. The RYGB procedure, the most complex of the bariatric interventions, has been extensively evaluated in order to define all the metabolic changes leading to rapid weight loss postoperatively. It was shown that it affects the gut peptide profile with a reduction in ghrelin and increase in GLP-1 and PYY (peptide YY) which promotes satiety and diminishes hunger [Bose M, 2010]. Interestingly, however, changes in brain activation, taste preferences and desire to eat have also been reported. Functional MRI studies (fMRI) by Ochner *et al* in female patients 1-month post-RYGB revealed a reduction in brain activation within the mesolimbic reward pathway in response to high calorie food cues as well as diminished visual and auditory whole-brain activation [Ochner C, 2011]. The altered perception of reward may play a role here as some RYGB patients report that high-fat and /or high-carbohydrate foods are no longer enjoyable post-surgery [Halimi K, 1981].

The long-term effects of bariatric surgery are accompanied by improved cardiovascular outcomes in this group of individuals which was also observed in our local population. My colleagues and collaborators, Min *et al* showed that bariatric surgery in individuals with impaired glucose metabolism, leads to significant reduction in predicted 10-year and lifetime CVD risk evaluated by QRISK score (24 and 24.5 % reduction, respectively) [Min T, 2017].

2.7.4 Faecal microbiota transplantation

In recent years more evidence has emerged regarding an important role of gut microbiota in the regulation of metabolism, insulin signalling and low-grade inflammation, suggesting involvement in the development of obesity [Baothman O, 2016]. Patients with T2DM were shown to have gut microbial dysbiosis: an increase in opportunistic pathogens and decrease in butyrate-producing bacteria [Qin J, 2012]. Interesting results from rodent studies showed that germ-free mice develop increased total body fat mass when colonised with microbiota from obese mice, compared to those colonised with 'lean' microbiota [Backhead F, 2004]. Results of human studies are unclear, not least due to the impracticalities and acceptance of this treatment. Hence, faecal transplantation is currently used in clinical practice only in the treatment of refractory, severe *Clostridium difficile* infection. However, van Reenen *et al*, conducted a double-blind, controlled trial in men with metabolic syndrome (n=18) who either received faecal transplant of their own faeces (placebo) or faeces donated by lean volunteers (intervention) which led to reduced fasting triglycerides and improved peripheral insulin sensitivity in the intervention arm [van Reenen, 2011]. Given the advent of 'natural' therapies in recent years, faecal transplantation may become more common in the near future once more evidence emerges.

2.8 Study hypothesis and aims

As discussed above, EVs are increasingly recognised as markers of diseases and potent biological vectors. Due to the lack of gold standard methods for EV isolation, and the inability to separate EV subpopulations of interest, more studies are required to understand the role of EVs in various processes such as the development of obesity and its associated complications. Although EVs have been found to be elevated in various conditions associated with obesity such as CVD and cancer, very few studies have examined EVs in the healthy state, and the effects of surgical or non-surgical treatment modalities on EV characteristics. Moreover, due to technical challenges, there are very few studies evaluating adipocyte-derived EVs obtained from human AT explants. Given how active AT is as an endocrine organ, characterisation of this particular population of EVs could provide valuable information regarding complex pathways behind obesity-driven systemic complications.

I hypothesised that EV profile in plasma from healthy subjects may vary regarding EV concentration, cellular origin and adipokine content depending on the BMI/waist circumference and that individuals who are overweight or obese as per the WHO criteria [WHO, 1995] may show subtle differences in their EV profile before overt metabolic consequences of increased adiposity develop. In particular, I postulated the obese subjects have increased expression of FABP4-expressing EVs (this adipokine has been used by our research group as a marker of AT-derived EVs in circulation) as well as platelet- and endothelial cell-derived EVs. I believed these changes would be accompanied by differences in circulating free adipokine levels such as adiponectin, IL6 and FABP4.

Secondly, I hypothesised that the circulating EV concentration, cellular origin and content of healthy subjects would differ significantly from that in individuals suffering from morbid obesity with established metabolic complications.

I also postulated that intervention aimed at reducing weight in morbidly obese subjects would lead to alteration in the EV concentration, cellular origin and EV-expressed adipokines, with decreased expression of those with proinflammatory properties.

Finally, given the differences in the biological properties of V-AT and S-AT, I hypothesised that EVs derived from these fat depots may show differences in their adipokine content.

To address this, the main aims of this thesis are:

- To evaluate circulating EVs in a cohort of metabolically healthy volunteers across a range of BMI
- To compare the EV profile of healthy versus severely obese subjects
- To assess the effects of weight loss on circulating EVs:
 - a) In patients undergoing bariatric surgery
 - b) In patients undergoing a lifestyle modification programme

Additionally, using *in vitro* experiments on subcutaneous and visceral AT explants I sought:

- To harvest and characterise human adipose tissue-derived EVs (ADEVs) from subcutaneous and visceral AT depots

Chapter 2

General Methods

2.1 Study approvals

All studies presented in Results Chapters (3-6) have received relevant ethical approvals, the details of which are described later.

2.2 Study support

The research work conducted for this MD thesis was supported by a Lewis Thomas Gibbon Jenkins of Britton Ferry Fellowship from the Royal College of Physicians, Wales. The research work was conducted at the following sites: Cardiff University (Wales Heart Research Institute; Centre for Endocrine and Diabetes Sciences), Cardiff Metropolitan University (Department of Biomedical Sciences), and Cardiff and Vale University Health Board (Out-patient and Biochemistry Department at University Hospital Llandough).

2.3 Study participants

As the study cohorts in each results chapter differ, their characteristics and exclusion and inclusion criteria are described in the relevant results chapters. All participants provided written informed consent.

2.4 Anthropometric measurements

For the cohort of healthy volunteers, the anthropometric measurements took place at the Clinical Research Facility, Wales Heart Research Institute, whilst for the patient cohorts they were undertaken as part of clinical evaluation in the relevant clinical centres. Height was measured using a Seca 242 measuring rod [Seca, Birmingham, UK] to the nearest cm and weight with Seca stand on scales [Seca, Birmingham, UK] to the nearest 0.1kg. Waist circumference was measured at minimal respiration by positioning the measuring tape immediately above the iliac crest in parallel to the floor. Hip circumference was established in the same manner around the widest portion of the buttocks.

Body Mass Index (BMI) was calculated as weight (kg) divided by height (meters) squared. The Waist-Hip Ratio (WHR) was established by dividing the waist circumference by the hip circumference (both in cm).

2.5 Isolation and characterisation of plasma-derived extracellular vesicles (EVs)

2.5.1 Differential ultracentrifugation

2.5.1.1 Background

EVs were predominantly isolated by differential ultracentrifugation (chapters 3-6) but additional isolation technique- size exclusion chromatography was used for the research work conducted in chapters 5 and 6 as described later. Differential ultracentrifugation was the most commonly used isolation approach at the time the research work described in this thesis was commenced.

Centrifugation leads to sedimentation of particles that are heavier than the solvent. Hence, differential centrifugation is a technique that selectively isolates the components of interest by using increasing centrifugation forces and duration.

Sedimentation coefficient depends on the mass and size of the particle and the viscosity of the medium that the particle is suspended in. The bigger the particle, the higher the sedimentation coefficient therefore sedimentation occurs faster. Differential centrifugation separates the particles of different sedimentation coefficient. The application of higher forces/spinning time allows smaller particles (with low sedimentation coefficient) to be obtained in the pellet [Livshits MA, 2015].

It is recommended that for differential centrifugation fixed angle rather than swinging bucket rotors are used due to a shorter particle migration path in the former. This facilitates a shorter running time which is more practical when running multiple samples [Livshits MA, 2015].

2.5.1.2. Experimental methodology

In my studies, venous blood from healthy volunteers and patients undergoing medical/surgical obesity management was drawn from a large peripheral vein using a 21G butterfly needle (Hospira, UK) into 3.2% (v/v) sodium citrate vacutainers which is the recommended anticoagulant by the Scientific Standardisation Committee of the International Society on Thrombosis and Haemostasis [Lacroix A, 2013]. The collected blood was handled gently, and the first centrifugation was typically performed within 10mins of venepuncture to avoid any artificial increase in EV levels associated with lengthy incubation prior to processing [Ayers L, 2011]. Acellular plasma was obtained following 2 centrifugation steps at 3000g for 10mins at room temperature (RT) (21°C). The obtained supernatant was then ultracentrifuged at 100000g for 1 hour at 4°C and the pellet was resuspended in 100µL of 0.22µm filtered x1 Phosphate Buffered Saline (PBS). (Fig. 2.1)

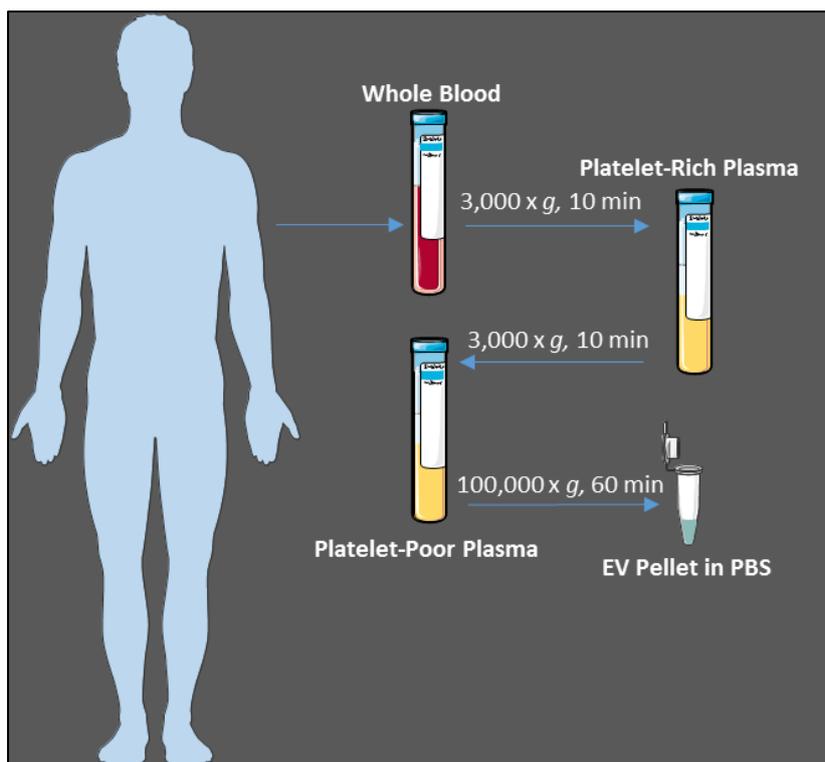


Fig.2.1: Isolation of EVs by differential centrifugation.

2.5.2 Size exclusion chromatography columns

2.5.2.1 Background

Due to developments in the EV research area when this study was ongoing, new methodologies were being implemented in order to improve EV sample purity. One such method was size exclusion chromatography (SEC), which was initially described in the 1950s and has gained renewed interest in recent years due to good reproducibility and speed [Hong P, 2012]. SEC has been 're-invented' for the purpose of EV isolation with numerous commercially available ready-made SEC column kits. It is a technique where molecules in a solution are separated based on their size to porous polymer beads packed into a column.

The thermodynamics of the chromatographic process are described by the following equation:

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} = RT \ln k$$

where ΔG° , ΔH° , and ΔS° are the standard free energy, enthalpy, and entropy differences, respectively; R is the gas constant; T is absolute temperature, and k is the partition coefficient.

However, in SEC the entropic processes are the ones driving the partitioning as there ideally should be no adsorption hence enthalpy is 0. Therefore, the equation describing SEC is:

$$\ln K_d = -\Delta S^\circ / R$$

K_d = thermodynamic retention factor

The thermodynamic retention factor is the fraction of intraparticle pore volume that is accessible to the analyte and is defined by:

$$K_d = \frac{V_r - V_o}{V_i}$$

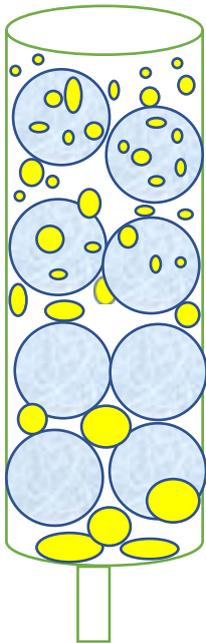
With V_r , V_o , and V_i representing the retention volumes of the analyte of interest, the interstitial volume, and the intra-particle volume. When the analyte is completely excluded from the pores of the stationary phase, K_d will equal 0, whilst when it fully accesses the intraparticle pores, it will be equal to 1 [Hong P, 2012]. In simple terms, in the SEC columns, sample components with small hydrodynamic radii pass through the pores in the column which results in late extraction, whilst those with large hydrodynamic radii are excluded from entering the pores [Li P, 2017].

2.5.2.2 Experimental methodology

This method of EV isolation from plasma was applied to samples from a cohort characterised in chapter 5 where Exo Spin Midi Columns (Cell Guidance Systems) were used as described by colleagues from our research group [Welton J, 2015]. Figure 2.2 illustrates the principles of this isolation technique. (Fig.2.2)



2x 500 μ L fractions of pure PBS and then 1mL plasma sample are loaded on the top of the column.



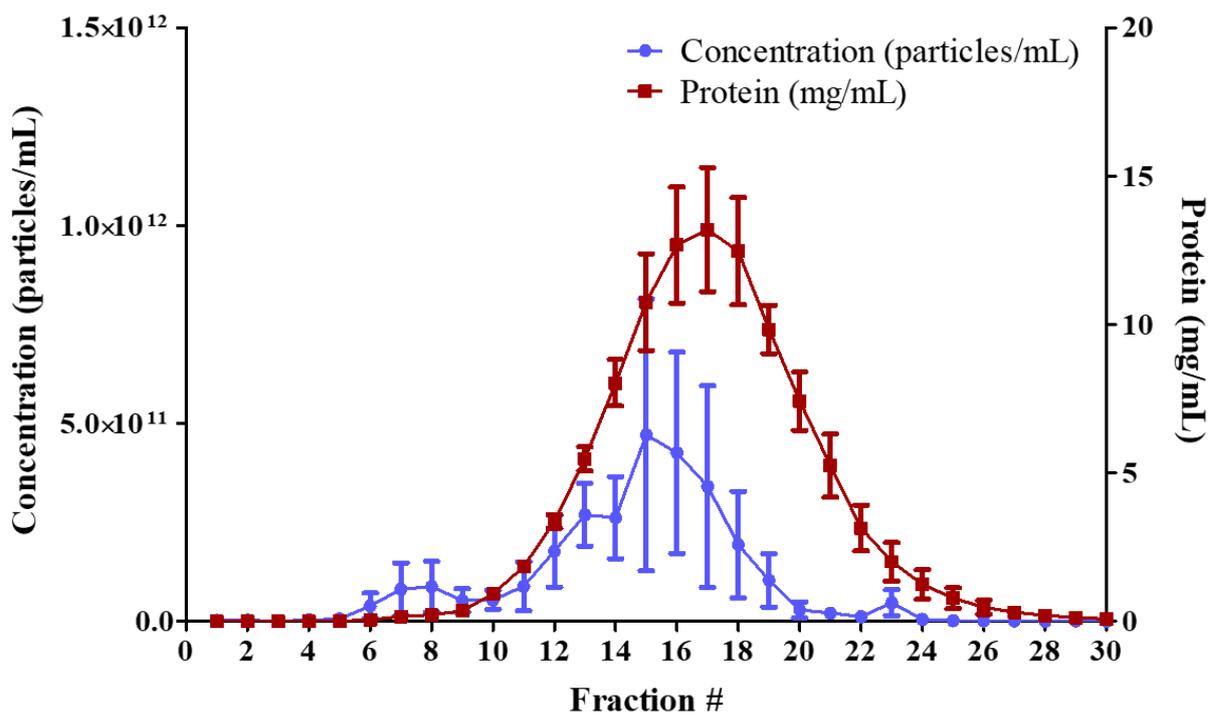
Larger particles cannot pass through the pores in the beads therefore they pass quickly through the column. The small particles pass through the pores therefore their passage through the column is slower.



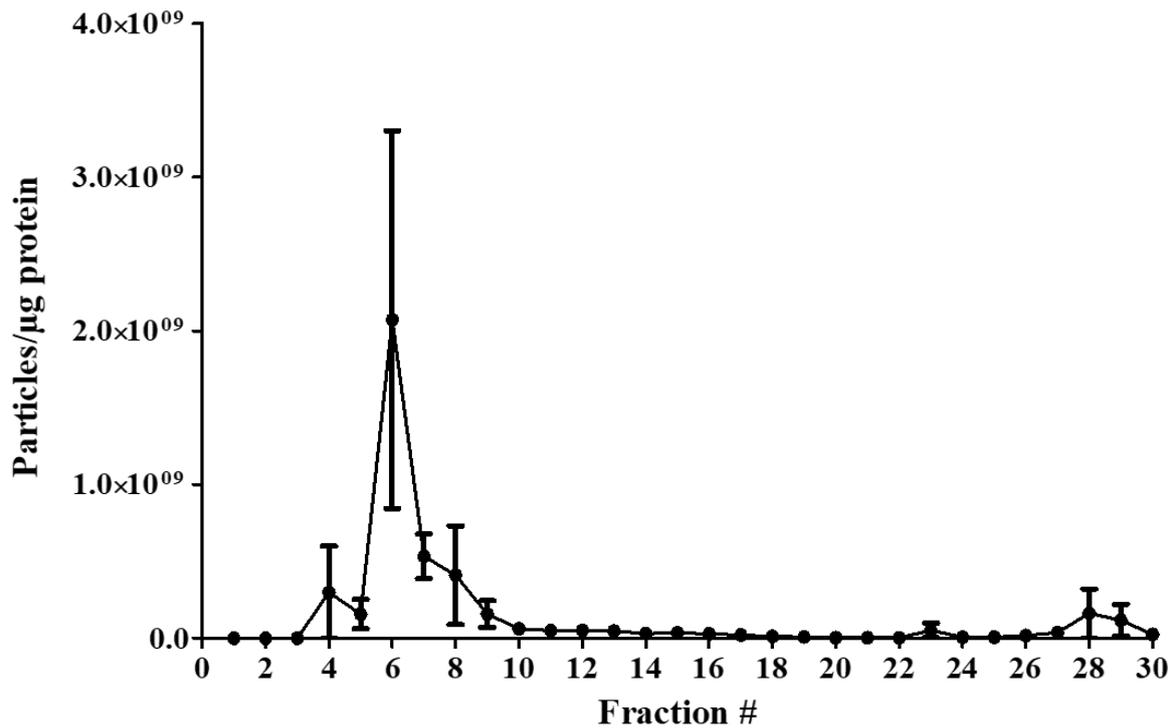
Fractions are collected at 500 μ L volume intervals (1-30) and are then analysed for protein concentration, particle count and EV marker presence.

Fig.2.2: Schematic principle of SEC columns.

Validation experiments were performed before this method was used to ensure the EV-rich fractions were being extracted in fractions 5-10 as described previously [Welton J, 2015]. Briefly, following equilibration of columns with 2x500 μ L filtered x1 PBS, 1mL of double spun (at 3000g for 10mins) acellular plasma was applied to the columns and fractions 1-30 x 500 μ L were collected. Initial analysis of particle concentration was undertaken using Nanoparticle Tracking Analysis software and each fraction's protein concentration measured by Nanodrop as described below. Figure 2.3 illustrates the peak of EVs eluting in the early fractions which is followed by a peak of protein in the later fractions. (Fig. 2.3)



a)



b)

Fig.2.3: Validation experiment of plasma EV isolation from healthy donors: a) protein (mg/mL) (Nanodrop) and nanoparticles/mL concentration (NTA) of 30 fractions obtained by SEC showed an early peak of EVs in fractions 5-10 followed by a protein peak in fractions 15-20 b) particle: protein ratio (concentration of nanoparticles divided by protein concentration) reveals a peak in early fractions which identifies the EV-rich fractions as described previously [Welton J, 2015].

These confirmatory experiments allowed me to run later experiments on clinical samples by focusing on fractions 5-10 where the majority of EVs should be present. These fractions were then pooled, and further analysis was performed as described in chapter 5 (section 5.3.5.1).

2.5.3 Storage of extracellular vesicles

For short-term (1-3 days) studies, EVs were stored at 4°C. When long-term storage was required, samples were frozen at a rate of -1°C/minute using a Mr Frosty (Thermo Fisher Scientific, UK) container and stored in -80°C. The latter appears to be an optimal storing temperature for preserving EVs as confirmed in the EV literature [Jayaram A, 2018, Lőrincz A, 2014] and the work of our colleague from the research group, Dr K Connolly [Connolly K, 2016].

2.5.4 Size and concentration analysis of extracellular vesicles

2.5.4.1 Background

EV size and concentration were established by Nanoparticle Tracking Analysis (NTA) using Nanosight LM10 (Malvern), software version 3.1. This method has proven to be very popular within the EV field in recent years in that it provides robust and repeatable results and enables data comparison between various research centres.

NTA enables direct real time visualisation of nanoparticles by using a finely focused laser beam to illuminate the EV sample in a suspension. (Fig. 2.4) The laser beam travels through a glass prism at a low angle which creates a thin beam of laser light at the interface of the glass-liquid layer and leads to illumination of the nanoparticles which as a result, scatter light. The light is then collected by 2 lenses connected to a sensitive electron multiplying charge coupled device (EMCCD) camera. This camera system is able to record videos of the particle movements at 30 frames per second with a view field of 100 µm x 80 µm [Carr B, 2014].

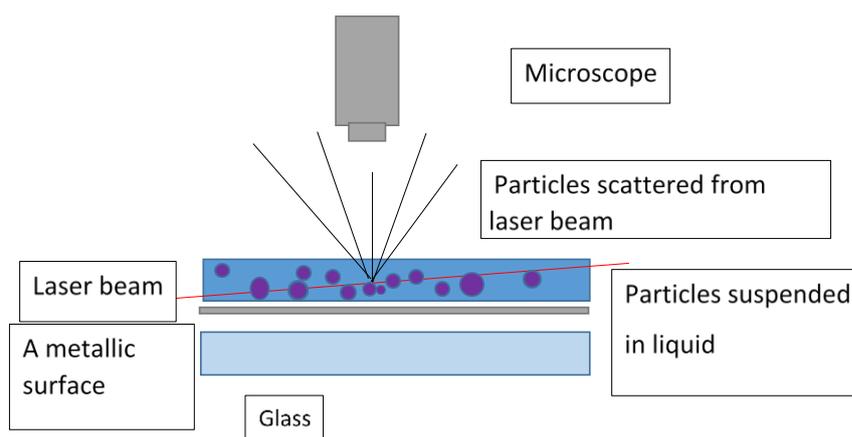


Fig.2.4: NTA operation: laser beam goes through a glass prism and is then refracted into particles containing fluid suspension which allows the particles to scatter light. A microscope with the attached camera then visualises the particles and traces their

Brownian motion over a set time which allows calculation of the size and concentration of EVs (adapted from Malvern, UK).

NTA determines the size and concentration of EVs by analysing the Brownian motion and light scattering properties of the nanoparticle suspension illuminated by a laser beam using a modified Stokes-Einstein equation where temperature (T) and viscosity (η) are constant parameters: [Dragovic RA, 2011] (Fig.2.5)

$$(x,y)^2 = D_t = \frac{k_B T t_s}{3\pi\eta d}$$

Fig.2.5: The modified Stokes-Einstein equation.

The modified Stokes-Einstein equation used in NTA software to analyse the size of individually tracked particles from their motion in 2 dimensions where k_B is the Boltzmann constant and $(x,y)^2$ is the mean-squared speed of a particle at a diluent temperature T , in a medium of viscosity η ; t_s =sampling time (ms); d = sphere equivalent hydrodynamic diameter (particle size).

Each individual particle is tracked during 60 second videos and, as smaller particles move faster than the bigger ones, NTA provides high resolution size distribution data. Moreover, concentration measurements are also undertaken based on a set volume of particle solution injected for each analysis. For biological samples, the lower limit of particle size detected using this method is 30nm [Carr B , 2014].

2.5.4.2 Experimental methodology

NanoSight LM10 software v 3.1 (Malvern, UK) was used with a 488nm (blue) laser installed. Prior to each set of measurements, a calibration measurement of 100nm polystyrene beads diluted 1:1000 was performed to ensure the results were reproducible. The pre- and post-analytical setting parameters were kept constant as described in Table 2.1 to allow comparison between various samples.

	Setting	Value
Pre-analytical	Camera shutter	450
	Camera level	12-16
	Camera gain	200-300
Post-analytical	Temperature	21-26° C
	Screen gain	10-15
	Detection threshold	5-8

Tab.2.1: Pre- and post- analytical settings used for EV sample analysis by NTA.

For each sample, 60 seconds videos were recorded in replicates of 5. The system was flushed with sterile water between sample measurements. Depending on starting plasma volume, EV samples were diluted in ultrapure water (Fresenius Kabi, UK) between 1:10000 to 1:50000 prior to NTA with the aim of obtaining a concentration of 10^8 - 10^9 particles/mL. The concentration of plasma EVs is presented as EVs/mL. Size distribution graphs were created by totalling the concentration of EVs/mL of plasma in each 100nm range (bin width).

2.5.5. Immunophenotyping of extracellular vesicles

2.5.5.1 Background

A Time Resolved Fluorescence (TRF) Immunoassay for EV characterisation was developed by our colleague, Prof Aled Clayton (Cardiff University) and has since been used by our EV research groups. [Webber J, 2014, Connolly K, 2015] It enabled me to establish EV cellular origin, cytokine and adipocyte-marker expression. This technique utilises lanthanides which are long-life fluorophores which following excitation emit light over a longer period of time compared to standard fluorophores (microseconds vs nanoseconds). Consequently, the background signal is reduced as the measurement commences after the background noise has decayed. (Fig.2.6) TRF using lanthanides rather than standard fluorophores is therefore more sensitive.

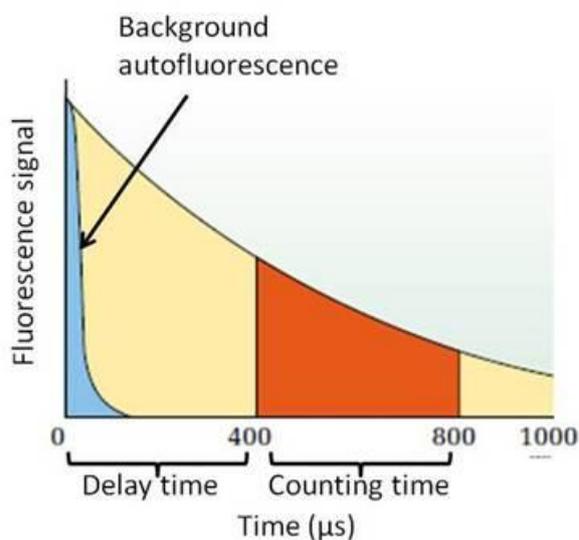


Fig.2.6: The principle of TRF using a long half-life fluorophore. Following excitation, the background fluorescence diminishes quickly in relation to the signal from lanthanide which allows a delay time before measurement of the emission signal is undertaken. Hence , background auto fluorescence is minimised (adapted from Perkin Elmer).

2.5.5.2 Experimental methodology

In the adaptation of this method by our group, high affinity protein binding 'sticky bottom' 96 well plates (Greiner Bio-One) were loaded with 5×10^{10} EVs/well (as established by NTA) diluted to a total volume of 100 μ L with x1 filtered PBS and incubated overnight at 4°C. Following a wash with Delfia Buffer (Perkin Elmer, Waltham, USA), non-specific sites were blocked with 1% (wt/vol) Bovine Serum Albumin (BSA) in PBS for 2 hours. After a further wash, primary antibodies were added in duplicates at 3 μ g/mL concentration (anti-CD41, anti-CD11b, anti-CD235a, anti-CD144, anti-CD9, anti-IL-6, anti-TNF α , anti-interferon γ , anti-adiponectin, anti-FABP4 and anti-PPAR γ , Tab.2.2). After overnight incubation in the cold room on a plate shaker and a further 3 washes, the secondary antibody (anti-rabbit IgG Biotin labelled, 1:2500 in 0.1% (wt/vol) BSA in PBS) (Perkin Elmer, UK) was added for 1 hour (room temperature (RT), plate shaker), followed by 3 further washes and staining with Europium-labelled Streptavidin for 45 mins (RT, plate shaker) (Perkin Elmer, UK). After the final 6 washes, Delfia Enhancement Solution (Perkin Elmer, UK) was then added to allow formation of fluorescent chelates. The resulting fluorescence emission was measured using a Clariostar spectrometer (BMG Labtech Ltd, Aylesbury, UK) configured to Time Resolved Fluorescence (TRF) where a narrow emission peak is produced with a large Stokes' shift and long decay time, thereby giving a good signal-to-noise ratio (each well received 400 flashes; measurement commenced after 400 μ s after the last flash and continued for 400 μ s; Fig 2.6).

During each experiment a set of Europium standards was applied in duplicate in order to set the gain adjustment, thus allowing comparison between various plates/experiments. MARS software (BMG Labtech, UK) was used for data analysis and results are presented in arbitrary TRF units (aTRF). All samples/markers were run in duplicate and the final aTRF value was calculated as the mean of 2 readings adjusted by subtracting the mean of negative control/background (signal from the EV-loaded wells where no primary antibody was added). Figure 2.7 illustrates the principle of the above described method.

All primary rabbit monoclonal antibodies were supplied by Abcam (Cambridge, UK) apart from anti-CD9 antibody which was provided by Cell Signalling Technology (Danvers, USA). For the analysis of internal EV content (IL6, TNF α , Interferon γ , adiponectin, FABP4, PPAR γ), RIPA Lysis Buffer (Santa Cruz Biotechnology Inc., USA) containing protease inhibitor, sodium orthovanadate and phenylmethyl sulfonyl fluoride (PMSF) was added for 1 hour (RT, plate shaker) before the addition of primary antibodies to enable the disruption of the EV membrane.

The optimal concentration of primary antibodies for this experiment was previously established by our group by comparison of TRF signals at 4 different concentrations of each primary antibody of

interest (10µg/ml, 3 µg/ml, 1 µg/ml and 0.1 µg/ml) added to a set number of EVs (5×10^{10}) with results indicating that saturation of TRF signal was achieved with 3 µg/ml and provided a reliable and replicable concentration for the purpose of this assay.

Primary marker	Source	Relevance
CD41	Abcam ab134131	Platelet marker
Cd11b	Abcam ab52478	Monocyte/macrophage marker
CD235a	Abcam ab129024	Erythrocyte marker
CD144	Abcam ab40772	Endothelial cell marker
CD9	Cell Signaling D3H4P	Exosomal marker
Interleukin-6	Abcam ab32530	Inflammatory pathway
TNFα	Abcam ab53450	Inflammatory pathway
Interferon γ	Abcam ab133566	Inflammatory pathway
Adiponectin	Abcam ab75989	Adipocyte marker
FABP4	Abcam ab92501	Adipocyte marker
PPAR γ	Abcam ab191407	Adipocyte marker
CD63	Santa Cruz Biotech.sc-15353	Exosomal marker

Tab. 2.2: Primary antibodies used in the EV immunophenotyping by TFR.

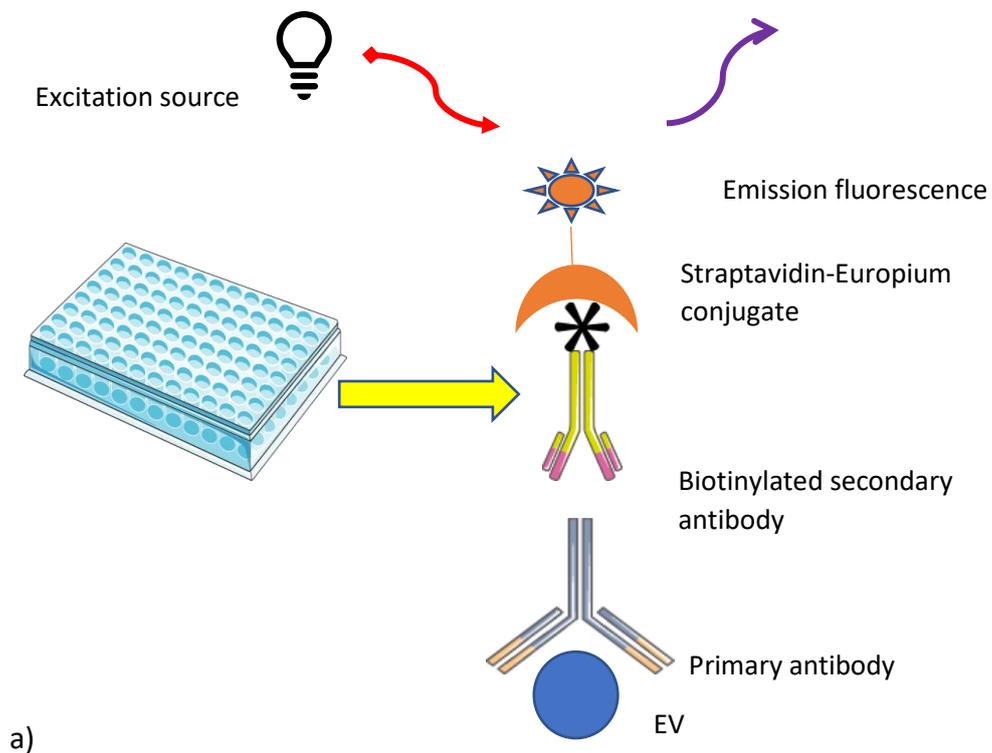
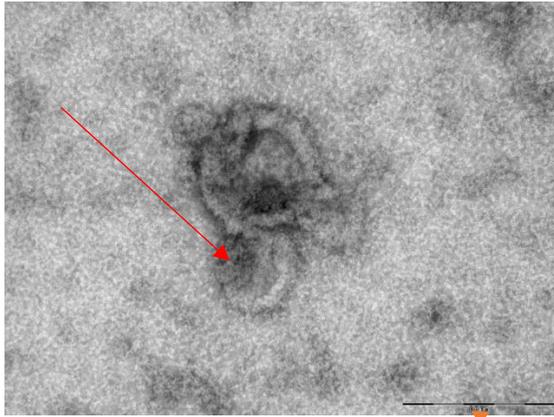


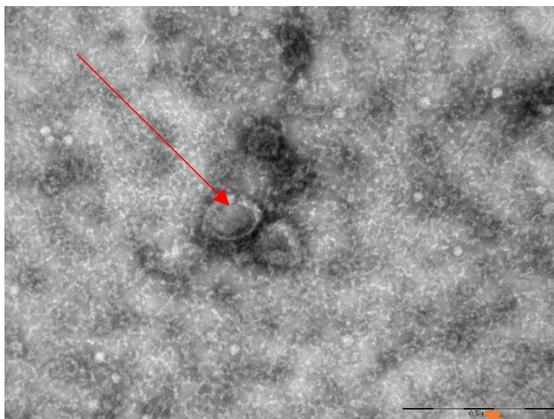
Fig. 2.7: TRF Immunoassay: a) the steps involved in TRF immunoassay: EVs are loaded into 96 well plates and exposed to primary antibodies. These then bind to a biotinylated secondary antibody and this complex is stained with Europium-labelled streptavidin. Finally, fluorescent chelates are formed. b) Fluorescence is measured in TRF mode by a Clariostar plate reader.

2.5.6 Electron microscopy

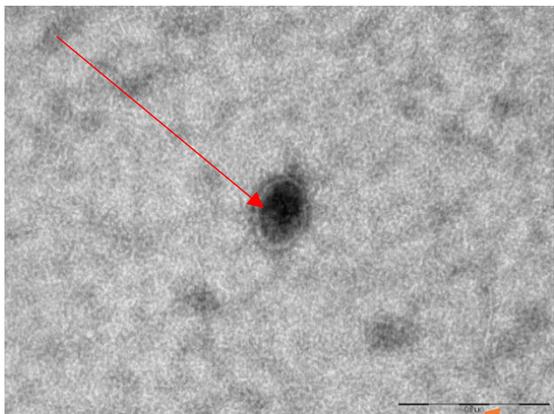
Electron microscopy of plasma-derived EVs isolated by differential centrifugation was performed in collaboration with Dr Christopher von Ruhland (Central Biotechnology Services, Cardiff University). Transmission electron microscopy was used to confirm EV presence and morphology following isolation. EVs were isolated as described above and resuspended in x1 sterile PBS and stored at 4°C until analysis was undertaken. EV droplets (50µL) were then adsorbed onto a carbon-coated grid for 30 mins and were later fixed with 1% glutaraldehyde (v/v) for 1 hour (RT). Following 3 washes in PBS for 1 minute and 6 washes of 10 minutes duration in water, EVs were stained negatively with 2% (w/v) uranyl acetate for 20 minutes. Following removal of excess stain, samples were air dried at RT and EVs were then visualised by a Philips CM12 TEM microscope (FEI Ltd, UK) at 80 kV. (Fig. 2.8)



a)



b)



c)

Fig.2.8: TEM images of plasma-derived EVs isolated (fresh) by differential centrifugation from 3 healthy donors (a-c); EVs are marked with red arrows; scale 500nm as marked with orange arrows.

2.6 Plasma measurements

For all the following assays, venous blood was collected into EDTA vacutainers and spun x2 at 3000g for 10mins immediately. Acellular plasma was then aliquoted and stored at -80°C until further analysis.

2.6.1 Measurement of plasma adipocytokines

An array of adipokines and cytokines was measured as described below using commercial kits. All samples were assayed in duplicate. A BMG Clariostar was used to determine the optical density at each assay's recommended wavelength.

2.6.1.1 Human plasma FABP4

The concentration of FABP4 in human plasma was established by a Quantikine ELISA Human FABP4 Immunoassay (DFBP40, R&D Systems, UK) which is a quantitative sandwich enzyme immunoassay with a mean minimum detectable (MDD) concentration of human FABP4 of 6.55pg/mL (range 2.7-14.2pg/mL). The reported assay range was 62.5 - 4,000 pg/mL and cross-reactivity was <0.5%. The assay only required 10µL of plasma.

Plasma samples were diluted 20-fold using Calibrator Diluent RD6-68 and assayed in duplicate. Samples and standards (4000pg/mL, 2000pg/mL, 1000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL and 62.5pg/mL) were added to 96 well microplates pre-coated with immobilised antibody which binds to FABP4 present in the sample. Following a wash with the wash buffer, a human FABP4 enzyme-linked polyclonal antibody was added. After a second wash, a substrate solution was added which led to colour development in the light protected wells in proportion to the amount of FABP4 present. Once the stop reagent had been added, the colour intensity was measured immediately at 450nm with the correction wavelength set at 570nm (BMG Clariostar, UK).

The duplicate readings for each standard, control and sample were averaged and the average zero standard optical density (O.D.) was subtracted from them. Following standard curve creation, a best fit curve was drawn through the points on the graph and the concentration of FABP was calculated.

2.6.1.2 Human Plasma PPAR γ

The concentration of human PPAR γ was measured using a Human PPAR γ Sandwich ELISA Kit (LSBio, Inc, USA, LS-F12376) with a detection range of 0.156-10ng/mL, lower limit of detection of less than 0.056ng/mL, intra-assay CV of <6.1% and inter-assay CV of <11.2%. Standards were set up at the following concentrations: 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.313ng/mL,

0.157ng/mL and 0ng/mL. Standards and undiluted plasma samples (100µL volume) were then added to pre-coated plates to allow binding to the capture antibody and incubated for 2 hours at 37°C. Following a wash, 100µl of biotin-conjugated antibody was added and after the excess antibody was washed away, Avidin-Horseradish Peroxidase (HRP) was added to bind with the biotin. Finally, a TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added which led to reaction with the HRP enzyme and colour development. This was then stopped by the addition of the sulfuric acid stop solution and measurement of the optical density (OD) at 450nm was undertaken. The standard curve was constructed by plotting the mean absorbance for each standard against the concentration and a best fit curve was drawn. The final concentration was calculated in the same manner as for FABP4.

2.6.1.3 Plasma IL6, interferon γ , TNF α and adiponectin

Assays for evaluation of IL6, TNF α , interferon γ and adiponectin concentration in human plasma were supplied by Abcam, UK (ab46042, ab100537, ab46087, ab99968, respectively). The characteristics of each kit are summarised in Table 2.3.

	IL6	TNF α	Interferony	Adiponectin
Detection method	Colorimetric	Colorimetric	Colorimetric	Colorimetric
Sample type	Serum, plasma, cell culture supernatant	Plasma, serum, cell culture supernatant	Plasma, cell culture supernatant	Plasma, serum, cell culture supernatant
Sensitivity	<0.8 pg/mL	<10pg/mL	<15pg/mL	<25pg/mL
Range	1.56-50 pg/mL	25-800pg/mL	20.6-15000pg/mL	Not provided
Recovery (mean% (range))	93%(72-123)	81%(74-90%)	86.82%(81-102)	90.29%(80-103)
Assay time	3hr 45mins	3hr 40min	5hr 30 mins	5hr 30mins
Species reactivity	Human	Human	Human	Human

Tab.3.3: The specifications of commercially available Abcam kits used for estimation of plasma concentration of IL6, TNF α , interferon γ and adiponectin.

Similarly to the above described methods, these were colorimetric ‘sandwich’ quantitative ELISA assays using the same methodology, including incubation steps and measurement settings. Undiluted plasma samples were measured in duplicate for IL6, TNF α and interferon γ , whilst plasma adiponectin measurement required 1:30000 dilution using a reagent provided. Absorbance was read by a BMG Clariostar spectrophotometer using 450nm as the primary wavelength and 620nm as the reference wavelength.

The table below summarise the concentrations of standard solution for each adipocytokine which was used for the standard curve preparation (Tab.2.4).

Standard #	IL6 (pg/mL)	TNF α (pg/mL)	Interferon γ (pg/mL)	Adiponectin(pg/mL)
1	50	800	15000	18000
2	25	400	5000	6000
3	12.5	200	1666.7	2000
4	6.25	100	555.6	666.7
5	3.125	50	185.2	222.2
6	1.56	25	61.7	74.07
7			20.6	24.69
Zero standard	0	0	0	0

Tab.2.4: The concentration of standards used in the Abcam assay kits used for measurement of plasma IL6, TNF α , interferon γ and adiponectin.

The final concentration of each adipokine was calculated from the standard curve as described above.

2.6.3 Plasma insulin measurement

A human insulin ELISA Kit (Abcam, UK, ab100578) was used to determine fasting insulin concentration using the same methodology as described above. Plasma samples were diluted 1:2. The kit had a detection range of 178.5-10713pg/mL with a minimum detectable dose of insulin of <50pg/mL and mean recovery of 99.03% (range 73-128). Standards were set at 167.47pg/mL, 334.96pg/mL, 669.91pg/mL, 1339.8pg/mL, 2679.67pg/mL, 5359.35pg/mL and 10718.71pg/mL. The plate was read at 450nm as previously.

2.6.4 Plasma glucose measurement

Fasting glucose concentration was established by the glucose oxidase method as previously described. [Dietzler DN, 1980] Briefly, this is a colorimetric method where glucose in the presence of air is oxidised by glucose oxidase to glucuronic acid which results in concomitant hydrogen peroxide release. The latter then undergoes decomposition by the peroxidase enzyme which results in oxygen release leading to oxidation of the chromogen which is then measured colorimetrically. Fifty microlitres of fasting plasma were assayed in duplicate for each healthy volunteer. The absorbance of samples and standards was read at 510nm using a Jenway 6705UV/Spectrophotometer. The glucose concentration of the samples (mg/dL) was calculated using the following formula:

$$\frac{\text{sample absorbance}}{\text{standard absorbance}} * \text{standard concentration}$$

The following calculation was used to convert glucose concentration (mg/dL) to mmol/L: mg/dL x 0.0555 = mmol/L.

2.6.5 HOMA-IR

The HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) was calculated as per the formula: HOMA-IR= fasting insulin(miU/L)*fasting glucose (mmol/L)/405. [Matthews DR, 1985]

2.7 Protein concentration

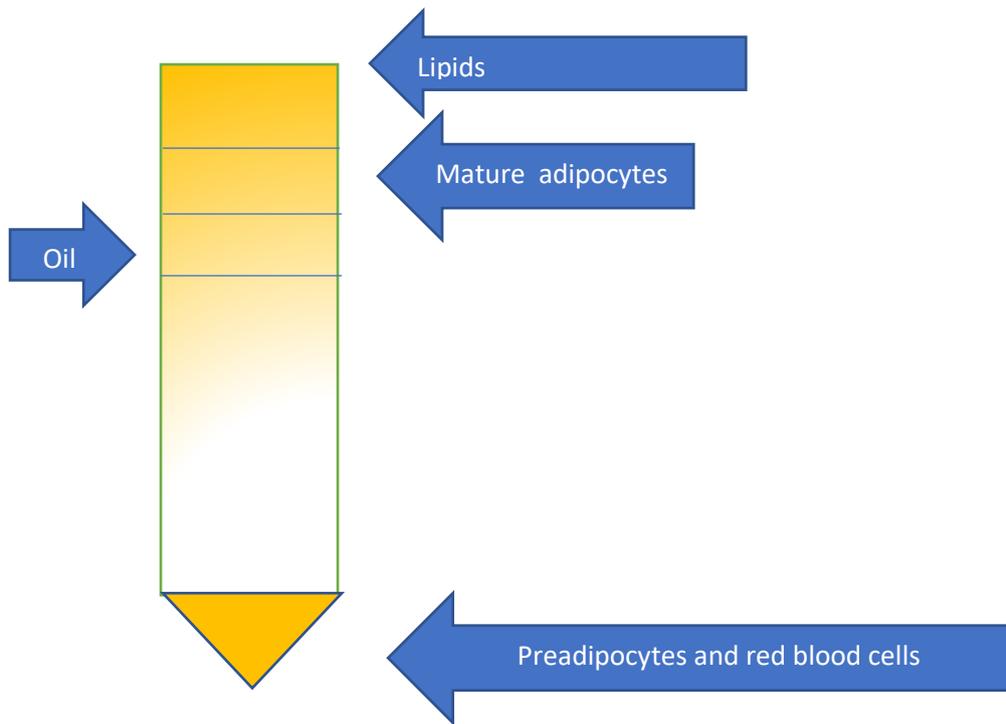
The protein concentration of the columned plasma-derived EVs and adipocyte-derived EVs from tissue culture was established using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific, UK) with a protein detection range of 0.1-400mg/dL. Following calibration with ultrapure water, 1µL of each column fraction was carefully pipetted onto the Nanodrop pedestal and following the arm closure, a sample column was formed and the optimal path length (0.05-1mm) found by the pedestal automatic movement. Each sample was assayed in duplicate. This method was chosen because of its time efficiency and small volume of sample required without any need to perform serial dilutions.

2.8 The isolation and characterisation of adipocyte-derived EVs (ADEVs) from primary adipocyte culture

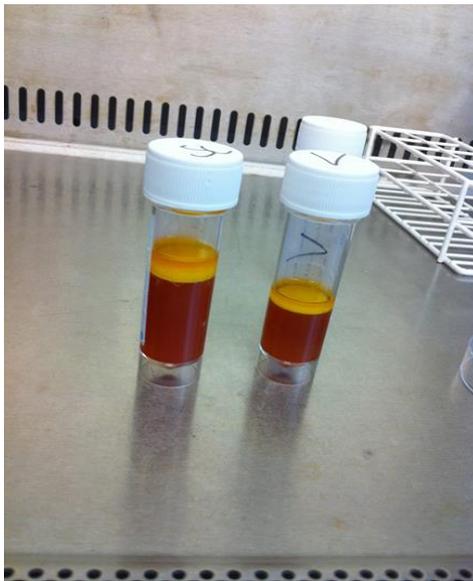
Samples of subcutaneous (abdominal wall) and visceral (omental or perinephric) fat were obtained from patients undergoing open or laparoscopic elective surgery at the University Hospital of Wales, Cardiff (REC ref. 06WSE03/37). Written informed consent was obtained from all study participants.

2.8.1. Sample preparation

Peri-operative samples were collected into sterile universal containers with no additives and immediately transferred to the tissue culture laboratory, where they were processed the same day. Firstly, fat tissue lumps were dissected into fine pieces using a disposable scalpel blade (size 11), (Swann Morton, UK). The tissue was then added to a mixture of collagenase type 2 (Gibco, 17101-015), 7.5% BSA (Bovine Serum Albumin, Sigma-Aldrich, A7906) and HbSS (Hank's balanced Salt Solution, Thermo Fisher, UK, 14025092) in order to perform tissue "digestion". The volume of each ingredient depended on the size of the fat tissue, with the following volumes required typically for a 0.7g sample: 7mLs HbSS, 3mLs 7.5%BSA, 1mL collagenase. The mixture was then incubated in a water bath at 37°C for 1 hour and shaken manually every 5 mins. Following this step, samples were centrifuged at 1500rpm for 5mins at 4°C which allowed separation of preadipocytes and RBC from mature adipocytes, oil and lipids. (Fig.2.9) Once oil, lipids and mature adipocytes were gently removed, the remaining preadipocyte pellet was resuspended in control medium and transferred to T25 tissue culture flasks.



a)



b)

Fig.2.9: Schematic illustration(a) and photograph (b) of separation process during preadipocyte isolation following adipose tissue digestion and 1500rpm centrifugation.

*Preadipocytes are pelleted at the bottom of the universal container.

Preadipocytes were maintained in control medium which was changed every 3-5 days at a maximum of 70-75% confluence between passages, and underwent differentiation following passage 3. (Fig.2.10) The components of control (CM) and differentiation medium (DM)(x2) are summarised in Table 2.5.

Component	CM	DM
DMEM (high glucose 4.5g/L, no glutamine, Lonza, BE12-614F)	85mLs	44.5mLs
Ham's F12 nutrient mix, Lonza, BE12-615F)	85mLs	44.5mLs
Sodium pyruvate	2mLs	1mL
Sodium bicarbonate	3mLs	1.5mL
FCS, Lonza	20mLs	20mLs
Penicillin/Streptomycin (Thermo-Fisher Scientific, 15140122)	4mLs	2mLs
Insulin (Sigma Aldrich, I-1882)	x	500nM (570µL)
Hydrocortisone (Sigma Aldrich, H-2270)	x	1µM (20µL)
Panthenonate (Sigma-Aldrich, P-5155)	x	17µM(200µL)
Biotin (Sigma Aldrich, B-4639)	x	33µM(64µL)
Triiodothyronine (Sigma Aldrich, T-5516)	x	1nM(1.4µL)
Pioglitazone	x	1µM(20µL)
Amphotericin B (Sigma Aldrich, A2942)	2mLs	1mL

Tab.2.5: Ingredients and their respective volumes required to prepare 200mLs of complete medium with 10%FCS and 100mLs of x2 differentiation medium mix.

The above described medium composition has been widely used by Prof Ludgate's group working on adipocyte tissue derived from orbits within the Centre for Endocrine and Diabetes Sciences, Cardiff University. However, I observed early on in my experiments that the visceral and subcutaneous tissue from my experiments was very prone to fungal infection, hence I modified my media by the addition of amphotericin B as a prophylactic measure.

Typically, it would take around 4-6 weeks from the day the sample was obtained to start the differentiation process. Preadipocytes were kept in differentiation medium for up to 21 days. (Fig.2.10)

2.8.2 Commercial preadipocyte culture

In addition to samples obtained from volunteers undergoing elective surgery, commercial visceral preadipocytes were obtained from Lonza, UK (PT-5005). This was due to the fact that the surgically obtained samples grew quite slowly and often developed fungal or bacterial infection towards the end of the experiments which led to sample loss at a great time and resources expense. The Lonza preadipocytes were grown in the same medium but showed faster growth rate between passages and no problems with sample contamination was noted.

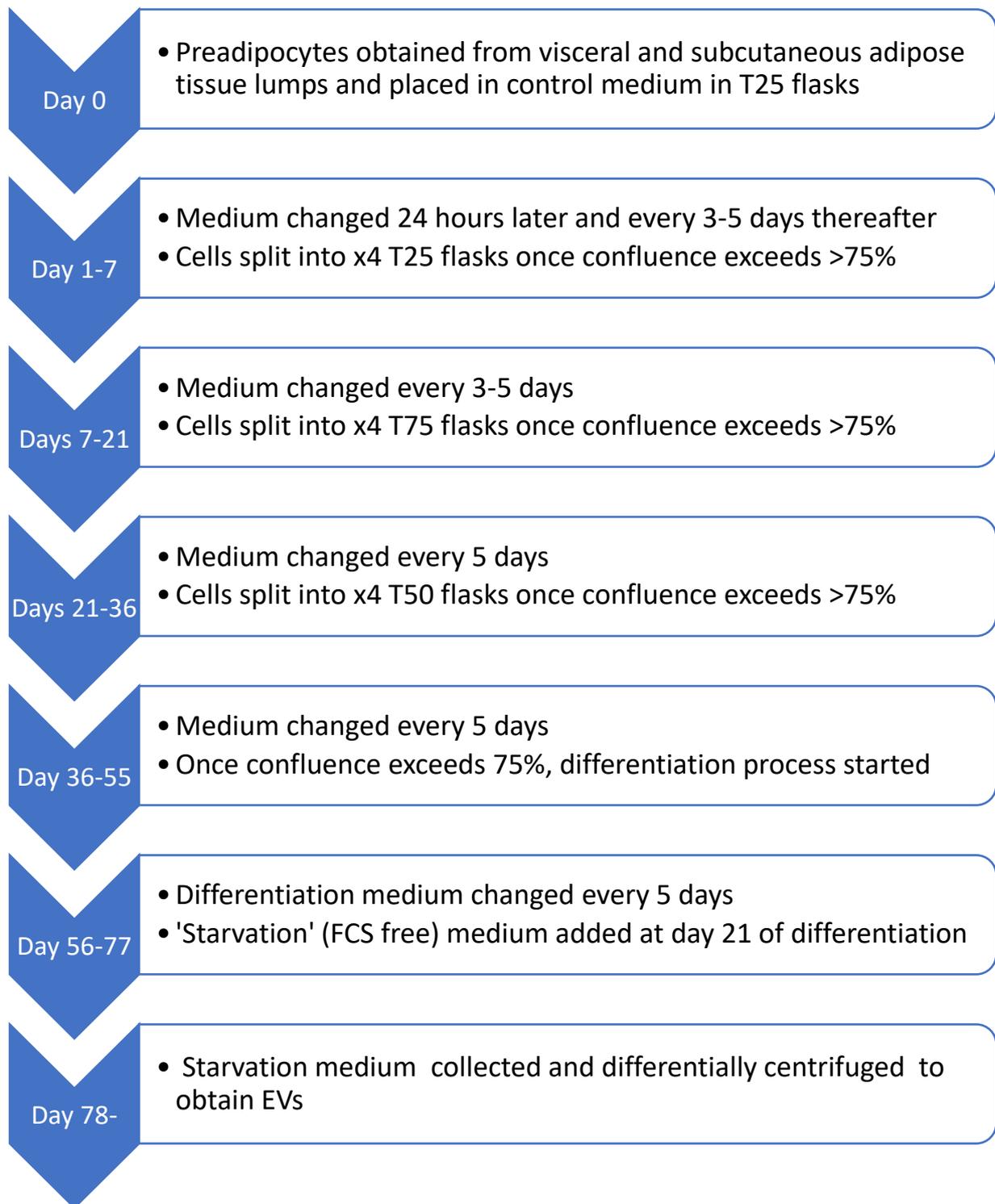


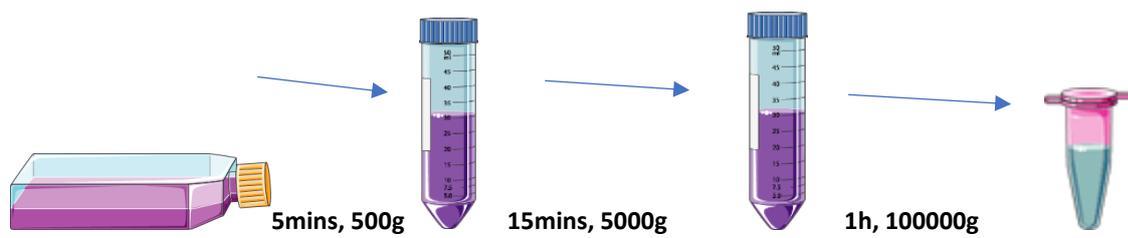
Fig.2.10: Timeline of primary adipocyte culture experiments from subcutaneous and visceral fat depot.

2.8.3 Adipocyte-derived EV isolation

2.8.3.1 Differential centrifugation

As mentioned above, prior to EV isolation, adipocyte cultures were pre-incubated in serum-free media for 24 hours. This is due to our observation [Connolly K, 2015] that was also described in the literature [Shelke V, 2014, Faure J, 2006] that isolating EVs from FCS- containing media may lead to overestimation of EV amount.

The removed FCS-free medium was first spun at 500g for 5 mins to remove any cells which was followed by a 15 mins spin at 5000g to remove cell debris and larger vesicles/apoptotic bodies. The final step was the same as for plasma derived EVs, namely ultracentrifugation at 100000g for 1hour at 4°C. The EV pellet was then resuspended in x1 filtered PBS. (Fig.2.11)



Conditioned culture medium

Fig.2.11: Differential centrifugation steps used to isolate adipocyte derived EVs (ADEVs).

2.8.3.2 Size exclusion chromatography columns

Exospin columns (Cell Guidance Systems, UK, EXO-01) were used to purify ADEV samples obtained by differential centrifugation prior to their content analysis. Due to smaller sample volume available, smaller SEC columns were chosen. One hundred μL EVs in x1 PBS samples were added to the columns after initial equilibration with x1 PBS and 6 fractions of 100 μL were collected. NTA revealed that EVs typically occur in fraction 3-4 with this column and sample type.

2.8.4 ADEV concentration and size

NTA was used as described above to establish ADEV size and concentration. Samples were diluted between 1:200-1:1000 in ultra-pure water prior to analysis.

2.8.5 Proteome Profiling

Human Adipokine Proteome Profiler Array Kit (R&D Systems, UK, ARY024) was used to establish adipocytokine expression in the adipocyte-derived EVs. In this method, capture antibodies for 58 human adipokines (Tab.2.6) are present in duplicate on a nitrocellulose membrane which also contains 3 duplicates of reference spots and 1 duplicate for negative controls. EV samples were first incubated with RIPA (1:1 volume ratio) and added to a 30 μL cocktail of biotinylated detection antibodies and diluted up to the volume of 1.5mL with supplied array buffers. The mixture was then added to wells where nitrocellulose membranes had been pre- incubated for 1 hour on a rocking platform shaker in 2mLs of an array buffer. Following overnight incubation on a rocking platform, membranes were washed x3 and incubated with Streptavidin-HRP for 30mins. Following 3 further washes, 1mL ChemiReagent Mix was pipetted onto each membrane and incubated for 1min. Films were then developed in a dark room in the autoradiography cassette following 1-hour exposure using photographic film (Amersham™ Hyperfilm ECL, GE Healthcare), Kodak™ -D19 developer and fixer by Sigma-Aldrich. Chemiluminescence signals were proportional to the amount of the adipokine bound (Fig. 2.12). This exposure time was chosen after validation experiments showed that shorter exposure time gives low signal which cannot be reliably read after scanning the membranes.

Developed films were then scanned and the average signal (pixel density) for each pair of adipokines was established using Quick Spots HImage+++ software (Western Vision, USA) which automatically subtracts the average signal of each pair of duplicate spots from the average signal of the negative spots.

Adiponectin	FGF basic	M-CSF
Angiopoietin-1	FGF-19	MIF
Angiopoietin-2	Fibrinogen	Myeloperoxidase
Angiopoietin-like 2	Growth Hormone	Nidogen-1/Entactin
Angiopoietin-like 3	HGF	Oncostatin M
BAFF/BLyS	ICAM-1	Pappalysin-1/PAPP-A
BMP-4	IGFBP-2	PBEF/Visfatin
Cathepsin D	IGFBP-3	PCSK9
Cathepsin L	IGFBP-4	Pentraxin-3
Cathepsin S	IGFBP-6	Pref-1/DLK-1/FA1
CCL2/MCP-1	IGFBP-rp1/IGFBP-7	RAGE
CCL5/RANTES	IL-1 beta	Resistin
Chemerin	IL-6	Serpin A12
Complement Factor D	IL-10	Serpin A8/AGT
C-Reactive Protein	IL-11	Serpin E1/PAI-1
CXCL8/IL-8	LAP (TGF- β 1)	TIMP-1
DPPIV/CD26	Leptin	TIMP-3
Endocan	LIF	TNF-alpha
EN-RAGE	Lipocalin-2/NGAL	VEGF
Fetuin B		

* BAFF/BLyS (B Cell Activating Factor/ B Lymphocyte Stimulator), BMP-4 (Bone Morphogenetic Protein 4), CCL2/MCP-1 (C C motif chemokine ligand 2/Monocyte Chemoattractant Protein 1), CCL5/RANTES (C C motif chemokine ligand 5/ Regulated Upon Activation Normal T-cell Expressed and Secreted), CXCL8/IL-8 (CXC motif ligand 8/Interleukin 8), DPPIV/CD26 (Dipeptidyl peptidase-4/cluster of differentiation 26), EN-RAGE (Extracellular Newly identified Receptor for Advanced Glycation End-products binding protein), FGF (Fibroblast Growth Factor), HGF (Hepatocyte Growth Factor), ICAM-1 (Intercellular adhesion molecule-1), IGFBP (Insulin Like Growth Factor Binding Protein), LAP (TGF- β 1) (Latency Associated Peptide (Transforming Growth Factor β 1)), LIF (Leukaemia Inhibitory Factor), NGAL (Neutrophil Gelatinase-associated Lipocalin), M-CSF (Macrophage Colony Stimulating Factor), MIF (Macrophage Migration Inhibitory Factor), PAPA-A (Pregnancy-associated Plasma Protein A), PBEF (Pre B cell Enhancing Factor), PCSK9 (Proprotein convertase subtilisin/nexin type 9), Pref-1/DLK-1/FA1 (Preadipocyte Factor 1/delta like 1/ foetal antigen 1), RAGE (Receptor for Advanced Glycation Endproducts), AGT (angiotensinogen), PAI-1 (Plasminogen Activator Inhibitor-1), TIMP (tissue inhibitor of metalloproteinases), VEGF (Vascular endothelial growth factor).

Tab.2.6:

Alphabetical list of adipokines detected using the Proteome Profiler Assay.

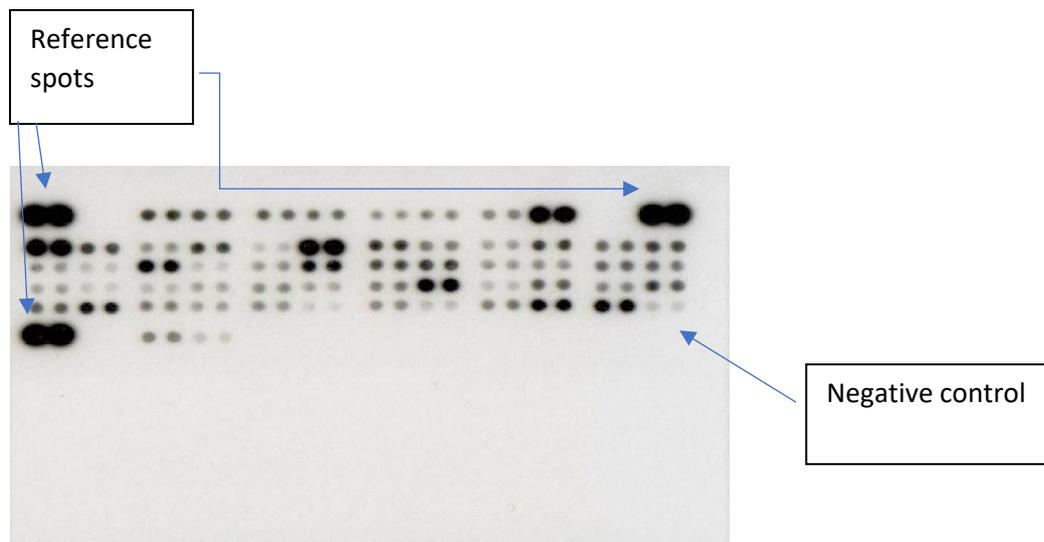


Fig.2.12: Example of a result obtained following incubation of visceral adipose tissue derived EVs obtained at day 21 of differentiation using the adipokine proteome profiler assay.

2.8.6 Oil Red O staining

Oil Red O was prepared from a 0.5% stock solution (w/v in isopropanol) at 3:2(v/v) dilution with distilled water (dH₂O) and filtered after 15mins through Whatman paper.

For this measurement, human adipocytes were cultured in 6 well plates and washed in sterile PBS before staining. Formaldehyde (10%, v/v in PBS) was then used to fix the cells over a 15 minute period at room temperature (RT) which was followed by a wash with dH₂O. Cells were then stained for 15 mins at RT with prepared Oil Red O solution as described above. Sixty percent isopropanol (v/v in PBS) was used to remove excess stain and following 2 washes with sterile PBS, cells were photographed (Nikon Diaphot microscope, Nikon) at 10x magnification (View Finder, Software, version 3.0.1, Better Light Inc. USA). The intracellular stain was extracted following two further washes with PBS by using 100% isopropanol. A BMG Clariostar was used to measure optical density at 490nm.

2.9 Statistical analysis

Graph Pad Prism (versions 6.0 and 7.0, GraphPad Software Inc. USA) software was used for statistical analysis and graph creation. Details of specific statistical analysis are described in the relevant results chapters. Data are presented as mean (SD) unless stated otherwise. A p value of <0.05 was accepted as statistically significant.

Chapter 3

Characterisation of circulating extracellular vesicles and plasma adipocytokines in healthy lean and obese subjects

Perspective

At the time this study was commenced, our group had established standardised protocols of EV isolation from plasma of various patient groups by differential centrifugation, which was then the most commonly used approach in the literature. We also had regular access to Nanosight (NTA) equipment, and developed Time Resolved Fluorescence Immunoassay for analysis of EV content and cellular origin. Moreover, our research group recently characterised adipocyte-derived EVs (ADEVs), and subsequently developed an interest in developing evidence for the presence of ADEVs in the circulation and validation of potential ADEV markers in plasma. Pilot results at that time confirmed by Western blotting the presence of FABP4 and PPAR γ in human plasma-derived EV samples, corresponding with findings from 3T3 (adipocyte) cells and 3T3-derived EVs. I therefore decided that a natural follow-up would be to analyse plasma EV samples from healthy volunteers across a range of BMIs and to evaluate them for the presence of the proposed adipocyte markers: adiponectin, FABP4 and PPAR γ as described by Connolly KD *et al* [Connolly K, 2015], in addition to 'traditional' markers of cellular EV origin relating to the principal blood components: erythrocytes, leukocytes and platelets as well as endothelial cells.

3.1 Introduction

Extracellular vesicles have emerged as potential disease biomarkers in recent years but relatively little is known about their characteristics in a healthy state. Most papers to date have predominantly used healthy cohorts as controls to the diseased populations, looking at expression of particular disease markers relevant in given clinical contexts, such as early detection of malignancy or autoimmune disorders, or for treatment response monitoring.

Over the last two decades it has become apparent that EVs are very heterogeneous; hence, in order to advance their use in the clinical setting, not only must a more standardised approach to their isolation and analysis be introduced, but also an understanding of the 'normal' EV profile and reference ranges should be established.

In the recent ISEV statement paper, Witwer *et al* [Witwer K, 2013] placed an emphasis on the importance of an organised, reproducible approach to sample collection and processing in order to minimise the effects of extraneous factors on study findings. Furthermore, little is known about the effects of the circadian rhythm, food and prior exercise/resting status, age and gender on circulating EV concentration and subtype. Until our understanding of the effects of these factors on circulating plasma EVs is improved, the application of EV measurements in clinical practice should not commence. One of my first study goals was therefore to characterise circulating plasma EVs in a cohort of healthy volunteers.

There is also relatively little research on plasma-derived EVs in the context of obesity *per se*. Given the growing interest in circulating EVs as disease biomarkers and the rising prevalence of obesity, with its well established cardiometabolic and inflammatory consequences [Redinger R, 2007], I wanted to evaluate if differences in circulating plasma EVs could be observed between healthy volunteers across the BMI range. If such differences were observed, this might help distinguish early obese 'at risk' individuals from those with so-called 'healthy obesity'. In time, this might translate into stratification of cardiovascular disease prevention and treatment.

I therefore aimed to characterise circulating plasma EVs from a cohort of metabolically healthy volunteers with no overt indicators of disease using a structured approach to sample collection and analysis. I also utilised an array of EV cellular origin markers, based on the established literature, for platelets, monocytes/macrophages, erythrocytes and endothelial cells (CD41, CD11b, CD235a, CD144, respectively), as well as new markers proposed by our group for the detection of circulating

ADEVs in plasma (FABP4, PPAR and adiponectin) and EV-expressed cytokines (IL6, TNF α , interferon γ).

3.2 Aims

The aims of this chapter were to characterise a cohort of volunteers without known cardio-metabolic complications across a range of BMI (lean, overweight, obese) with respect to:

1. Anthropometric measurements (BMI, weight, waist, waist-hip ratio (WHR), age, gender)
2. Glucose metabolism (fasting glucose, fasting insulin, HOMA-IR) and circulating plasma adipokines (IL6, TNF α , Interferon γ , adiponectin, FABP4, PPAR γ)
3. EV concentration, cellular origin and adipokine content using the same panel of adipokines as for 'free' plasma measurements
4. Evaluation of the effect of increased BMI on circulating EV profile
5. Evaluation of gender-related differences in circulating EVs and plasma adipokine profile

3.3 Scientific question/hypothesis

Does EV concentration and content alter in response to increased BMI in healthy individuals with no formal diagnosis of cardiometabolic disorder?

Does gender affect the circulating EV profile?

3.4 Methods

Disclosure: Recruitment of volunteers, consenting, anthropometric measurements, sample collection and laboratory analysis were performed by myself. Thus, I was not blind to BMI category or gender whilst undertaking the laboratory work. However, such an approach ensured standardisation of the research protocol and minimised inter-user variability whilst performing the laboratory assays.

3.4.1 Recruitment/ Ethics

Healthy non-smoking volunteers on no regular prescribed/ 'over the counter' medications or herbal supplements were invited to take part in this study. Ethical approval was obtained from the School of Medicine, Cardiff University (SoMRec 14/53).

All volunteers attended the Clinical Research Facility at the Wales Heart Research Institute between the 9 and 11am to avoid any potential variation in EV concentration and profile due to circadian rhythm. They underwent anthropometric measurements and donated a fasting venous blood sample.

The inclusion criteria were:

- No known chronic illness
- Non-smoking
- Not on any regular medication
- Able to provide written, informed consent
- Able to attend following an 8 hour overnight fast.

Exclusion criteria included:

- Pregnancy/post-partum
- Recent surgery or major illness
- Recent/current viral/bacterial infection
- Unable to provide written, informed consent
- Unable to attend in a fasting state

3.4.2 Anthropometric measurements

3.4.2.1. BMI (Body Mass Index)

Body Mass Index was calculated as weight (kg) divided by height (m) squared. Height was established using a Seca 242 measuring rod [Seca, Birmingham, UK] and weight was measured by a Seca floor scale [Seca Birmingham, UK].

3.4.2.2 Waist: Hip Ratio

The Waist: Hip Ratio (WHR) was determined by dividing the waist circumference (cm) by the hip circumference (cm). Waist circumference was measured by a tape measure positioned parallel to the floor immediately above the iliac crest at minimal respiration. The hip circumference was measured around the widest portion of the buttocks.

3.4.3 Blood Pressure Measurement

Following a brief period of rest, 2 blood pressure readings were obtained 5 minutes apart using a validated Omron HEM-9907 blood pressure monitoring device [Omron Healthcare UK Ltd, Milton Keynes, UK]. The blood pressure cuff was inflated on the non-dominant arm of each subject. The mean of 2 readings was then calculated.

3.4.4 Venepuncture

Venepuncture was performed using a 22G 'butterfly' needle inserted into a large peripheral vein (typically in the antecubital fossa). Samples for plasma markers were taken into EDTA vacutainers whilst samples for EV isolation were taken into citrate vacutainers as heparin-based anticoagulants are discouraged by the International Society of Extracellular Vesicles (ISEV) as heparin can bind EVs, affect platelets activation and give false-negative PCR readings [Witwer K, 2103].

3.4.5 Plasma analysis

Following a double centrifugation at 3000g for 10 minutes at 4°C, acellular plasma was stored at -80°C until recruitment was completed. All samples were analysed simultaneously in duplicates.

3.4.5.1 Fasting glucose

Glucose concentration was measured using a glucose oxidase assay as previously described in Methods chapter section 2.6.4 [Dietzler DN, 1980]. Fifty microliters of fasting plasma were assayed in duplicate for each healthy volunteer.

3.4.5.2 Fasting insulin

A human ELISA sandwich kit was used (Abcam, ab100578) as per the manufacturer's instructions. The same methodology was applied as for the human adipokine measurements (Methods chapter, section 2.6.3). Plasma was diluted 1:2 for this assay.

3.4.5.3 HOMA-IR

HOMA-IR was calculated as described in the Methods chapter, section 2.6.5

3.4.5.4 Plasma adipokines

Sandwich ELISA assays were used for these measurements as described in chapter 2 section 2. Samples were assayed in duplicate. Plasma IL6, Interferon γ , TNF α and adiponectin were evaluated using Human ELISA kits provided by Abcam (ab46042, ab100537, ab46087, ab99968 respectively). Plasma FABP4 concentration was evaluated by a Human Quantikine FABP4 ELISA kit (R&D Systems, DFBP40). A human PPAR γ Sandwich ELISA kit was used for plasma PPAR γ evaluation (LifeSpan BioSciences, Inc, LS-F12376). Plasma samples were diluted 1:20 for FABP4 and 1:30000 for adiponectin assays whereas it was measured undiluted for the remaining ones. A BMG Clariostar plate reader was used to determine optical densities at 450nm wavelength (with 540nm correction).

3.4.6 EV isolation and analysis

3.4.6.1 EV isolation

EVs were isolated by differential centrifugation as described in the Methods chapter, section 2.5.1.2.

3.4.6.2 EV concentration and size

Nanoparticle Tracking Analysis (NTA) was undertaken using NanoSight LM10 as described in Methods, section 2.5.4. All samples were measured within 48 hours of isolation. Following that, samples were gradually frozen to -80°C (1°C/hr using a 'Mr Frosty' cell freezing device) and stored for further analysis which was typically performed within 1-3 weeks of sample collection.

3.4.6.3 EV cellular origin and content

Time Resolved Fluorescence –based immunoassay was used for this analysis as described in Methods, section 1.3. Samples were assayed in duplicates and fluorescence was measured using a BMG Fluorostar Optima plate reader.

3.4.7 Statistical analysis

GraphPad version 6.0 was used for statistical analysis. Normally distributed variables are presented as mean (SD) whereas non-normally distributed variables are shown as median (range). The Shapiro-Wilk test was used to test for normality. A P value of <0.05 was accepted as statistically significant. When comparing normally distributed variables, t-test was used whilst for not normally distributed ones- the Mann Whitney test. The Kruskal Wallis test was used when more than 2 groups were compared with post-test Dunn's analysis. The Spearman correlation analysis was applied to test for correlations.

3.5 Results

3.5.1 Baseline anthropometric and metabolic characteristics of the healthy cohort

A total of 49 healthy subjects were recruited: 26 female (mean age 33.8 ± 11.5 years) and 23 male (mean age 33 ± 7.6 years), $p = ns$. Of these, 22 subjects had BMI $\leq 25 \text{ kg/m}^2$ (range $16-25 \text{ kg/m}^2$), 13 had BMI between $25-30 \text{ kg/m}^2$ (overweight) and 14 had BMI above 30 kg/m^2 (obese; range $30.3-42 \text{ kg/m}^2$). Therefore 55% of the evaluated cohort were overweight or obese.

The anthropometric and metabolic characteristics are summarized in Table 3.1.

Age (years)	Lean (BMI≤25kg/m ²)	Overweight (BMI 25-30kg/m ²)	Obese (BMI>30kg/m ²)	P value
Gender (M/F)	M=9, F=13	M=6, F=8	M=8, F=5	
BMI (kg/m ²)	21.86 (2)	27.7 (1.56)	35.3 (3.8)	^{a,b,c} p<0.005
Weight (kg)	64.2 (9)	79.5 (11.4)	105.9 (16.8)	^{a,b,c} p<0.005
Waist circumference (cm)	F: 76.9 (9.9) M: 82.2 (4)	F: 84.6 (8.8) M: 100.5 (6.75)	F: 109.4 (19.3) M: 118.5 (11.9)	F: ^a p=ns, ^{b,c} p<0.01 M: ^{a,b,c} p<0.01
WHR (waist:hip ratio)	F: 0.76 (0.05) M: 0.835 (0.03)	F: 0.77 (0.08) M: 0.92 (0.07)	F: 0.91 (0.06) M: 0.98 (0.07)	F: ^a p=ns, ^{b,c} p<0.01 M: ^a p<0.05, ^b p<0.005, ^c p=ns
Resting systolic BP (mmHg)	123.6 (12.9)	121.1 (13.4)	124.5 (15.7)	^{a,b,c} p=ns
Fasting glucose (mmol/L)	5.5 (0.7)	4.8 (1.7)	5.7 (0.87)	^{a,b,c} p=ns
Fasting insulin (mIU/mL)	3.36 (3.26)	4.6 (6.8)	5.4 (8.7)	^{a,b,c} p=ns
HOMA-IR	0.82 (0.75)	1.08 (1.5)	1.31 (1.95)	^{a,b,c} p=ns
Age (years)	32.6 (12.5)	33.5 (8.1)	34.7 (5.4)	^{a,b,c} p=ns

Tab.3.1: Anthropometric and metabolic characteristics of the healthy volunteer cohort. Waist circumference and WHR ratio are presented separately for male and female volunteers. ^a overweight vs lean ^b obese vs lean ^c obese vs overweight

The average BMI in the female cohort was $25.7 \pm 5.5 \text{ kg/m}^2$ compared to $28.6 \pm 6.4 \text{ kg/m}^2$ in the male cohort, with the average weight and WHR $70 \pm 15.5 \text{ kg}$ vs $90 \pm 23 \text{ kg}$ and 0.79 ± 0.08 vs 0.9 ± 0.08 , respectively.

The distribution of BMI in male and female cohorts is presented in Figure 3.1a and 3.1b respectively.

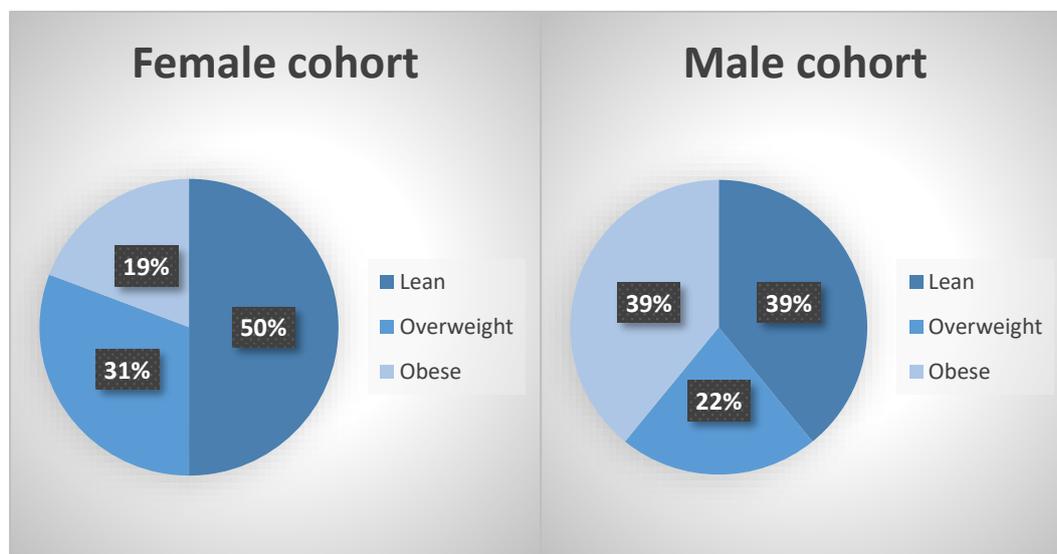


Fig.3.1: The BMI distribution: a) percentage BMI distribution in the healthy female cohort b) percentage BMI distribution in the male cohort.

3.5.2 Glucose homeostasis

The mean fasting glucose concentration remained within the normal range for evaluated subjects (Table 3.1). Fasting plasma insulin, as expected, was low in this cohort. Although, there are no clearly agreed cut-off values for HOMA-IR which define insulin resistance (since it is influenced by gender, age and ethnicity), it is notable that the average HOMA-IR in my obese cohort was below cut-off thresholds conventionally used for diagnosing insulin resistance in the literature (which vary from 1.5 to 2.77) [Gayoso-Diz P, 2013].

3.5.3 Circulating plasma cytokine and adipokine profile

Measurements of these adipocytokines were performed in 40 out of 49 subjects; reasons include not enough plasma available, sample compromised or failure of assay.

3.5.3.1 Interleukin 6

The median IL6 concentration was 1.49 pg/mL (0.43-7.4pg/mL). IL6 was significantly higher in healthy male volunteers compared to females (1.9 vs 1.1pg/mL, $p<0.05$). Plasma IL6 correlated with fasting insulin and HOMA-IR. Moreover, positive correlations were observed with EV concentration/mL and WHR ($r= 0.35$ and $r=0.4$ respectively, $p<0.05$) and negative correlation with plasma adiponectin ($r=-0.47$, $p<0.005$). There were no correlations observed with BMI, age or EV- IL6 expression ($r= 0.29$, $p=0.0065$, $r=0.14$ $p=0.39$ and $r=0.39$, $p=0.076$).

3.5.3.2 TNF α

As expected, plasma TNF α concentration in this cohort of metabolically healthy volunteers was low, with a median concentration of 47.7pg/mL (range 2-833pg/mL) and no differences between males and females (49pg/mL vs 47pg/mL, $p=0.82$). In 6 subjects the concentration of this cytokine was below detection threshold of the assay used therefore they were excluded.

3.5.3.3 Interferon γ

Circulating plasma interferon γ was expected to be very low and difficult to detect in plasma of healthy donors. In our cohort, only 17 subjects had detectable levels of this cytokine with a median concentration of 98pg/mL (range 10-604pg/mL) with the assay's sensitivity threshold being 15pg/mL.

3.5.3.4 Adiponectin

In contrast to the above, all subjects had detectable levels of circulating adiponectin, with a median concentration of 203.4 $\mu\text{g/mL}$ (range 115.1-822.9).

Interestingly, plasma adiponectin was higher in women than in men (272.3 $\mu\text{g/mL}$ vs 128 $\mu\text{g/mL}$, $p<0.005$). However, no difference was observed between lean, overweight and obese BMI cohorts as plasma adiponectin did not correlate with BMI ($r=-0.2$, $p=0.2$), neither did it correlate with HOMA-IR, fasting insulin or age (respectively $r=-0.2$, $r=-0.19$, $r=-0.04$, $p=ns$). However, it did correlate negatively with WHR and waist circumference ($r=-0.46$ and $r=-0.35$, $p<0.05$). As discussed above plasma adiponectin correlated negatively with plasma IL6 concentration but no relationship with other plasma adipocytokines was observed. Similarly to above described adipocytokines, there was no correlation observed between plasma and EV-associated adiponectin ($r= -0.12$, $p=0.45$). Interestingly however, plasma adiponectin correlated negatively with various EV markers as summarised in Figure 3.2.

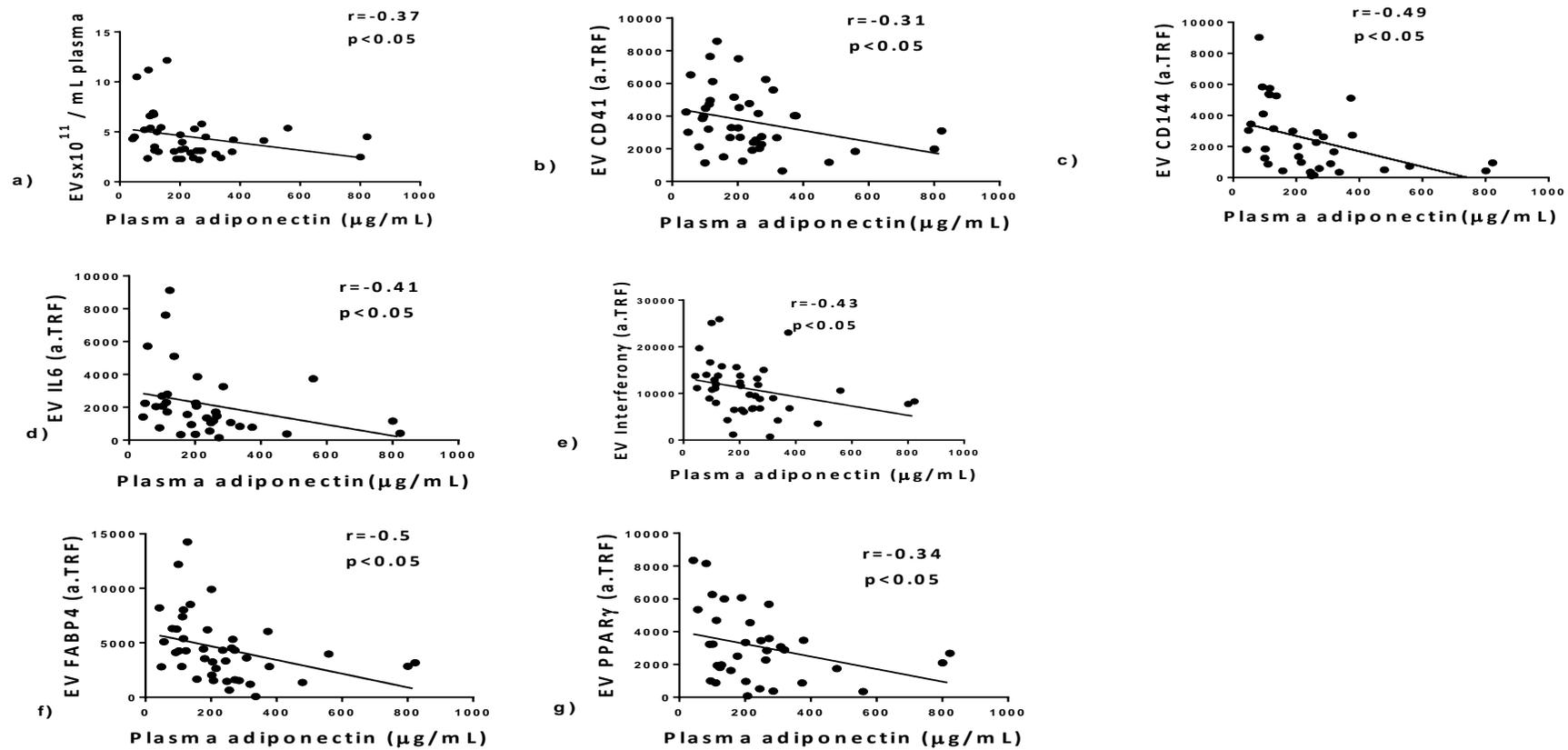
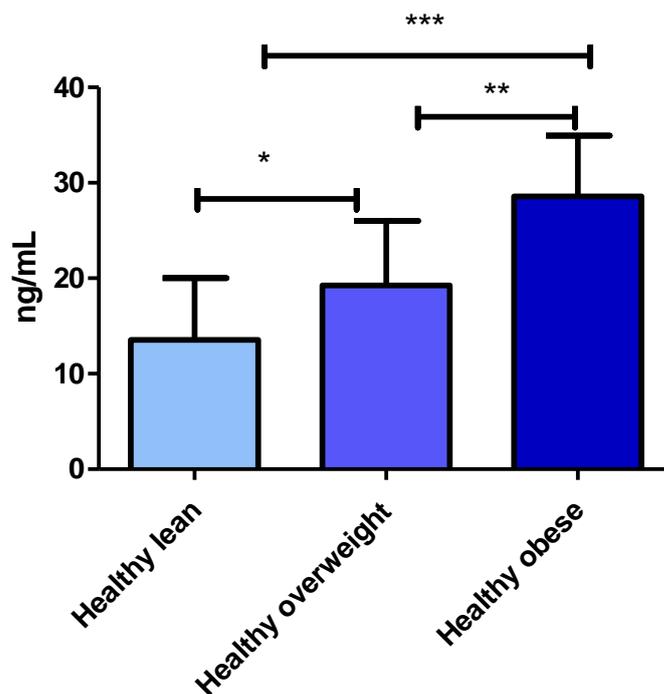


Fig. 3.2 Spearman correlation analysis of plasma adiponectin and EV concentration/mL of plasma (a) and EV expressed cellular markers (b,c) and adipokines (d-g).

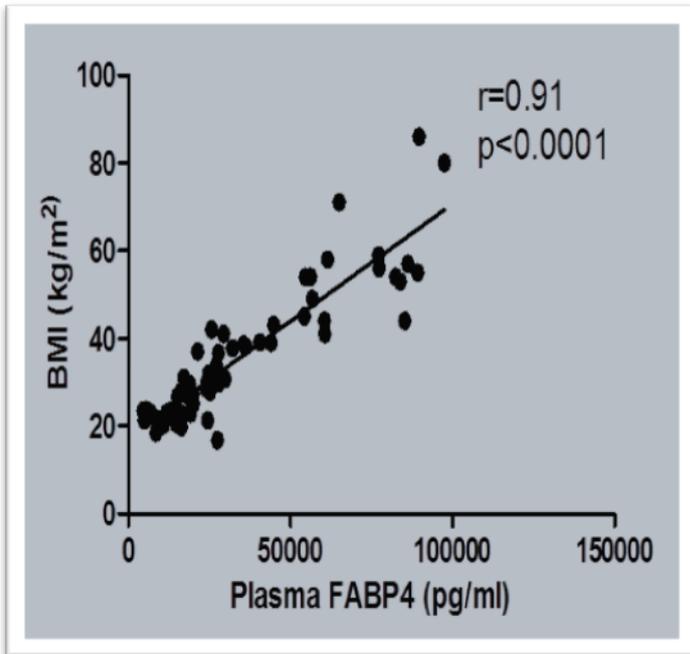
3.5.3.5 Fatty Acid Binding Protein 4

Similarly to the adiponectin assay, all healthy subjects had detectable levels of circulating plasma FABP4 with a mean concentration of 19.9 ± 9.07 ng/mL. There were no gender differences observed (mean plasma FABP4 concentration in women vs men: 21.8 ± 7.8 vs 18.1 ± 9.8 ng/mL, $p=ns$) but significant differences between healthy lean and healthy overweight and obese volunteers were observed (13.5 ± 6.4 vs 19.2 ± 6.7 vs 28.5 ± 6.3 ng/mL, all $p < 0.05$) (Fig. 3.3a). Therefore, as expected, plasma FABP4 concentration correlated significantly with BMI (Fig. 3.3b), weight and waist ($r=0.68$, $r=0.56$, $r=0.59$ all $p < 0.0001$), as well as WHR and age ($r=0.36$, $p < 0.05$ for both). There were no correlations observed with any of the glucose metabolism measurements, other plasma adipocytokines or EV concentration or markers, including EV FABP4 (all $p=ns$).

Plasma FABP4 concentration



a)



b)

Fig.3.3. a) Plasma FABP4 concentration (mean (SD)) in healthy lean, overweight and obese volunteers. * $p<0.05$, ** $p<0.01$, * $p<0.005$ b) Spearman analysis of correlation between plasma FABP4 and BMI from all cohorts evaluated in this thesis**

3.5.3.6 PPAR γ

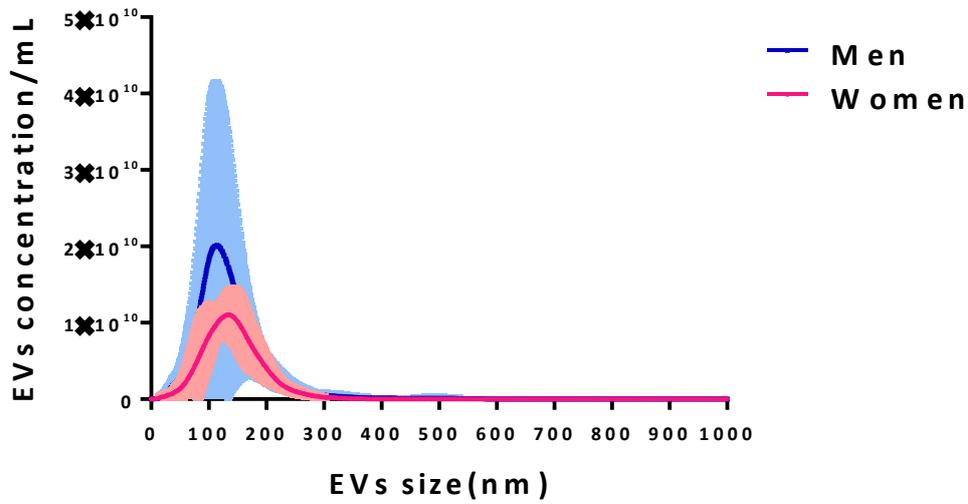
The presence of free PPAR γ in plasma was expected to be very low however the assay was performed to mirror the EV expressed PPAR γ analysis. Unfortunately, in 29 out of 40 subjects the levels were below the detection threshold of the assay which had a sensitivity threshold of 0.156ng/mL according to the product data sheet. Amongst those 11 subjects who had detectable levels of plasma PPAR γ (M=8, F=3, lean=4, obese= 7) the median concentration obtained was 0.37ng/mL (0.09-6.3).

3.5.4 Characterisation of circulating plasma EVs in the healthy population

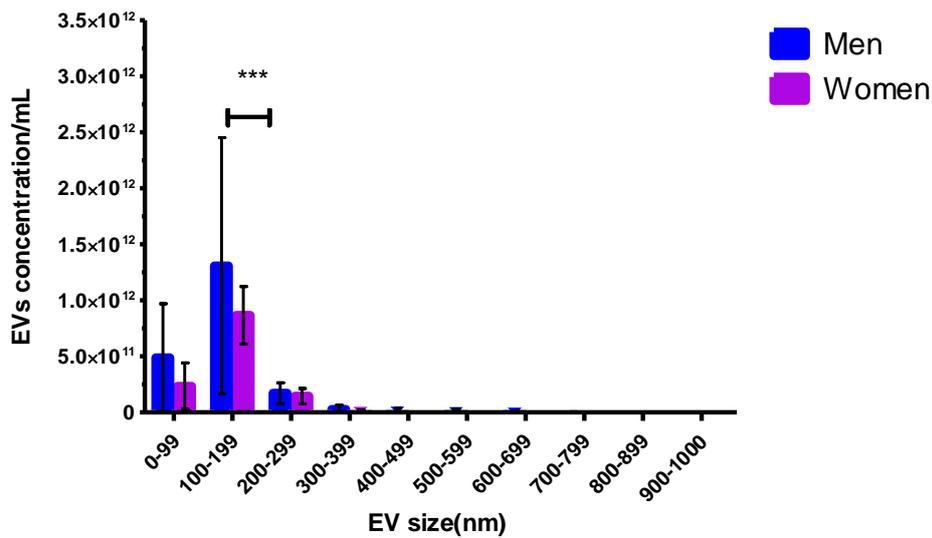
3.5.4.1 EV concentration and size distribution

There was no statistically significant difference in median EV concentration between healthy lean and healthy overweight and obese individuals (3.3×10^{11} EVs /mL (2.2-12.15) vs 4.1×10^{11} EVs/mL (2.3-8.7) vs 4.7×10^{11} EVs/mL (2.3-11.2), $p=0.64$).

The size distribution analysis did not reveal any differences either. In contrast, male volunteers had a higher concentration of EVs within a subgroup ranging from 100 to 200nm in diameter ($p < 0.01$, Fig.3.4) though no differences were seen in the median plasma concentrations (4.5×10^{11} EVs/mL (2.2-12.5) vs 3.7×10^{11} EVs/mL (2.3-8.7), $p=0.3$).



a)



b)

Fig.3.4 a-b Differences in size distribution of plasma EVs between male and female healthy volunteers. Male subjects had significantly higher EV concentration within the exosomal range (100-200nm in diameter). *= $p < 0.005$.**

The correlations between EV concentration/mL of plasma and other parameters are summarised in Table 3.2.

EVs conc./mL of plasma	r	P value
BMI	0.15	0.33
WHR	0.14	0.37
Age	0.15	0.34
HOMA-IR	0.39	0.01
Glucose	0.007	0.96
Plasma IL6	0.35	0.02
EV-IL6	0.4	0.01
Plasma Adiponectin	-0.37	0.01
EV-adiponectin	0.14	0.39
Plasma FABP4	0.12	0.42
EV FABP4	0.17	0.27
EV PPAR γ	0.13	0.42

Tab.3.2: Spearman correlation analysis between EV concentration/mL of plasma and other evaluated parameters.

3.5.4.2. EV cellular origin

The expression of the following cell-of-origin markers was evaluated : CD41(platelets); CD11b (monocytes/macrophages) , CD235a (erythrocytes) and CD144 (endothelial cells) as well as the exosomal marker: CD9.

There were no differences in the expression of these markers between healthy lean, overweight and obese individuals ($p=0.96$, $p=0.31$, $p=0.26$, $p=0.97$, $p=0.84$, respectively) (Fig 3.5a). It is likely related to the large individual variations in expression of those markers across the analysed groups of healthy volunteers which given the sample size makes it difficult to detect any obvious differences.

Interestingly, when results were compared by gender, the expression of CD144 was significantly higher in males vs females, whilst there was no statistically significant difference for CD41 ($p=0.07$).

There was no difference in the expression of the erythrocyte marker or CD9 ($p=0.14$ and $p=0.37$, respectively) but female subjects had higher CD11b signals ($p=0.02$)(Fig 3.6a).

3.4.3.3 EV adipocytokine expression

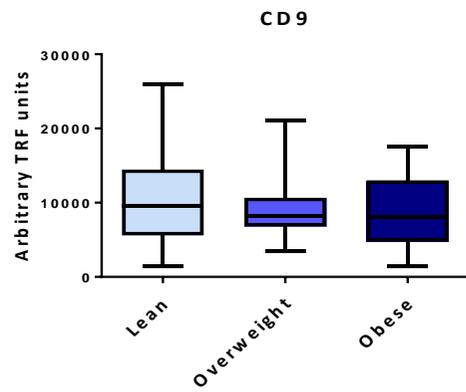
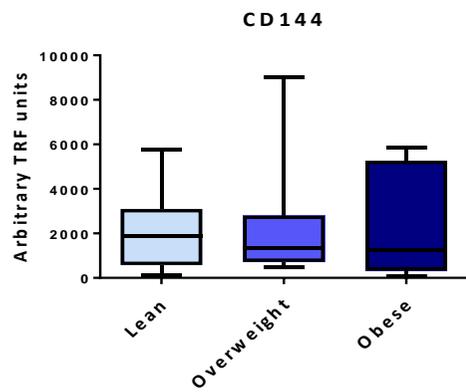
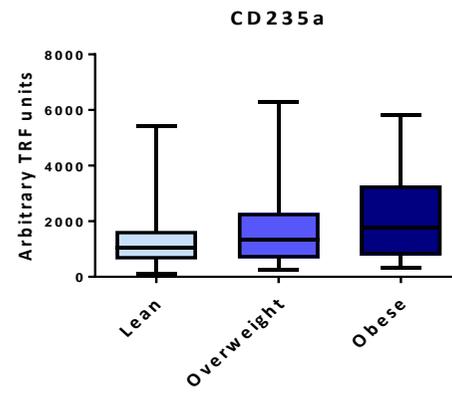
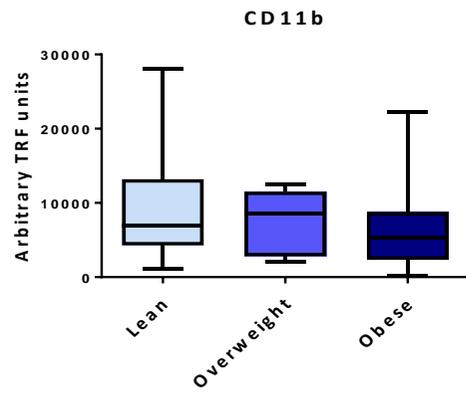
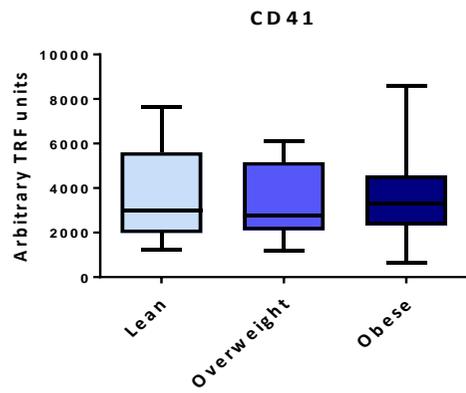
Analysis of the expression of the following adipocytokines was performed: Interleukin 6, TNF α , Interferon γ , adiponectin, FABP4 and PPAR γ - to match the plasma adipocytokine analysis.

No differences were observed with regard to expression of these adipocytokines in EVs isolated from lean, overweight and obese individuals ($p=0.75$, $p=0.95$, $p=0.53$, $p=0.36$, $p=0.35$, $p=0.7$, respectively). This remained true even when obese and overweight healthy individuals were grouped together or waist circumference rather than BMI was used to define body habitus.

Interestingly, gender differences were observed with significantly higher signals from EV-expressed IL6, Interferon γ and FABP4 in males ($p= 0.01$, $p=0.0006$, $p=0.0013$) but no differences for TNF α , adiponectin or PPAR γ ($p=0.57$, $p=0.15$, $p=0.44$ respectively)(Fig. 3.6b).

The higher expression of EV-IL6 in male subjects was in keeping with higher plasma-free IL6 expression but correlation between plasma and EV IL6 was not significant ($p=0.07$). As mentioned above, no direct correlation was observed between the plasma and EV-expressed TNF α , Interferon γ , adiponectin, FABP4 ($p=0.2$, $p=0.86$, $p=0.74$ and $p=0.71$). This suggests that the process of EV packaging with adipocytokines is controlled by mechanisms different to those that regulate secretion of plasma 'free' adipocytokines.

Adiponectin-Receptor- 1 was also found to be expressed within plasma derived EVs with median TRF signals of 19542 (478-50485 a.TRF) but it did not correlate with plasma or EV-expressed adiponectin or BMI ($p=0.93$, $p=0.23$, $p=0.39$ respectively).



a)

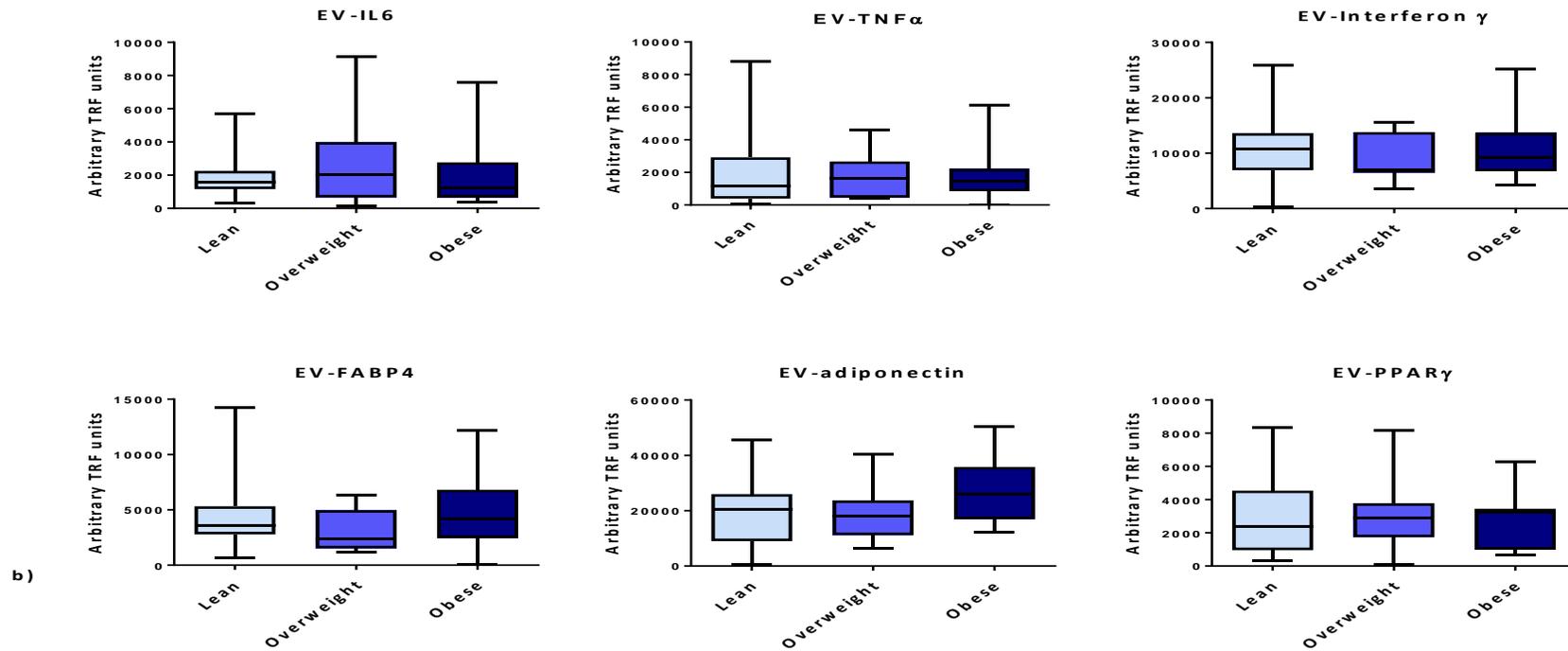
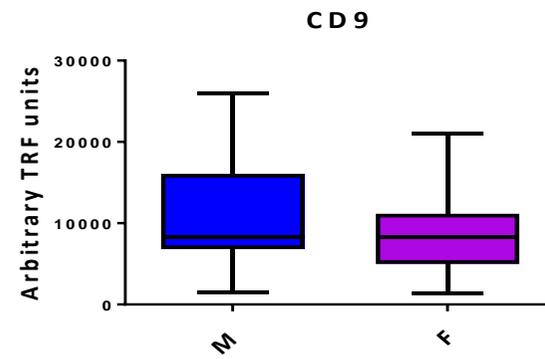
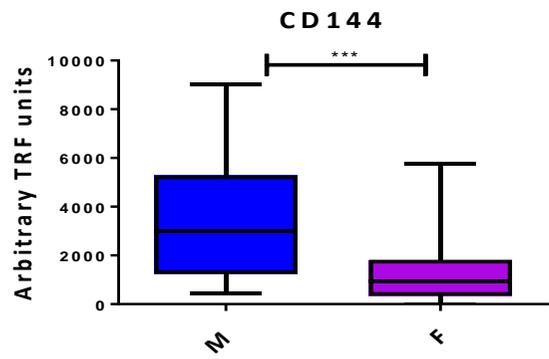
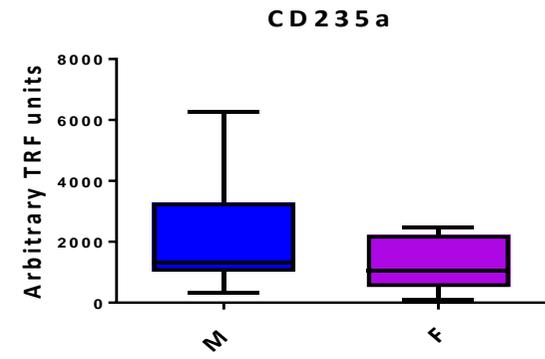
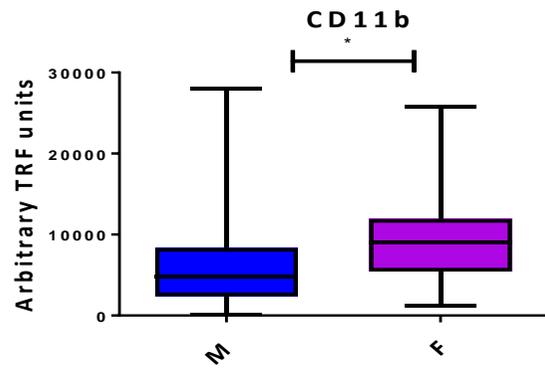
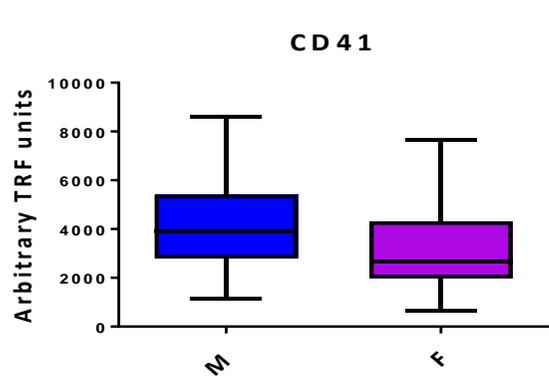
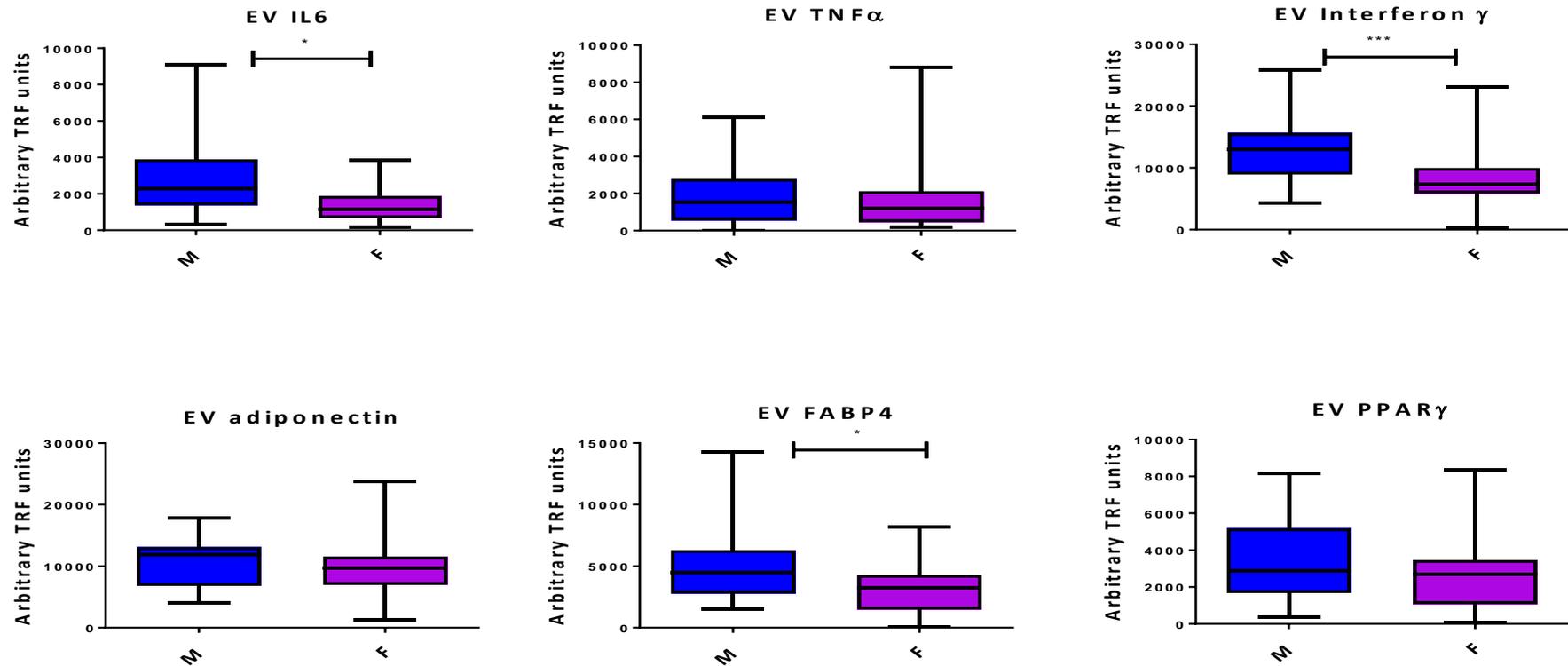


Fig.3.5 a) Expression of cell of origin markers (CD41 platelets, CD11b monocyte/macrophages, CD235a erythrocytes, CD144 endothelial cells) in plasma EVs isolated from healthy lean, overweight or obese individuals. b) EVs adipocytokine expression in lean, overweight and obese individuals.



a)



b)

Fig.3.6 a) Effects of gender on expression of cell-of-origin markers (CD41 platelets, CD11b monocyte/macrophages, CD235a erythrocytes, CD144 endothelial cells). * $p < 0.05$, *** $p < 0.005$ b) Effects of gender on EV adipokine expression. * $p < 0.05$, *** $p < 0.005$

3.5.4.4 Expression of the exosomal marker CD9

There was no difference in expression of CD9 between healthy volunteers, irrespective of their BMI or gender. However, interesting significant correlations were observed between CD9 and EV-expressed FABP4, interferon γ , and adiponectin across the BMI range, suggesting that exosomes (rather than microvesicles) may be the primary vesicular carriers for these molecules (Fig.3.7).

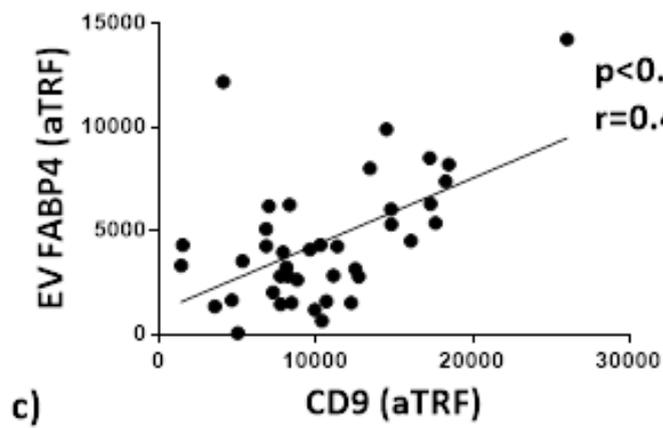
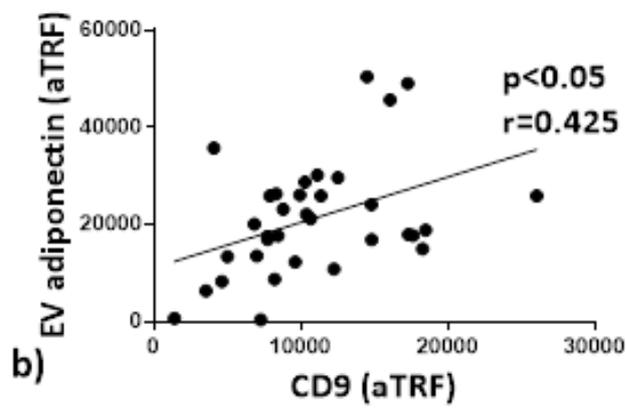
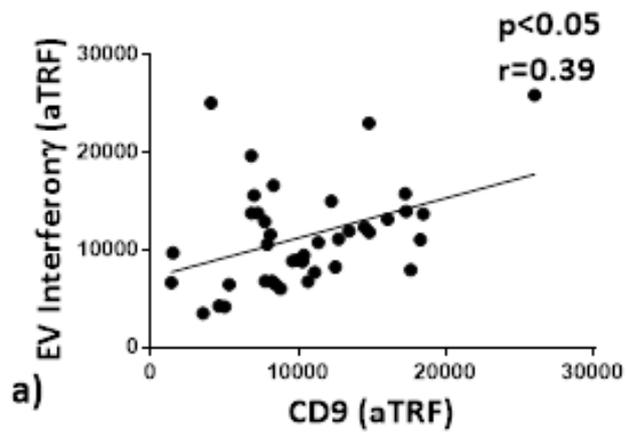


Fig.3.7: Spearman correlation analysis of the exosomal marker CD9 with EV-expressed interferon γ , adiponectin and FABP4 (a-c).

3.6 Discussion

3.6.1 Anthropometric measurements and glucose metabolism

Body Mass Index (BMI) and Waist-Hip Ratio (WHR) correlate strongly with body fat and the cut-off point for the healthy volunteers in this study dividing them into healthy weight/lean, overweight and obese groups was based on the World Health Organisation (WHO) and National Institute for Health and Clinical Excellence (NICE) definitions [WHO, 1995, NICE, 2006]. These cut-off points were chosen based on established relationships between mortality and increased BMI; current guidelines suggest that health intervention is required when BMI is 25kg/m² or more [WHO, 1999, NICE, 2005].

However, a significant limitation in using BMI is the fact that it reflects 'overall' adiposity, whilst it is visceral adiposity in particular that has emerged as an independent predictor of health outcomes [WHO, 1999, NICE, 2006]. The latter is easily measured by waist circumference. However, since commonly used cut-off points to define cardiometabolic risk differ for men and women (102cm and 88cm, respectively), I decided to use BMI rather than waist circumference to divide my cohort into subgroups. The recruited subjects were well matched with regard to age and despite significant BMI, weight and waist circumference differences, no differences were observed between them with respect to glucose metabolism (fasting glucose, insulin and HOMA-IR). On the one hand, this observation confirms the 'healthy obese' status of the recruited subjects. On the other, it shows that changes in glucose metabolism in obese subjects may be a late sign of underlying metabolic dysfunction. This would be in keeping with the current assumption that about half of the estimated 415 million of people with type 2 diabetes worldwide are unaware of their condition [Beagley J, 2014]. In recent years, the term 'metabolically healthy obesity' has gained lots of attention following observations that some obese individuals may be somewhat protected from developing the cardiometabolic consequences of increased adiposity. However, it is presently unclear how best such subjects can be distinguished at an early stage from 'high risk' obese subjects using traditional markers of metabolic health [Stefan N, 2013]. A recent meta-analysis by Lotta *et al* which evaluated a total of 140845 individuals from various studies according to lean, overweight and obese BMI categories, showed that current traditional binary markers of metabolic health reported in literature (such as the presence or absence of metabolic syndrome, insulin resistance or both, or cardiorespiratory fitness) have limited predictive performance in establishing the long-term risk of developing type 2 diabetes [Lotta L, 2015]. The so-called metabolically healthy obese subjects still had a high absolute risk of developing type 2 diabetes (10-year cumulative incidence 3.1% [95% CI 2.6–3.5]) [Lotta L, 2015]. Therefore, greater emphasis has been placed in recent years on developing more complex machine learning algorithms to predict the risk of cardiometabolic disease [Olivera A,

2017] as well as the development and introduction to clinical practice of more complex and individualized metabolomics/proteomics profile [Peddinti G, 2017]. This aligns with the aims and results of my study as discussed below.

3.6.2 Plasma adipocytokines

As discussed in chapter 1, adipose tissue is an active endocrine organ secreting a variety of molecules playing a crucial role in the regulation of metabolic homeostasis. The chronic low-grade systemic inflammation associated with obesity is likely to occur secondary to the imbalance in secretion of pro-inflammatory vs. anti-inflammatory cytokines from adipose tissue [Ouchi N, 2011]. These adipokines are believed to play an important role in the intercellular cross-talk regulating inflammation and metabolism. However, the pathways regulating these processes are not yet fully understood. Below, I discuss the relevance of my analysis of plasma IL6, TNF α , adiponectin and FABP4 concentration. As plasma Interferon γ and PPAR γ levels were very low and measurable in only a small proportion of subjects, I will not discuss them further in the context of this study.

3.6.2.1 Plasma IL6

Plasma IL6 levels are well known to be increased in metabolic syndrome/obesity and type 2 diabetes mellitus (T2DM). Indeed, IL6 concentrations may help predict the risk of type 2 diabetes development [Pradhan AD, 2001]. Moreover, IL6 levels decrease following a lifestyle intervention aiming at weight reduction (which also leads to increase in plasma adiponectin levels) [Esposito K, 2003]. It is believed that around one third of circulating IL6 is secreted by adipose tissue and this cytokine correlates with adiposity [Fried SK, 1998], which was shown by its correlation with WHR in our cohort. IL6 also has an effect on insulin signalling as it exacerbates insulin resistance in hepatocytes [Senn JJ, 2003].

IL6 and TNF α hinder the secretion of adiponectin [Kern PA, 1996], which was supported by the negative correlation observed between IL6 and adiponectin in my cohort. In my study, apart from association between IL6 and overall plasma EV concentration, we did not observe any correlation with any other EV markers. However, a previous study by Ueba *et al* evaluating Japanese healthy volunteers showed that platelet-derived EVs correlated with plasma IL6 concentration as well as CRP [Ueba T, 2010].

There do not appear to be gender-related reference ranges for IL6 but in my cohort, interestingly, plasma IL6 was marginally higher in men. In contrast to my subjects, who were relatively young, a study by Campesi *et al* revealed that plasma IL6 (after correction for body weight) was significantly higher in post- vs. premenopausal women and also higher compared to men >45 years of age [Campesi I, 2016].

3.6.2.2. Plasma TNF α

Similarly to IL6, plasma levels of TNF α are increased in obese individuals and decrease following weight loss interventions [Kern PA, 1995]. Many animal models have shown that this cytokine plays a crucial role in regulation of insulin sensitivity as it attenuates insulin-stimulated tyrosine phosphorylation of the insulin receptor in muscle and adipose tissues, which leads to insulin resistance [Hotamisligil GS, 1994]. It is therefore not surprising that in my data as well as in the literature [Hivert MF, 2008] plasma TNF α correlates with markers of insulin resistance, even when HOMA-IR is still within normal range (as it is in this cohort). This indicates the involvement of this cytokine in processes regulating metabolic homeostasis.

Plasma cytokines did not correlate with BMI or age in my cohort, which as stated above was relatively young and there were not many severely obese volunteers recruited (the highest BMI was 42kg/m²).

3.6.2.3. Plasma Adiponectin

Adiponectin is an anti-inflammatory, anti-atherogenic adipokine that is known to be present in abundance in the circulation. Though this protein is primarily secreted by adipocytes, it paradoxically decreases in obese subjects and is reduced in patients with T2DM [Hotta K, 2000] suggesting its secretion is disturbed in those clinical circumstances. The secretion of this adipokine is in fact decreased by IL6 and TNF α (which are elevated in obesity driven chronic systemic inflammation) [Ouchi N, 2011] and reduced levels of adiponectin can be considered as a marker of visceral adiposity. Indeed, in our cohort plasma adiponectin showed negative correlation with WHR (Fig.3.2). It also correlated negatively with FABP4- expressing EVs, which we believe, are predominately secreted by adipocytes.

Moreover, hypoadiponectinaemia is associated with insulin resistance: in previous studies on Rhesus monkeys, a decline in plasma adiponectin concentration occurred before hyperinsulinaemia developed [Hotta K, 2001]. This might explain why no correlation was observed with HOMA-IR/fasting insulin in my healthy volunteers who even in the obese arm had no apparent biochemical evidence of disturbed glucose homeostasis suggestive of impending insulin resistance.

Interestingly, men had significantly lower plasma adiponectin levels compared to women in my study. This is in keeping with findings from a study by Nishizawa *et al* who in an attempt to explain this phenomenon showed that *in vitro* testosterone reduces adiponectin secretion from 3T3 cells and that castration leads to increase of this adipokine in the mice models [Nishizawa H, 2002]. This has led to a conclusion that androgens are involved in reducing circulating plasma adiponectin levels and might thus contribute to the known increased risk of cardiovascular disease and insulin

resistance in male subjects. The interplay between adiponectin and hypothalamo-pituitary-gonadal (HPG) axis has only begun to be explored in recent years. In fact, it has been shown that adiponectin and adiponectin receptors are present in HPG axis cells participating in gameto- and steroidogenesis and regulated by factors such as age, gender, nutrition and hormonal status [Rak A, 2017].

Finally, I also found interesting correlations between plasma adiponectin and EV markers. Firstly, plasma adiponectin correlated negatively with circulating EV concentration. This might suggest a possible negative feedback mechanism between the two as adiponectin is known as the 'anti-inflammatory' adipokine whereas increased circulating EVs concentration are found in various pathological disorders associated with increased inflammation such as CVD [Agouni A, 2008, Helbing T, 2014]. EV secretion is known to be affected by a rise in intracellular calcium [Pasquet JM, 1996] hence calcium ionophores directly trigger EV release [Hess C, 1999]. On the other hand, recent studies have shown that increased concentrations of extracellular calcium and phosphate also stimulate EV release (from macrophages) which have high calcification potential and can be involved in excessive intimal and medial calcification which subsequently leads to CVD development [New SE, 2013]. It was also reported by Kapustin *et al* that vascular smooth muscle cell calcification is affected by exosome secretion regulated by sphingomyelin phosphodiesterase 3 that is induced by increased extracellular calcium levels [Kapustin A, 2015]. Thus both *in vitro* and *in vivo* (in chronic kidney disease patients) high extracellular calcium levels were associated with increased secretion of EVs with a 'calcifying' potential [New SE, 2013]. High plasma adiponectin levels have anti-atherogenic potential as they suppress TNF- α -induced mRNA expression of various adhesion molecules, decrease lipid accumulation in macrophages and decrease proliferation of vascular smooth muscle cells [Ouchi N, 1999]. Adiponectin is known to lead to increase in intracellular calcium [Ouchi N, 2011]. Therefore, its higher levels possibly inhibit secretion of those EVs subpopulation that have detrimental effects on the vascular endothelium, with a consequent protective effect against CVD development. This would be in keeping with the findings by Zoccali *et al* who reported that plasma adiponectin inversely correlates with the incidence of ischaemic heart disease in CKD patients (who are known to have high plasma calcium levels) [Zoccali C, 2002]. The mechanism behind the inverse relationship between plasma adiponectin and EV concentration is likely to be more complex but I believe that alterations in calcium homeostasis may play an important role here.

In my study, I also found that plasma adiponectin correlated negatively with endothelial- and platelet-derived EVs subpopulations which are known to be increased in subjects with CVD [Agouni A, 2008, Helbing T, 2014]. This suggests that this adipokine may have an important effect on EV-driven intracellular communication affecting/protecting the vascular endothelium.

A previous study by Kranendonk *et al* evaluating correlations between plasma adiponectin and “atherothrombotic” EV markers (cystatin C, CD14, serpin G1 and F2) in subjects with clinically evident cardiovascular disease did not reveal any relationship between adiponectin and those specific EV expressed markers [Kranendonk ME, 2014]. This may indicate that once overt cardiovascular disease is present (which is associated with hypoadiponectinaemia), the negative feedback between adiponectin and the release of EVs, which have proinflammatory and proatherogenic potential, is disturbed.

It should also be noted that the plasma EVs in my cohort were expressing adiponectin receptor-1 by TRF immunoassay which may suggest that this adipokine is not only involved in regulating EV secretion but may directly affect EV biology by binding to its receptor on them. More detailed studies in this area are required which were outside the scope of this project.

3.6.2.4 Plasma Fatty Acid Binding Protein 4 (FABP4)

FABP4 is emerging as an early and potent biomarker of cardiovascular and metabolic disease and has been detected not only in adipocytes but also in macrophages [Furuhasi M, 2015]. High circulating levels of this adipokine are associated with insulin resistance, type 2 diabetes and atherosclerosis [Furuhasi M, 2015]. It is involved in intracellular lipid cargo, glucose metabolism and inflammatory response [Furuhasi M, 2015, Hotamisligil GS, 2015]. My data showed a strong correlation between BMI, weight and waist circumference and plasma FABP4. Moreover, changes in plasma concentration of this adipokine were observed between metabolically healthy lean, overweight and obese individuals before any changes in traditional glucose metabolism markers such as HOMA-IR or fasting glucose or proinflammatory cytokines associated with obesity such as IL-6 are seen. This suggests that plasma FABP4 could be utilised in the future as a marker of impending cardiometabolic dysfunction. It would be of great interest to follow up my healthy cohort over a 5- or 10-year period to evaluate the predictive value of high plasma FABP4 for the development of cardiovascular disease or type 2 diabetes mellitus. Evidently, this was not possible due to time restrictions in my project but interesting data from other research centres on the use of this adipokine are emerging. A study by Miedema *et al* evaluated the association between plasma free fatty acids (FFA) and plasma FABP4 and total and cardiovascular mortality in older adults (mean age=75, n=4770, median follow up 11.8 years). Interestingly, they found that it was plasma FFA and not plasma FABP4 that was better associated with cardiovascular and non-cardiovascular mortality [Miedema MD, 2014]. However, the study by Xu *et al* where 495 non-diabetic adults were followed up for 5 years showed that subjects with higher baseline plasma FABP4 levels developed a progressively worse cardiometabolic risk profile and increasing risk of metabolic syndrome independently of adiposity and insulin resistance [Xu A, 2007]. Also, von Eynatten *et al* showed that plasma FABP4 in subjects with clinically overt CVD

was, over a 10-year follow-up, associated with increased risk of secondary cardiac events and cardiovascular death [von Eynatten M, 2012]. Therefore, there is a growing interest in clinical application of this adipokine in various risk stratification approaches. A recent study by Lindt *et al* evaluating various proteomics that could be used to predict the risk of atrial fibrillation (AF) highlights the 'predictive' potential of plasma FABP4 [Lind L, 2016] whilst Ningh *et al* suggest it could also be a useful biochemical marker to estimate the risk of developing gestational diabetes [Ningh H, 2016]. Moreover, a recent study by Tu *at al* suggests that plasma FABP4 can also be used to predict the outcome from an acute ischaemic stroke [Tu W, 2017]. It is therefore quite likely that plasma FABP4 may soon become incorporated into risk stratification pathways given that it is relatively easy to measure using commercially available assays. However, the important research questions that remain unanswered are: 1) whether lowering plasma FABP4 can affect primary and secondary CVD outcomes and 2) how this can be achieved pharmacologically. Advances in our understanding of this protein may, in my opinion, have a significant impact on our approach to the management of cardiometabolic disorders in years ahead.

3.6.3 Circulating plasma EVs in healthy volunteers

My study has shown that in metabolically healthy individuals the circulating EV profile is unaffected by increasing BMI/weight. It was observed not only with regard to the expression of markers of cellular origin of the main plasma-derived EV families (platelets, monocytes/macrophages, erythrocytes, endothelial cells) but also with regard to their adipokine content. This indicates that EV secretion and packaging could be controlled by mechanism(s) maintaining metabolic homeostasis in the healthy state and until this is disturbed, EV profiles of so called "healthy obese" individuals do not differ from "healthy lean" individuals. As discussed earlier, EVs have gained lots of attention in the recent years as potential disease biomarkers but it must be remembered that cells release EVs in both health and disease states and the fact that increase in various EVs has been associated with many pathological disorders may have slightly drawn the attention away from the fact that EVs probably also play an important role in physiological intercellular signalling in order to maintain health. Therefore, in contrast to what I initially hypothesized, circulating EVs cannot at present, at least using current methodology, be used as biomarkers of impending metabolic disorder if obesity alone is present. They do, however, appear to reflect overt cardiometabolic disease as evident in the literature and by my further work described in chapter 4 and 5 which characterises EVs in severely obese subjects who developed obesity driven comorbidities.

Interestingly, I observed that within a healthy cohort there are differences in circulating plasma EV profiles that suggest EVs in men have more pro-inflammatory potential which would be in keeping with the increased CVD risk associated with male gender. Although there were slightly more males in

my study in the overweight or obese category (Fig.3.1), the lack of correlation between EV characteristics and BMI/WHR suggest that there are different mechanisms, other than adiposity alone, influencing these gender-related differences. This observation, apart from potential interpretation in the CVD risk stratification context, is hopefully a useful addition to the knowledge required to establish accurate gender-related reference ranges for healthy plasma EV profiles.

Below, I have discussed some recent studies, which further elaborate on this in the context of my results.

3.6.3.1 Plasma EVs and obesity

As mentioned previously, Kranendonk *et al* recently evaluated a cohort of 1012 patients with clinically manifest vascular disease. Isolated plasma EVs were evaluated for the expression of cystatin C, serpin G1 (C1-inhibitor), serpin F2 (alpha-2-antiplasmin) and CD14 (monocytes) markers [Kranendonk ME, 2014]. This study, in keeping with my findings, showed that it is not the adipose tissue excess but rather adipose tissue dysfunction and the presence of metabolic complications that drives the differences in circulating plasma EVs. When vascular disease subjects in this study were divided into subgroups based on BMI (<30 and >30kg/m²), visceral obesity (waist circumference >88cm for females and >102cm for males) or visceral adipose tissue thickness, no differences in the concentration of the above 4 EV-expressed markers were observed between the groups [Kranendonk ME, 2014]. Only when overt metabolic complications occurred (such as dyslipidaemia, presence of metabolic syndrome or T2DM), the EV profile started to differ, showing positive correlations between EVs expressing cystatin C and high CRP, and negative associations with HDL. Moreover, the EV –cystatin C levels were associated with 57% higher odds for developing the metabolic syndrome. There were, however, no gender-related differences in the EV profiles observed [Kranendonk ME, 2014].

Similarly, Zhang *et al* evaluated the impact of type 2 diabetes and obesity in combination and separately on absolute EV concentration as well as EV expression of platelet, leukocyte and monocyte markers. They showed that subjects with diabetes had higher concentrations of platelet-derived as well as fibrinogen-positive, tissue factor-positive and P-selectin positive EVs, with no differences in monocyte or leukocyte markers. Obesity did not affect any of the EV profile and even within subjects with diabetes, the concentration of platelet-derived EVs did not differ between lean and obese diabetic patients [Zhang X, 2014].

3.6.3.2 Plasma EVs and gender

A recent study by Baek *et al* characterised EVs from healthy males and females over 40 years of age using a sandwich ELISA-based EV array developed by this group [Baek R, 2016]. Samples were evaluated for 37 various EV markers/EV associated proteins (which apart from CD9 differed from those used in my study). The results showed that there was a wide variation in expression of markers amongst individuals. There was no effect on the EV profile of gender *per se*, but smoking appeared to increase CD9 expression as well as CD151 (another tetraspanin), and decreased Epithelial Cell Adhesion Molecule expression (EpCAM). However, when gender was further split by smoking status, it was seen that male smokers had higher level of CD171 (L1CAM) (cell adhesion molecule), PD-L1 (Programmed Death Ligand-1) and TSG101 (Tumor susceptibility gene 101 proteins) compared to female smokers or male non-smokers. Female smokers had also significantly lower TSG101 and CD146 (MUC18; cell surface glycoprotein) compared to non-smokers. Certain proteins were also significantly lower in female compared to male smokers such as AREG (amphiregulin), MUC1 (polymorphic epithelial mucin), CD146, CD13 (membrane alanyl aminopeptidase) and TSG 101. Aging was associated with a decrease in oestrogen receptor (AREG) in both genders but only significantly in males [Beak R, 2016].

Gustaffson *et al* evaluated gender-related differences in plasma EVs from healthy men and women (aged 20-70) isolated by differential centrifugation and evaluated by flow cytometry (hence only identifying EVs >200nm in diameter) [Gustaffson CM, 2015]. This was in contrast to the above Danish study, which used an array of exosomal markers (CD9, CD63, and CD81) in order to specifically characterise smaller EVs. The study found that platelet-derived EVs were the most abundant in both male and female plasma. Platelet- and endothelial- EVs as well as P-selectin and phosphatidylserine-expressing EVs were found to be higher in women compared to men. However, when only premenopausal females were evaluated and compared to age-matched males, lower erythrocyte and stem/progenitor cell EV signals were observed. Tissue factor (TF)-positive EVs were lower in postmenopausal women and erythrocyte derived ones were higher, with the latter marker correlating positively with age in females. Males showed a positive correlation of adipocyte-derived EVs with age [Gustaffson CM, 2015]. These findings contrast with my results where my analysis of percentage distribution showed the highest proportion of monocyte/macrophages EVs followed by platelets-, erythrocyte- and endothelial-derived EVs. This discrepancy may be due to the use of different methodology as measurements did not include small EVs (exosomes) in the Gustaffson study (which used flow cytometry alone). Both Gustafsson's study and my data indicate, however, that FABP4-expressing EVs (potentially representing an adipocyte origin) were higher in males. I also believe that some of the discrepancies between my results and the above cited studies are due to

the fact that my cohort was in fact very young (mean age 33, only 2 postmenopausal subjects included) and with no history of smoking.

The EV differences in women merit further consideration and may have future practical implications. A recent study by Toth B *et al* aimed to evaluate if the increased risk of thromboembolic events in young women might relate to gender differences in plasma EV profile. They found (using flowcytometry) that annexin V –binding EVs, platelet-derived EVs, P -and E- selectin expressing EVs were higher in women and that the luteal phase of the menstrual cycle was associated with increased platelet- and endothelial-derived EVs [Toth B, 2007]. Further research evaluating differences between pre- and postmenopausal women as well as EV fluctuations in response to the menstrual cycle is required in order to establish accurate reference ranges.

3.6.4 Exosomes as carriers of adipokines

Finally, it is important to note that my study showed an association between CD9 (exosomal marker) and adipokines such as FABP4, interferon γ and adiponectin. This might suggest that CD9-expressing small EVs (<150nm in diameter) are the main carriers of these adipokines within the total EV population. This is a potentially important observation as exosomes have a distinct secretory pathway from other EVs (as discussed in chapter 1). Importantly, corresponding plasma adipokine markers did not correlate with that found in EV, again implying specific packaging. The recent efforts from various EV research centres in the last few years have been focused on developing reproducible methodology to isolate specific subpopulations of EVs from plasma/cell medium, which in the longer term could be used in novel pharmacological therapeutic and diagnostic approaches. Various functions have been so far described for exosomes, sometimes contraindicatory, which suggest that EVs of different intracellular origin may have different function and this should be reflected in their composition [Kowal J, 2014]. The obvious barrier at present is a lack of a gold standard approach to EV isolation in general, and even less so with specific EV subpopulations which is an area our group is also working on using magnetic beads and co-labelled antibodies [Connolly KD, 2018]. Recent work by Kowal *et al* on dendritic cell-derived EVs proposed a protocol combining differential centrifugation followed by immune-labelling or ioixanol gradient separation and extensive proteomic analysis, allowing EVs of different sizes and cellular compartmental origin to be distinguished [Kowal J, 2016]. This was a pilot study and given how labour intensive such an approach is, it appears that this approach might not easily translate to application on a larger scale. Secondly, as EVs are a hugely heterogeneous group we still have not understood the exact pathways controlling specific EV subpopulation secretion and packaging. Given how crucial EVs appear in intercellular signalling, potential methods allowing control of their content and uptake could have revolutionary effects in driving novel pharmacotherapies. There are already some promising studies in

dermatology, vaccines and oncology [Fais S, 2016] but all are based on loading 'nanoparticles' with a certain therapeutic exogenous agent rather than controlling endogenous EV secretion. These obstacles need to be overcome before EVs can be practically used as therapeutic agents.

My data showing that specific EVs subpopulations carry adipokines associated with obesity and inflammation form a useful addition to what is already known.

3.7 Summary

3.7.1 Study limitations

The main limitation of the study is that the cohort was relatively young and in my overweight/obese subgroup I did not have many participants with class III obesity ($BMI > 40 \text{ kg/m}^2$). The study would benefit from a larger cohort size and a follow-up analysis after a 5 to 10-year period of observation. Moreover, since my study was performed, there is more interest in using precipitation assays or size exclusion for more precise isolation of a purified EV fraction.

3.7.2 Study strengths

Most EV studies so far have focused on characterisation of the EV profile in various pathological states such as malignancy, autoimmune disease or cardiovascular disease and used the healthy cohorts for comparison purposes mainly. Data on "healthy EVs" is lacking and this study by its strict selection of healthy, non-smoking and relatively young individuals adds to our understanding of what a 'healthy EV' profile consists of. All samples were handled in a standardised reproducible manner, which limits any variation that might arise from different sample timing, processing or storage conditions.

3.7.3 Conclusions

The main findings from this study are outlined below:

- The EV profile is similar in lean subjects and in people with obesity alone without any other features of the metabolic syndrome. Therefore, EVs are not early markers of metabolic dysfunction but should be regarded as important messengers in intercellular communication maintaining the physiological milieu in the healthy state
- Healthy men have a more 'proinflammatory' EV profile, which may explain in part why they are predisposed to cardiometabolic disease compared to women
- Plasma 'free' and EV-expressed adipokines do not correlate which suggests that different secretory pathways may be involved in their release into the circulation.
- Plasma FABP4 correlates strongly with BMI and may be regarded as a marker of impending metabolic dysfunction

- Given that my study showed no differences in circulating EVs in individuals of varying BMI who have not yet developed any metabolic consequences of increased adiposity, longitudinal studies evaluating the change in EV profile over a prolonged period of time would be of interest. Due to obvious time restrictions I was not able to perform such an analysis.

Chapter 4

Characterisation of cell-derived extracellular vesicles in morbidly obese individuals undergoing bariatric surgery

Perspective

Following the work undertaken on EV characterisation in healthy volunteers, I intended to undertake similar analysis on a cohort of morbidly obese individuals and to evaluate the effects of weight loss on the EV profile. Whilst the study described in chapter 5 was being developed, an opportunity for a scientific collaboration with Professor Jeffrey Stephens from the local bariatric surgery research centre became available. This allowed me to add an interventional arm to my work with the additional advantage of accessing samples from a study that was already established, and hence immediate sample availability. However, a disadvantage was the limited volume of plasma available, since many other metabolic effects of bariatric surgery were being evaluated by other researchers in this study. Following a period of discussions with my supervisors, I decided to perform the analysis of those samples despite some methodological limitations encountered which are discussed later.

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4.1 Introduction

Bariatric surgery markedly reduces fat mass but the effects on circulating extracellular vesicles are unclear. As EVs are secreted by a variety of cells, they may mediate some of the cardiometabolic benefits accompanying weight loss. However, it is unclear how EVs, particularly adipocyte-derived EVs, alter in response to bariatric surgery.

As mentioned previously, adipose tissue is no longer regarded simply as a passive energy store but as an active endocrine organ that communicates with other tissues by secreting a variety of molecules including leptin, adiponectin, cytokines, growth factors and complement proteins. The obesity pandemic has led to a greater adoption of bariatric surgery as a treatment for morbid obesity and its secondary complications [Batterham RL, 2016]. Bariatric surgery exerts multiple effects on adipose tissue, including reducing adipocyte size, improving sensitivity to insulin and catecholamine-mediated lipolysis, increasing levels of adiponectin and decreasing levels of leptin, CRP, IL-6, TNF α and Monocyte Chemoattractant Protein-1 (MCP-1)[Frikke-Schmidt H, 2016]. However, the pathways regulating these changes are not fully understood.

Adipocyte-derived EVs (AD-EVs) are likely to play important roles in paracrine and endocrine communication within adipose tissue and possibly the circulation but comparatively little is known about their function. They probably represent only a small percentage of the total population of EVs present in the circulation [Ertunc ME, 2015], at least in the healthy state, but are known to affect target cell activity [Ogawa R, 2010, Aoki N, 2010] such as increasing lipid storage in small adipocytes [Muller G, 2011].

Fatty Acid Binding Protein 4 (FABP4) is one of the most abundant cytoplasmic proteins within adipocytes, whose function extends beyond lipid transport to include regulation of glucose metabolism and inflammatory responses [Hotamisligil GS, 2015, Furuhashi M, 2015]. Indeed, its circulating concentration correlates strongly with the risk of metabolic disease [Furuhashi M, 2015]. Recent data in 3T3-L1 adipocytes and mouse adipose tissue demonstrate that lipolytic stimuli trigger accumulation of FABP4 within intracellular multivesicular bodies (MVB), and its subsequent secretion via the non-classical pathway in vesicles expressing exosomal markers (CD63 and ALG-2 interacting protein X(ALIX)) [Ertunc ME, 2015]. It appears, however, that only a small proportion of total FABP4 is transported this way [Hotamisligil GS, 2015, Ertunc ME, 2015]. Although it is currently not clear what the precise biological function of such EVs is, they are likely to play an important role in intercellular

communication at both a paracrine and systemic level and may influence how adiposity contributes to the development of obesity-associated comorbidities.

Since lipolysis triggers EV secretion from adipocytes, conditions associated with substantial weight loss might result in increased secretion of adipocyte-derived EVs into the circulation. I therefore hypothesised that bariatric surgery would result in increased expression of adipocyte-derived proteins, including FABP4, not only in a free form but also in association with EVs.

4.2 Aims

The aim of this study was to characterise a cohort of patients undergoing bariatric surgery at baseline and 2 follow-up time points (1 and 6 months) with regard to the following variables:

- Anthropometric measurements (weight, BMI, % weight loss)
- Plasma-derived EV concentration, cellular origin and adipocytokine content
- Plasma concentration of FABP4, IL-6, fasting glucose, insulin, HOMA-IR

4.3 Methods

4.3.1 Study population

Study participants were recruited from patients undergoing planned bariatric surgery at the Welsh Institute of Metabolic and Obesity Surgery (WIMOS). At the time of recruitment for this study, individual suitability for receiving bariatric surgery in Wales was evaluated using the Swansea modified Dudley Bariatric Surgery Comorbidity Score (DUBASCO) (Table 4.1) and funding was approved for those scoring ≥ 12 . This scoring system takes into account BMI, age and comorbidities but negative scores are also allocated if any of the following is present: failed weight loss following gastric balloon insertion (-1 point), established chronic cardiovascular disease/ejection fraction $< 40\%$ (-2 points), chronic illness with a prognosis of less than 5 years (-3 points), inability to mobilise more than 10 metres (-4 points) and estimated glomerular filtration rate (eGFR) < 40 ml/min/m² (-4 points). People with previous complex gastrointestinal surgery, ongoing psychiatric illness or untreated eating disorders were deemed unsuitable to receive this treatment.

Score	1	2	3	4
BMI (kg/m ²)	≥ 35.0-40.0	≥ 40.0-50.0	> 50.0-60.0	≥ 60.0
Age (years)	≥ 60	51-60	41-50	≤ 40
Co morbidities				
Type 2 diabetes (duration <15 years)	IGT/IFG	Optimal glycaemic control (HbA1c ≤7.5%)	Suboptimal glycaemic control (HbA1c 7.6-10%)	Poor glycaemic control (HbA1c >9%) or on insulin
Hypertension	Well controlled on 1 medication (BP <140/90 mmHg)	Well controlled on 2 medications (BP <140/90 mmHg)	Poorly controlled on 2 medications (BP ≥ 140/90 mmHg)	Poorly controlled on more than 2 medications (BP ≥ 140/90 mmHg)
Dyslipidaemia	Requiring no treatment	Well controlled on 1 medication (TC ≤5 mmol/l, TG ≤1.7 mmol/l)	Well controlled on 2 medications (TC ≤5 mmol/l, TG ≤1.7 mmol/l)	Poorly controlled on 2 medications (TC >5 mmol/l, TG >1.7 mmol/l)
Sleep Apnoea	Neck circumference >43 cm or snorer	OSA, no CPAP	OSA, Epworth <15 on CPAP	OSA, Epworth >15 on CPAP
Total				
Gross Score				
Negative score ^a				Reason for negative score:
Net Score				

BP: blood pressure; TC: total cholesterol; TG: triglyceride; OSA: obstructive sleep apnoea; CPAP: continuous positive airway pressure; Epworth: Epworth sleepiness scale

Tab.4.1: The Swansea modified DUBASCO score.

The study eligibility criteria in those who were suitable for bariatric surgery were:

- Age 20-60 years
- BMI >40kg/m²
- Physical fitness for surgery
- Type 2 diabetes mellitus (T2DM) or impaired glucose tolerance (IGT) diagnosed previously or prior to the study by the 75g oral glucose tolerance test (OGTT) according to the American Diabetes Association (ADA) criteria [ADA 2015].

4.3.2 Types of intervention

Study participants received one of the following treatment modalities: adjustable laparoscopic gastric banding (GB), laparoscopic sleeve gastrectomy (SG) or biliopancreatic diversion (BPD).

Gastric banding (GB) is a comparatively straightforward and lower risk procedure which involves placement of a tight adjustable band distal to the stomach entrance in order to create a pouch. The silicon ring within the band is connected to an infusion port placed within the subcutaneous tissue. This allows the degree of gastric restriction to be increased by infusion of 0.9% saline through the port which will lead to decreased band diameter.

Sleeve gastrectomy (SG) is also a laparoscopic procedure which converts the stomach into a tube-like structure by removing approximately 80% of it using endoscopic stapling devices. In contrast to GB, it is not reversible. Both GB and SG were traditionally considered as 'restrictive' types of bariatric surgery i.e. only affecting the amount of food that can be ingested. However, in recent years there has been some data emerging which indicates these procedures may also lead to weight loss by other mechanisms such as induction of satiety by gastric cardia compression from the gastric band [Burton, 2011] or changes in gut hormone profile in subjects undergoing sleeve gastrectomy [Colomb, 2015]. For the purpose of this study, subjects undergoing these procedures were, however, grouped together as those undergoing 'restrictive' surgery.

Finally, biliopancreatic diversion (BPD) is a malabsorptive procedure whereby subtotal gastrectomy and bypass of most of the small bowel (except for the distal 100cm) is performed. It therefore leads to significant weight loss but is also associated with a risk of malnutrition [Topart P, 2017].

The choice of operation for an individual was decided by the multidisciplinary specialist team and was not affected by participation in this study.

4.3.3. Study design

As mentioned above, participants were reviewed at 3 time-points: 1 week prior to planned bariatric surgery, and at 1 and 6 months postoperatively. During those visits demographic information (age, gender), clinical measurements (weight, BMI) and fasting blood samples were obtained. All participants were given a unique study number.

4.3.4 Ethical approval

The study was approved by the Local Research Ethics Committee, Research and Development Office and Cardiff Metropolitan University (LREC No: 06/WMW02/7, R&D: S06GenMed569). All participants provided written informed consent. A material transfer agreement between Swansea University and Cardiff Metropolitan University was established in order to perform the EV analysis.

4.3.5 Clinical measurements

Clinical measurements (weight, BMI) were performed as described in the Methods chapter (section 2.4.).

4.3.6 Sample collection

Fasting venous blood was collected from a large peripheral vein into EDTA vacutainers. Samples were immediately spun twice at 3000g for 10mins to render plasma acellular, then frozen and stored at -80°C until further analysis.

4.3.7 EV isolation and characterisation

EVs were isolated by differential centrifugation at the Department of Biomedical Sciences at Cardiff Metropolitan University and measured by Nanoparticles Tracking Analysis (Nanosight, Malvern, UK) as previously described (Methods chapter, section 2.5). Since only 2mLs of plasma were available from each participant at each time-point, only 5×10^9 EVs /well were available to perform the TRF immunoassay using the markers described in the previous chapter for EV cellular origin and content (CD41: platelets, CD11b: monocytes/macrophages, CD235a: erythrocytes, CD144: endothelial cells, CD9: exosomes, IL-6, TNF α , Interferon- γ , adiponectin, FABP4 and PPAR γ). The primary antibodies used in this assay were obtained from the same supplier and used in the same concentration as described in the Methods chapter (Table 2.2). However, since I used a different EV loading number in the TRF immunoassay, no direct comparison between this cohort and subjects described in chapter 3 was performed.

4.3.8 Plasma measurements

Plasma FABP4 concentration was established using a human FABP4 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), and plasma free fatty acids were measured by a Colorimetric Quantification Assay Kit (ab65341, Abcam, Cambridge, UK) in the Biomedical Sciences Laboratories at the Cardiff Metropolitan University. Measurements of fasting glucose were undertaken on the day of sample collection at the Department of Medical Biochemistry, Morriston Hospital using a Roche Modular P800 Analyser. A Roche Modular E170 analyser was used to measure fasting insulin. HOMA-IR was calculated as described in Methods chapter (section 2.6.5). Plasma IL-6 concentration

was evaluated by a Quantikine ELISA human IL-6 kit (R&D Systems, Minneapolis, MN, USA) at the Diabetes Research Group at the Institute of Life Science, Swansea University Medical School. All samples were assayed in duplicate.

4.3.9 Statistical analysis

4.3.9.1 Sample size

This was a proof of concept study that had the primary aim of determining the change in plasma and EV-expressed FABP4. There are very limited data available in relation to FABP4 in plasma following bariatric surgery and, to my knowledge, no studies investigating EV FABP4 in this context. As I expected a substantial 20% change in plasma FABP4 over the three sampling points and a range of 10-15% (based on previous variation measured from plasma) [Stejskal D, 2008], a power calculation suggested that I would need at least 15 subjects to detect this difference (one-way ANOVA at 3 levels; $p=0.05$; $\alpha=0.8$). As this study was limited to a previous sample size of 20, my study was adequately powered.

4.3.9.2 Statistical tests

Normally distributed variables were reported as mean \pm SD whereas non-normally distributed variables were summarised as medians with interquartile ranges (IQR). For normally distributed variables the differences at different time points were analysed using repeated measures ANOVA with Bonferroni's multiple comparisons post-test analysis. For changes in variables with non-Gaussian distribution, the Kruskal-Wallis test was applied with post-test Dunn's comparison. Spearman's correlation analysis was applied to detect significant correlations between evaluated parameters. Statistical significance was accepted at $p<0.05$ and Graph Pad version 6.0 was used.

4.3.10 My involvement

Participant recruitment, clinical measurements, sample collection, analysis of glucose homeostasis and plasma IL-6 measurement were performed by Prof J. Stephens, Dr SL Prior and Dr T Min from the Diabetes Research Group, Swansea University. My role included undertaking EV isolation and analysis and measurement of plasma FABP4 and FFA at Cardiff Metropolitan University. I remained blind to surgical therapy group and all other measurements until all experiments were completed.

4.4 Results

4.4.1 Population characteristics

In total, 20 participants completed the study. There were thirteen female and seven male patients, with a mean age of 50.7 ± 8 years and a baseline mean body weight and BMI of 151.3 ± 31 kg and 54 ± 12.6 kg/m², respectively. The anthropometric and metabolic characteristics are summarised in Table 4.2. As anticipated, all patients undergoing bariatric surgery achieved significant % total weight loss (%TWL) (-8.3% at 1 month and -20% at 6 months to mean values of 138.8 ± 28 kg and 121.6 ± 26 kg, respectively; $p < 0.001$) and BMI reduction of -10% and -18.5% (to mean values of 48.9 ± 11 and 44 ± 10.2 kg/m², respectively; $p < 0.001$). Thirteen of the patients underwent restrictive laparoscopic surgery (sleeve gastrectomy (SG) $n=10$, gastric banding (GB) $n=3$) and seven an open malabsorptive procedure (biliopancreatic diversion (BPD)).

	Baseline (n=20)	1 month post-surgery	P (vs. baseline)	6 months post- surgery	P (vs. baseline)
Age (years)	50.7 (8)				
Gender (M/F)	M=7, F=13				
Weight (kg)	151.3 (31)	138.8 (28)	<0.001	121.6 (26)	<0.001
BMI (kg/m ²)	54 (12.6)	48.9 (11)	<0.001	44 (10.2)	<0.001
Glucose (mmol/L)	7.9 (3.2)	6.2 (1.5)	<0.05	6.1 (3.0)	0.092
IGT subgroup	5.8 (0.7)	5.5 (1.2)	ns	6.1 (4.2)	<0.05
T2DM subgroup	9.9 (3.4)	6.9 (1.6)	<0.05	6.2 (1.2)	<0.001
Insulin (mIU/L)	111.7 (74.7)	103.3 (76.5)	ns	74.7 (57.5)	0.054
IGT subgroup	104.4 (52.5)	106.2 (72.9)	ns	79.3 (67.6)	ns
T2DM subgroup	119 (95)	100.5 (84.1)	ns	69.7 (47.4)	0.054
HOMA-IR	2.40 (1.37)	1.97 (1.36)	ns	1.60 (1.68)	<0.001
IGT subgroup	2.25 (0.7)	2 (1.4)	ns	1.8 (2.1)	ns
T2DM subgroup	2.5 (1.8)	1.9 (1.3)	ns	1.37 (0.9)	<0.05
IL-6 (ng/mL)	9.5 (8.3)	11.1 (11.3)	ns	14.7 (24.6)	ns
FABP4 (ng/mL)	67.8 (18.5)	82.7 (17.7)	<0.05	62.7 (19.9)	ns

Tab.4.2: Anthropometric and metabolic characteristics of patients undergoing bariatric surgery at baseline, 1- and 6-months' follow-up. ns=non-significant.

4.4.2 EV concentration, cellular origin and content

There were no significant differences in mean EV concentration/mL of plasma between baseline ($1.44 \pm 0.9 \times 10^{11}$ nanoparticles/mL), 1 month ($1.3 \pm 0.7 \times 10^{11}$) and 6 months postoperatively ($1 \pm 0.4 \times 10^{11}$) (Figure 4.1a). However, detailed analysis of EV concentration within different size ranges (measured using a 100nm band width) revealed a significant reduction in EVs measuring between 100 and 200nm in diameter at 6 months compared to baseline ($p < 0.01$, Fig 4.1b).

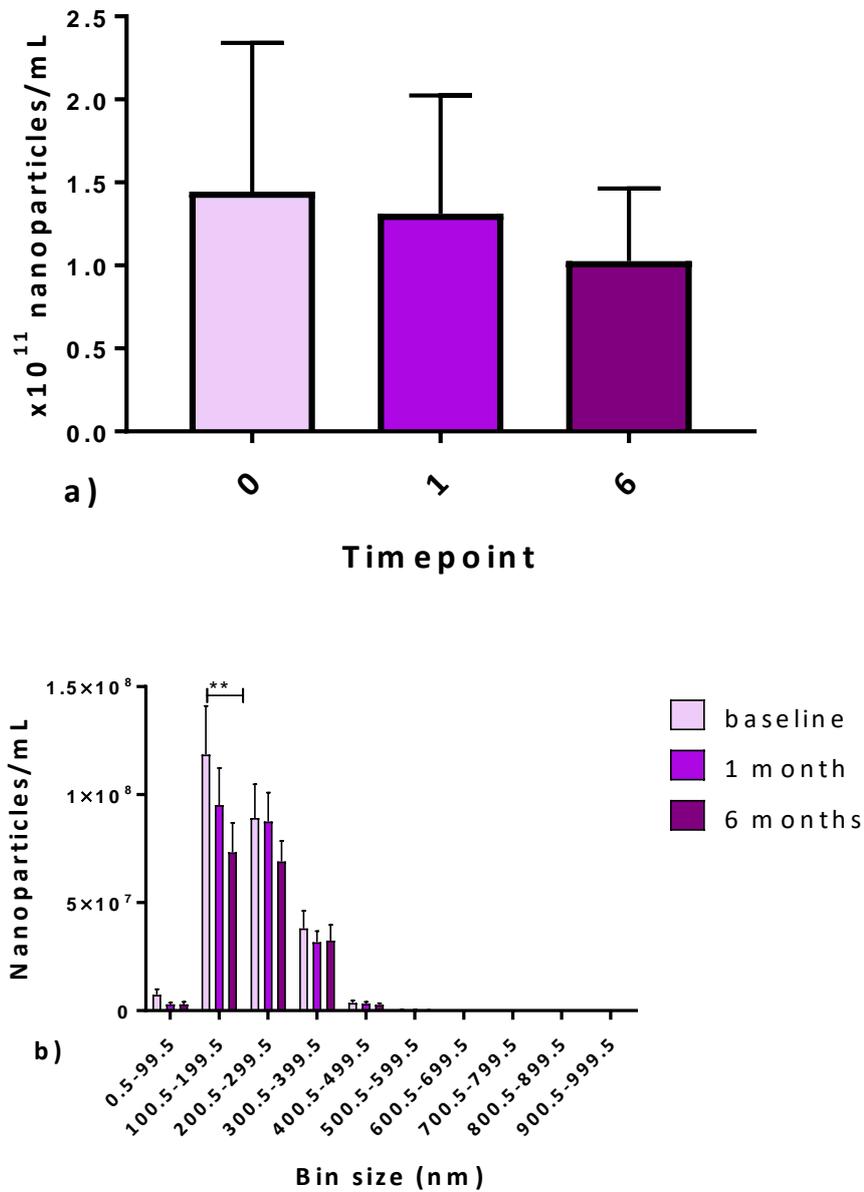


Fig.4.1: EV concentration/mL plasma and size distribution at 3 timepoints : a) EV concentration/mL of plasma at baseline, 1 month and 6 months' follow-up (mean±SEM) b) Two-way ANOVA with Bonferroni post-test revealed significant decrease in EVs sized 100.5-199.6nm in diameter at 6 months' follow-up compared to baseline (mean±SD), p<0.01().**

The relative distribution of the main EV cell-of-origin markers (CD41 (platelets), CD11b (monocytes/macrophages), CD235a (erythrocytes), CD144 (endothelial cells)) did not change in response to surgery (Fig.4.2).

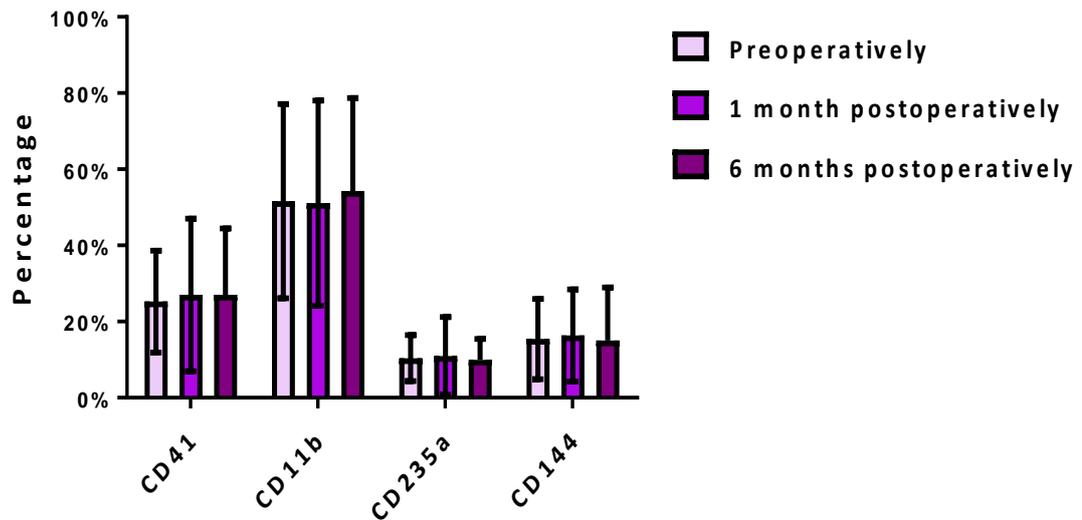


Fig.4.2: Bariatric surgery did not alter EV cellular origin. Relative expression at baseline, 1 and 6 months postoperatively: CD41 (platelets): 25±13% vs 27±20% and 27±17%; CD11b (macrophages/monocytes): 51± 25% vs 51±26% and 54±24%; CD235a (erythrocytes): 10±6% vs 11±10% and 9±5%; CD144 (endothelial cells): 15±10% vs 16±12% and 15±13%, respectively, all p=non-significant.

Following surgery, there were no differences in median TRF signal for the exosomal marker CD9, EV-

expressed adiponectin, IL-6, TNF α , interferon γ or PPAR γ (Fig 4.3).

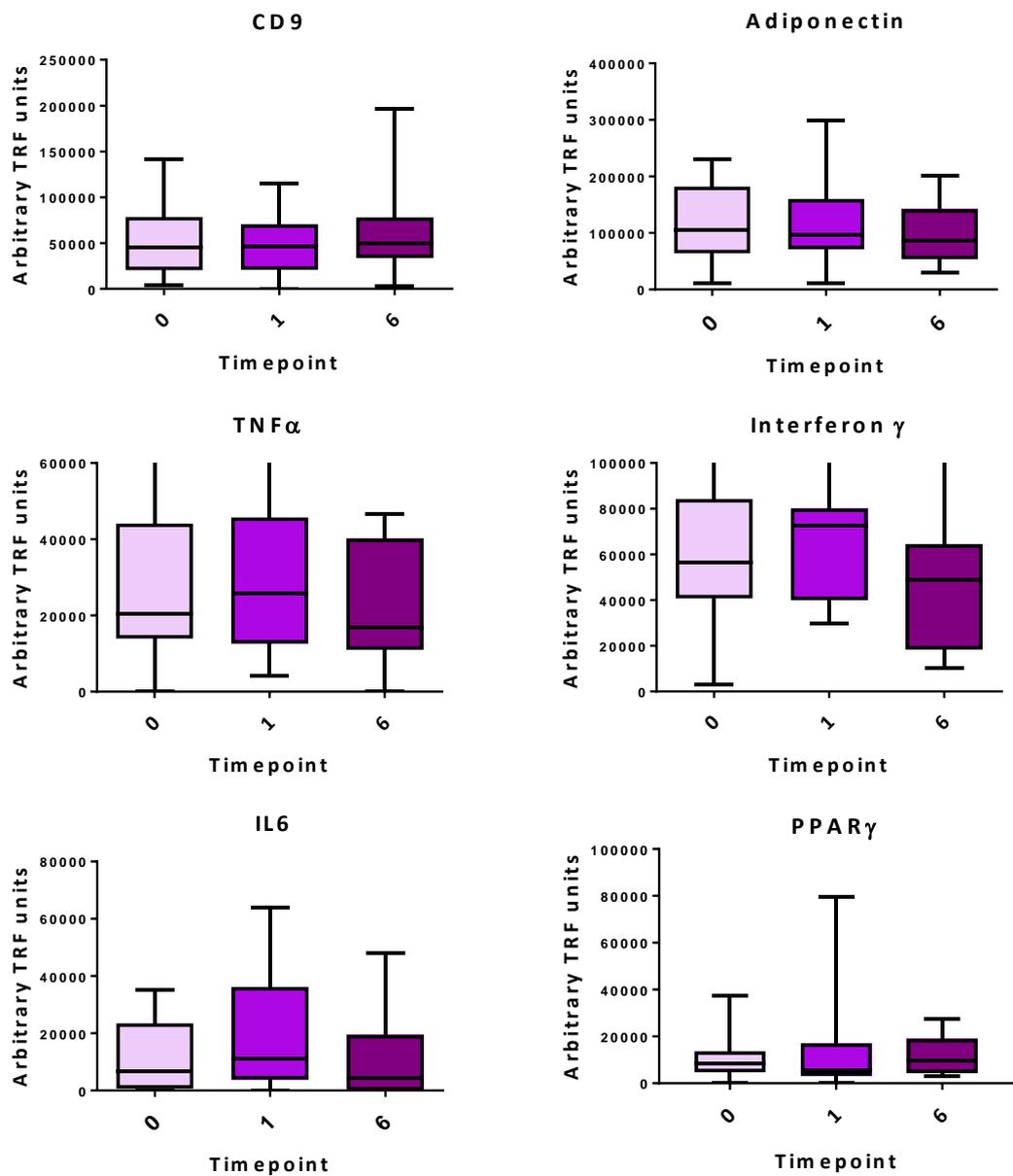
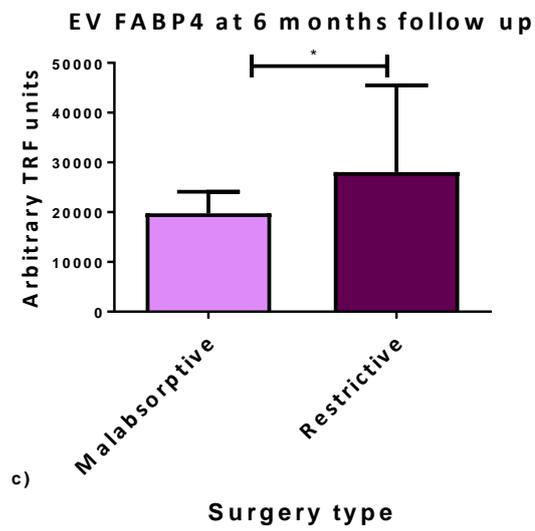
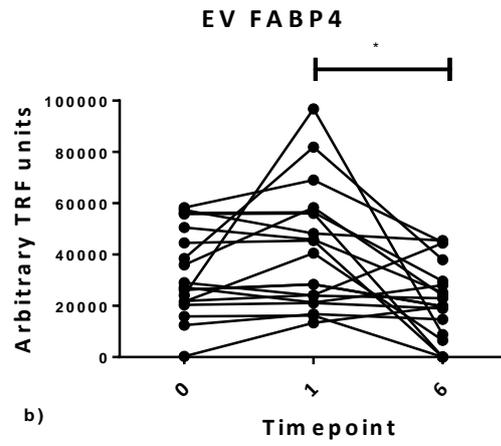
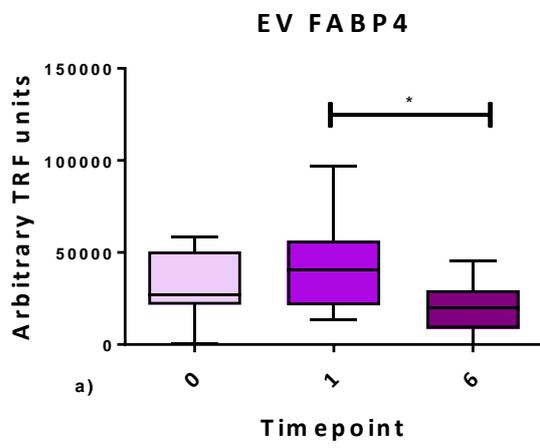


Fig.4.3: Median EV expression of CD9, adiponectin, TNF α , interferon- γ , IL-6 and PPAR γ), p=ns.

However, the FABP4 EV signal increased (non-significantly) at 1 month before falling significantly by 6 months ($p < 0.01$) (Fig. 4.4a, 4.4b). The median FABP4 EV signal at 6 months' follow-up was significantly lower in the group who underwent biliopancreatic diversion surgery ($n=7$) compared to those who had sleeve gastrectomy or gastric banding ($n=13$) (19775 (6565-24094) vs 28057 (14683-45459) a.TRF, $p < 0.05$) (Fig. 4c), with no differences in EV FABP4 expression between those groups at baseline or 1 month (26302 (21641-55806) vs 38483 (285-100213) a.TRF, $p=0.51$ and 40571 (23923-96781) vs 45422 (13383-81841) a.TRF, $p=0.6$, respectively).

A significant correlation was observed between the EV signal for CD9 and each of FABP4 ($r=0.5$, $p < 0.0005$), adiponectin ($r=0.59$, $p < 0.0001$), TNF- α ($r=0.53$, $p < 0.0001$) and interferon γ ($r=0.41$, $p < 0.005$) (Figures 4.4 d-g), suggesting that these adipocytokines are transported by an EV population which is specifically of exosomal origin.



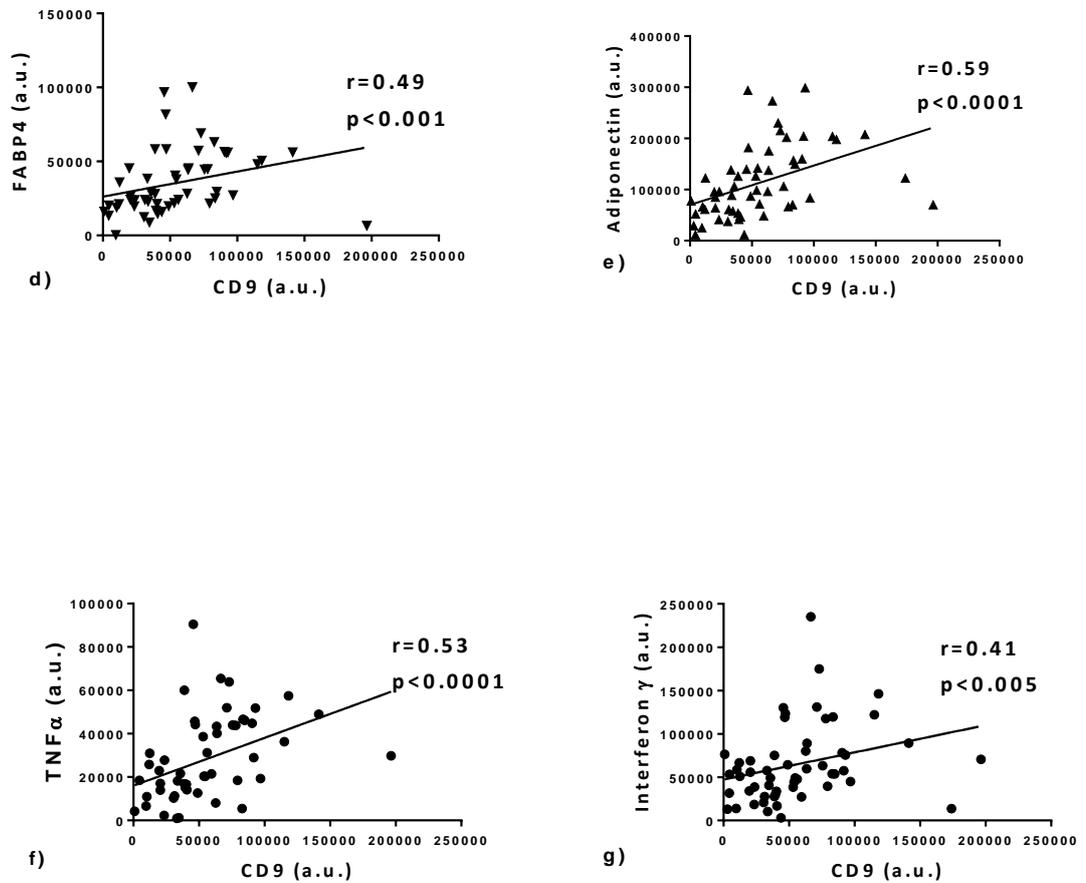


Fig. 4.4: a) Changes in EV FABP4 (median with IQR) reveal significantly lower TRF signal at 6 months' follow-up compared to 1-month ($p<0.05$) b) Individual pattern of changes in EV FABP4 TRF signal for all study participants at 3 timepoints c) Median EV FABP4 signal was significantly lower in the cohort that underwent biliopancreatic bypass diversion surgery at 6 months' follow-up ($p<0.05$). Spearman's correlation analysis across the whole cohort revealed significant correlation between the exosomal marker CD9 and EV-derived: FABP4 (d), adiponectin (e), TNF α (f) and interferon γ (g). *= $p<0.05$, **= $p<0.01$

4.4.3 Plasma FABP4

A similar pattern of change to that observed for EV FABP4 was observed with free plasma FABP4, with a significant rise at 1 month ($p < 0.001$ vs baseline) before a significant decrease by month 6 ($p < 0.001$ vs month 1) (Fig. 4.5a, 4.5b). Furthermore, we observed that plasma FABP4 concentration correlated with plasma free fatty acid concentration at 1 month follow-up ($r = 0.48$, $p < 0.05$, Fig. 4.5 e) but not at baseline or 6 months. Neither BMI nor plasma FABP4 correlated with EV FABP4 (Fig. 4.5c, 4.5d).

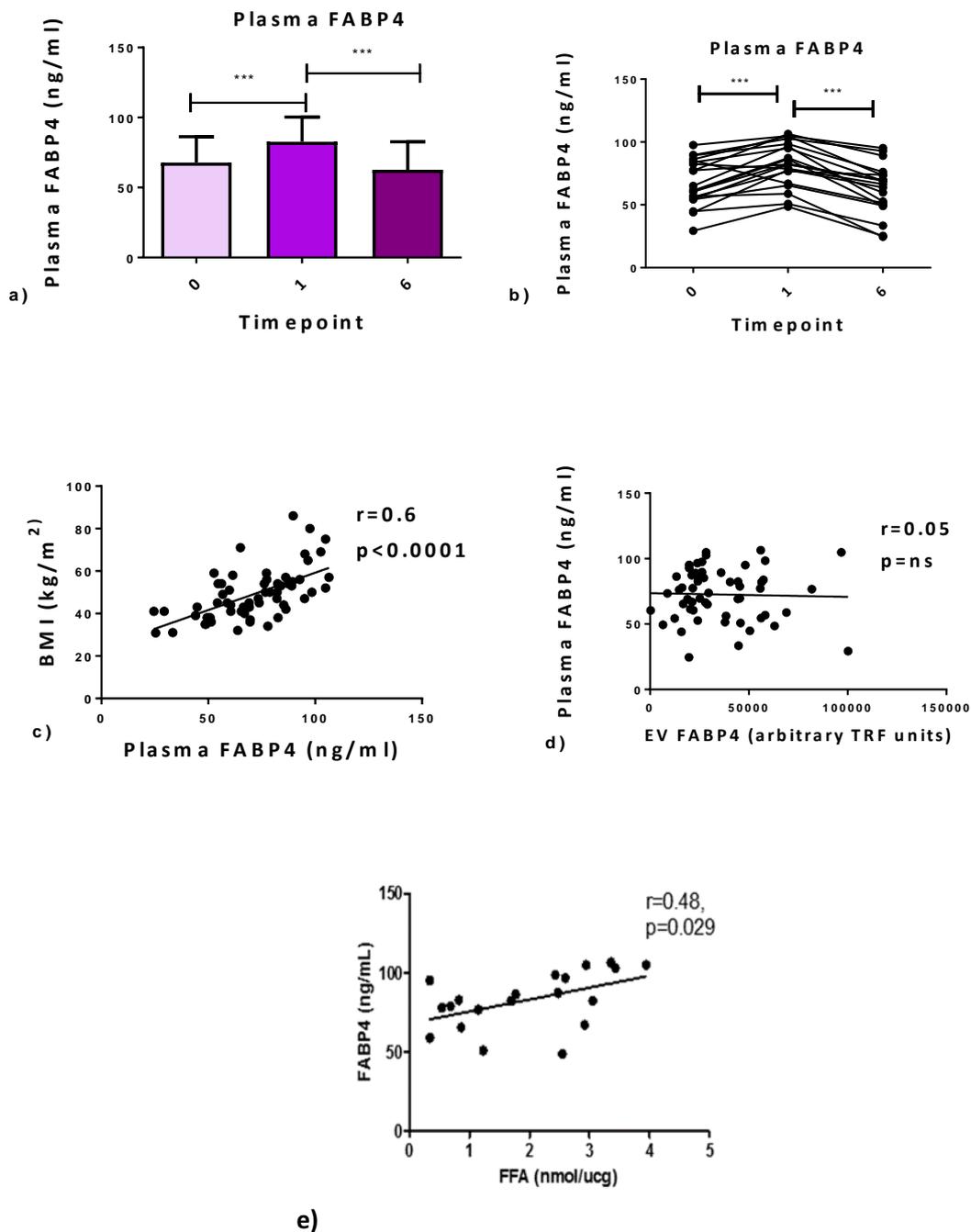


Fig.4.5: a) Plasma free FABP4 levels (means) were significantly higher at 1 month follow-up compared to baseline and 6 months' follow-up b) Plasma FABP4 concentration at 3 time points for all study participants (n=20) c) Spearman's correlation analysis revealed significant correlation between plasma FABP4 and BMI ($r=0.6$, $p<0.0001$) d) No correlation between EV-contained and plasma free FABP4 was observed ($r=0.05$, $p=ns$) e) Significant correlation was observed between plasma FABP4 and plasma free fatty acids at 1 month follow up ($r=0.48$, $p<0.05$). *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$

4.4.5 Additional analysis

In order to explore any relationship between EVs and weight loss, I subsequently subdivided this sample into two similar-sized groups: those who achieved more than 18% TWL at 6 months (n=9) and those who did not (n=11). Two significant differences were observed: significantly lower EV PPAR γ in the group that achieved greater weight loss at 6 months' follow-up (5015 (2979-12973) vs 20133 (7066-27583) a.TRF, p<0.05), and significantly higher EV TNF α at 1 month follow-up in the group that failed to achieve weight loss of >18% (3629 (8053-90438) vs 14650 (4177-44817) a.TRF, p<0.05)). In contrast, no relationships between any EV changes and improvements in glucose metabolism (defined as achievement of FPG less than 6mmol/L and/or HOMA-IR <1.4) were observed between the 12 subjects who achieved it and the 8 that did not (all p=ns).

Since differences in fat distribution and lipolysis between men and women have been described [Frühbeck G , 2014] and I observed gender differences in the healthy volunteer EV profile in chapter 3, I then analysed my data split by gender. There were no gender differences in FFA concentrations at any time-point. Plasma FABP4 concentration at 6 months' follow-up was higher in females (78.47 \pm 14.9 vs 48.27 \pm 21 ng/mL, p<0.05) despite no significant EV FABP4 or BMI differences between genders at this time-point (21354 (6565-44491) vs 24671 (14683-45459) a.TRF, p=0.54, 45.3 \pm 10.8 vs 41.5 \pm 9 kg/m², p=0.43, respectively). EV CD11b (monocyte/macrophages marker) was higher in females at all time-points (preoperatively: 56507 (26078-208501) vs 10729 (6400-74280) a.TRF, p<0.005); at 1 month: 65244 (15779-177945) vs 15362 (7669-85205) a.TRF, p=0.05 and at 6 months: 66257 (6361-164498) vs 23398 (10124-86499) a.TRF). Baseline EV PPAR γ was also higher in females (10400 (5268-37321) vs 4185 (616-7605) a.TRF, p<0.05); however, no differences were observed post-surgery (p=ns). No other gender differences were observed in other EV markers

4.5 Discussion

This study shows that bariatric surgery is followed by dynamic changes in the expression of EV-associated as well as free plasma FABP4. Since FABP4 is predominantly expressed in adipocytes, our finding of altered EV-expressed FABP4 after surgery is likely to reflect changes in adipocyte EV secretion. As demonstrated by Ertunc *et al*, lipolysis triggers a non-classical pathway of FABP4 secretion within EVs, and these EVs may be intended to play a different role in intercellular communication compared to their free circulating form [Ertunc ME, 2015]. Bariatric surgery is known to rapidly reduce visceral and subcutaneous lipid depots, accompanied by an increase in plasma free fatty acids and beta-hydroxybutyrate levels, reflecting increased lipolysis [Johansson L, 2008]. Previously, increased expression of lipolytic genes within adipose tissue following bariatric intervention was demonstrated, including triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and perilipin [Karki S, 2015]. ATGL, HSL and free fatty acids have been found to affect FABP4 secretion [Ertunc ME, 2015].

In this study, EVs expressing FABP4 and plasma free FABP4 showed a similar pattern of change with a transient peak in the early postoperative period followed by significant reduction at 6 months' follow-up compared to 1 month. Furthermore, plasma FABP4 levels at 1 month correlated with plasma free fatty acid levels. I therefore believe that the transient postoperative rise in EV and plasma FABP4 may reflect the dynamic changes occurring in adipose tissue following surgery, including reduced total fat mass and adipocyte size, and increased lipolysis, albeit that a limitation of my work is that I did not measure plasma glycerol due to limited volume of plasma available as discussed earlier. Previous studies have shown similar changes in circulating FABP4 levels following weight loss [Furuhasi M, 2016, Comerford KB, 2014]. Stejskal *et al* indicated that this pattern of an initial rise in plasma FABP4 with subsequent normalisation to baseline levels was observed particularly in individuals who achieved sustained weight loss [Stejskal D, 2008]. However, I am unaware of any previous study evaluating EV-expressed and plasma free FABP4 simultaneously in the context of bariatric surgery.

Open bariatric surgery might involve more adipose tissue damage compared to a laparoscopic approach. However, at 1 month follow-up I did not observe differences in plasma and EV FABP4 in the BPD group compared to the SG/GB group. Nevertheless, it is entirely possible that differences in plasma- and EV-FABP4 between open and laparoscopic surgery might be apparent at very early time-points post-surgery and this would be an interesting area to explore in future studies.

I did not find a correlation between plasma FABP4 and the EV FABP4 TRF signal, nor between EV FABP4 and BMI. This might relate to the fact that adipocyte-derived FABP4-containing EVs probably carry only a small proportion of the total FABP4 secreted by adipocytes [Hotamisigli GS 2015, Ertunc ME, 2015]. Moreover, adipocyte-derived EVs containing FABP4 could also be taken up by neighbouring cells in adipose tissue in a paracrine manner, as shown in previous studies [Aoki N, 2010]. Therefore, establishing a correlation between BMI and both plasma soluble FABP4 and EV-contained FABP4 is difficult, at least using current methodology used for plasma EV isolation. In contrast, EV-expressed TNF α was greatest at 1 month in subjects showing less weight loss. Whilst mechanisms other than weight loss may contribute to the changes in EV FABP4, it is tempting to speculate that EVs in patients showing the greatest weight loss exhibit a reduced inflammatory profile. However, this remains to be fully elucidated.

Previous studies examining the effects of bariatric surgery on circulating EVs have largely focused on changes in EV concentration and cell-of-origin expression, such as platelets, monocytes or endothelial cells. Campello *et al* evaluated changes in a range of EV subtypes in 20 patients undergoing sleeve gastrectomy and found a significant decline in endothelial-, platelet-, and leukocyte-derived EVs and in annexin V, tissue factor and CD36-expressing EVs at 12 months' follow-up [Campello E, 2016]. The percentage reduction in EV subtypes correlated with the reduction in BMI. Similarly, Cheng *et al* found a reduction in platelet-, endothelial-, monocyte- and tissue factor-expressing EVs in patients following Roux-en-Y gastric bypass surgery, with the monocyte EVs showing an association with HbA1c and BMI reductions [Cheng V, 2013]. In contrast, Stepanian *et al* found no effect of significant weight loss (mean BMI decrease of 24%, n=32, including 27 patients who had gastric bypass or sleeve gastrectomy) on total-, platelet- or endothelial-derived EVs, despite improvements in HOMA-IR, lipid profile and CRP [Stepanian A, 2013]. This is in keeping with my findings, since I found no effect on platelet, leukocyte, erythrocyte or endothelial EVs nor overall EV concentration irrespective of the type of bariatric surgery performed and despite improvements in weight and glycaemic control. However, it should be noted that all of these referenced studies used flow cytometry to evaluate EV concentration and origin/protein expression. This approach is not sensitive enough to distinguish between different populations of EVs and to detect very small EVs (<200nm), as the analysis gate in standard flow cytometers is usually determined using 1 μ m beads. Therefore, the exosomal EV range which showed very interesting biological correlation in my data cannot be fully evaluated using flow cytometry alone. Identification of more precise changes pertaining to adipobiology that take place following the surgical approach are in line with the usefulness of new criteria to define success of bariatric and metabolic surgery that are being put forward [Frühbeck G, 2015]. Despite the significant weight loss achieved by all study participants,

the average 6 months' follow-up BMI remained within the class III obesity range (Table 4.2) which may partially explain why no significant differences in more of the EV markers were observed and why the baseline and 6 months' follow-up plasma and EV-FABP4 results did not differ. Individuals who were selected to undergo BPD surgery had a higher average BMI at all 3 time-points compared to the SG/GB cohort (baseline 66.2 ± 12.7 vs 47.6 ± 6.3 kg/m²; at 1 month 59.7 ± 10 vs 43 ± 6.2 kg/m²; at 6 months 54.2 ± 8.1 vs 38.5 ± 6.1 kg/m², all $p < 0.001$), which corresponded with equally higher concentration of plasma FABP4 in BPD group (baseline 83.2 ± 10.7 vs 59.5 ± 16.3 ng/mL, at 1 month 99.4 ± 8.4 vs 73.6 ± 14.4 ng/mL; at 6 months 78.3 ± 15.1 vs 54.2 ± 17.2 ng/mL, all $p < 0.001$). Interestingly, however, the 6 months' follow-up EV FABP4 signal was significantly lower in the BPD arm compared to the SG/GB one (Fig 4.4c), despite no differences at baseline or 1 month follow-up between these groups. Given that FABP4-expressing EVs are likely to derive from adipocytes, this may suggest that BPD has a greater effect on postoperative processes within adipose tissue regulating the secretion of this group of EVs. Although the mechanism behind this and the biological relevance of this phenomenon is not clear at present, this finding may be in keeping with previous reports indicating that this form of metabolic surgery might be more effective in reversing obesity-driven comorbidities such as type 2 diabetes, hypertension and dyslipidaemia [Celio AC, 2016, Lee SK, 2016]. Finally, it should be noted that although increased circulating adipocyte-derived EVs suggest disturbed adipose tissue homeostasis, their full biological role has not been fully investigated and they may, depending on clinical circumstances, play a deleterious as well as protective role, as has been recently shown for circulating endothelial EVs in the context of vascular disease [Dignat-George F, 2011].

4.6 Study limitations

Similar to previous research evaluating EV changes post-bariatric surgery, the main limitation of this study includes the relatively small sample size ($n=20$). The study may therefore be underpowered to show significant differences in other EV cytokines. Secondly, many of the participating patients were taking concomitant medications at the time of study enrolment, including diabetes medication as, in contrast to most previous studies, I did not exclude patients with chronic illnesses such as hypertension or dyslipidaemia, which could have interfered with the EV readings (as only patients with obesity accompanied by comorbidities as per the DUBASCO scoring system qualify for bariatric surgery in Wales). Thirdly, this study had a relatively short follow-up; studies with a longer follow-up period are needed in the future to fully describe the chronic changes in EVs post-bariatric surgery. Fourthly, it would have been helpful to have included a control group of patients undergoing non-bariatric surgery for comparison. Lastly, direct comparisons between clinical studies evaluating changes in circulating EVs are limited by the fact that establishing a gold standard method for EV

isolation to be adopted across laboratories has only recently been a focus of the International Society of Extracellular Vesicles (ISEV) [Witwer K, 2013], although my adopted approach is one of the most commonly accepted protocols that I have followed to yield consistent samples and results. This approach was not used in the studies by other authors [Campello E, 2016, Cheng V, 2013, Stepanian A, 2013] and similarly the EV analysis in the studies I cited above was performed by flow cytometry which is typically not sensitive enough to detect EVs of exosomal origin and differs from the immunophenotyping method which was adapted for EV analysis specifically [Webber J, 2014, Connolly K, 2015].

Due to the limited amount of plasma volume available, I was also limited by the number of EVs generated which led to alteration in my TRF immunoassay protocol and made it impossible to compare the EVs characteristics to those from the healthy volunteer cohort. I was also unable to undertake additional plasma measurements such as glycerol concentration which could have added more useful information to this study.

4.7 Conclusions

To my knowledge, this study is the first to report concomitant fluctuations in plasma free FABP4 in addition to exosome-associated FABP4 secretion in the follow-up period after bariatric surgery which may relate, at least in part, to increased lipolysis. These changes are likely to play an important role both in paracrine communication within adipose tissue and endocrine communication with other target tissues, since EVs are able to affect recipient cells' function and gene expression. However, further research is required to understand the precise mechanisms regulating secretion and content of adipocyte-derived EVs.

Chapter 5

The effects of a multidisciplinary weight loss programme on circulating extracellular vesicles

Perspective

Following the work undertaken on characterisation of EVs derived from healthy volunteers and people undergoing bariatric surgery, I wanted to complement my work by adding a third volunteer/patient group undertaking a lifestyle or dietary intervention to induce weight loss. During this time, a new multidisciplinary tier 3 weight loss management service was being established by Dr Dev Datta at University Hospital Llandough (UHL). Engagement in such multidisciplinary intervention is now mandatory for all patients who wish to be considered for bariatric surgery in Wales, with the aim of helping patients achieve weight loss and potentially avoiding surgical intervention.

The development of this service fitted well with the aims of the study I wanted to perform and did not require any additional resources. It also allowed me to run the EV analysis on 'fresh' samples which were processed and stored in the same way as the samples from results chapter 3. This would facilitate direct comparison of the results from the morbidly obese individuals with the healthy volunteer cohort.

As there was almost a 2-year time gap between recruitment and analysis of study participants from chapters 3 and 5, new methods had been advocated for EV processing, such as size exclusion chromatography columns for sample purification. Therefore, for the analysis of the samples in this chapter, I decided to employ both methods: differential ultracentrifugation alone as described in chapter 3 and 4 and column-based size exclusion chromatography.

5.1 Introduction

As discussed in previous chapters, EVs are being increasingly recognised as disease biomarkers and vectors of paracrine communication. They appear to be involved not only in intercellular communication but also in maintaining tissue homeostasis.

The growing pandemic of obesity puts increased burden on the health services due to associated comorbidities such as cardiovascular disease, type 2 diabetes mellitus, several malignancies, mobility problems and also social stigma and isolation, which may lead to further deterioration in health and quality of life. In the UK, the economic cost of people being overweight or obese was estimated at £16 billion in 2007, a figure which is expected to rise further by 2050. [NICE, 2014] Interventions to reduce this burden are therefore critical.

The current management of obesity is very complex and challenging. Approaches vary from lifestyle interventions incorporating dietary changes and/or increased physical activity, pharmacological therapies (which have not been very promising to date due to low efficacy or side-effects), to invasive management in the form of bariatric surgery (which carries risks involved with the procedure itself and postoperative complications and comorbidities).

Typically, an initial weight loss target of between 5 and 10% of initial body weight is recommended [Rossner S, 1997]. For the intervention to be regarded as successful, the results should be maintained for at least a year [Wing R, 2001]. Maintenance of the weight lost post-intervention is crucial to achieve long-term health benefits such as a decreased incidence of type 2 diabetes mellitus. This was evident in landmark trials such as the Diabetes Prevention Program (DPP) and its 10-year follow-up data [Knowler WC, 2009]. Lifestyle interventions (diet and exercise) have also been shown to reduce ectopic fat accumulation in overweight and obese individuals and thus prevent the development of insulin resistance [Hens W, 2015].

It is now accepted that a multidisciplinary team approach to weight loss management is preferable in order to address the breadth of treatment approaches that are required, such as psychological counselling, dietary support and advice, individually tailored physical exercise regimens and/or pharmacotherapy and management of coexisting comorbidities [Montesi L, 2016].

This is in keeping with the recent NICE (National Institute for Health and Care Excellence) guideline recommendations which give specific directions on the establishment and composition of such multidisciplinary services. The Department of Health has been recommended to commission local weight management programmes which are completed by at least 60% of participants with a target

average weight loss of 3% total body weight, with at least 30% of participants losing 5% or more of their initial body weight [NICE, 2014].

Although more long-term studies are required to evaluate the long-lasting effects of participating in such programmes, the obvious advantage of these interventions is that they are believed to be safe and unlikely to cause any harm to patients. For this reason, and in view of increasing demand on bariatric surgery services, the Welsh Institute of Metabolic and Obesity Surgery (WIMOS) requires all patients in Wales to complete a 2-year lifestyle intervention before they are considered for bariatric surgery referral.

In the context of my work, current data on the effects of a structured weight loss programme on circulating extracellular vesicles are very limited with only one previous manuscript evaluating changes in adipocyte-derived EVs in this context [Eguchi A, 2016]. Evaluating the changes in EV concentration and characteristics along with the associated changes in the metabolic and anthropometric measurements would contribute to our understanding of the role of circulating EVs in adiposity-driven metabolic derangements and would help to establish whether they could be used as future early disease biomarkers in this clinical context.

Due to time restrictions, my study specifically focused on changes in EV characteristics following 6 months' participation in the 2-year programme offered to patients in Cardiff and the Vale of Glamorgan.

5.2 Aims

The aims of this chapter were to characterise a cohort of individuals undergoing a tier 3 weight loss management programme with respect to:

1. Anthropometric measurements: weight, BMI, age, gender
2. Baseline and follow-up metabolic profile: HbA1c, lipid profile, thyroid function, circulating plasma adipocytokines: IL6, TNF α , adiponectin, FABP4
3. Evaluation of psychological wellbeing/health (by a questionnaire)
4. Plasma-derived EV concentration, cellular origin and adipocytokine content (as described in previous chapters) pre- and post-intervention using 2 different isolation methods: differential ultracentrifugation and size exclusion chromatography columns
5. Comparison of circulating plasma EV concentration between severely obese individuals and healthy volunteers

5.3 Methods

5.3.1 Study design

This study was designed as a 24-week non-randomised observational study evaluating the effects of a multidisciplinary weight loss management plan on circulating EVs in patients with morbid obesity. Participants were recruited from patients who were referred to the newly established level 2/3 weight management service at Cardiff and Vale University Health Board. The structure of this service and the care delivery pathways remained unchanged and were not affected by an individual's participation in the study. As per established practice, referrals to the obesity management clinic were reviewed by the lead physician, Dr Dev Datta, and/or discussed at the Multidisciplinary Team Meeting consisting of Dietitians, a Specialist Nurse, Physiotherapist/ Occupational Therapist and a Psychologist at the University Hospital Llandough. Only patients qualifying for tier 3 weight loss management support were included in this study as those attending tier 2 dietician and specialist nurse-led services were seen at community-based GP surgeries, hence it would not be logistically possible to obtain and process the blood samples for EV isolation in such a setting.

Patients were evaluated at the point of first contact with the service (baseline) and around 22-24 weeks (6 months) after intervention commencement. Participation in the study only required the patients to donate additional blood samples for EV isolation during already scheduled routine appointments with the lead physician, and was usually coordinated with the timing of their routine baseline and follow-up blood samples that constituted part of their general clinical care.

The CAV Adult Weight Management Services Patient Pathway is attached in the Appendix section.

Flow chart/visit summary

Visit 1 (Cardiff and Vale UHB, Llandough Hospital, Out-patient Department)

- Eligibility check and informed consent
- Standard clinical consultation (incl. weight, BMI, BP measurement)
- Blood sampling

Visit 2 at ~24 weeks (Cardiff and Vale UHB, Llandough Hospital, Out-patient Department)

- Standard clinical consultation (incl. weight, BMI, BP measurement)
- Blood sampling

Between visits, participants underwent a multidisciplinary weight loss management programme as per the usual clinical care plan already in place. Briefly, this consists of frequent appointments/telephone encounters with the dietician and a specialist nurse, and when required, separate sessions with a psychologist with the expertise in management of eating disorders. In addition to that, regular visits to the Consultant Led clinic are scheduled at 2-3 monthly intervals.

5.3.2 Study population

Participants were recruited from a cohort of patients undergoing the tier 3 weight loss management programme at UHL. The referral criteria to attend this programme were:

- Individuals with BMI $>40\text{kg}/\text{m}^2$ or
- Individuals with BMI $>35\text{kg}/\text{m}^2$ and comorbidities such as obstructive sleep apnoea (OSA), type 2 diabetes mellitus or impaired glucose tolerance (IGT), dyslipidaemia, hypertension, non-alcoholic fatty liver disease (NAFLD), polycystic ovary syndrome (PCOS), orthopaedic/mobility problems.

The exclusion criteria were:

- current acute illness
- pregnancy
- active malignancy
- inability to provide full written consent
- recent acute illness or surgery
- previous bariatric surgery

5.3.3 Ethical approval

The study received full ethical approval (REC approval number 16/EE/0342; East of England, Cambridge South Research Ethics Committee), local R&D (approval no: 16/JUL/6572) as well as Cardiff Metropolitan University ethical approval (CHS ethics no 8371). A 3-way material transfer agreement was prepared for samples to be stored and analysed at Cardiff Metropolitan University. All participants provided informed written consent.

5.3.4 Anthropometric measurements

Weight, height and BMI were measured as described in Methods chapter section 2.4 and were performed as part of patients' routine clinical care.

5.3.4 Sample collection, processing and storage

Venous blood was drawn from a large peripheral vein as described in the Methods chapter (section 2.5.1.2). Blood was then immediately centrifuged for 10mins at 3000g using a Heraeus Labofuge 400 (DJB Labcare) centrifuge at the UHL Biochemistry Department. The supernatant was collected and centrifuged again at 3000g for 10mins to render plasma acellular. Plasma was then aliquoted and transported to Cardiff Metropolitan University where it was stored at -80°C until further analysis.

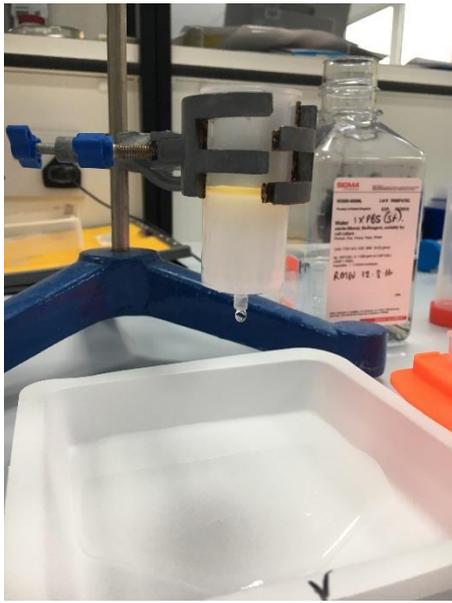
5.3.5 EV isolation, measurement and analysis.

EVs were isolated from thawed plasma by ultracentrifugation at 100,000g using a Sorvall centrifuge and Surespin 360 rotor. EV concentration, size, cellular origin and content were measured as described in the Methods chapter (sections 2.5.4. and 2.5.5). For the analysis of this cohort, an additional exosomal marker antibody (anti-CD63, rabbit polyclonal, 200 µg/mL)(sc-15353, Santa Cruz Biotechnology) was used alongside anti-CD9. Otherwise, all primary antibodies used were from the same supplier and the same concentration as described previously (Methods chapter, section 2.5.5.2), except for anti-IL6 antibody used for size exclusion chromatography EV samples which was purchased from Thermo Fisher Scientific (IL-6 Antibody (4H16L21), ABfinity™ Rabbit Monoclonal, 701028) due to a supply problem from Abcam.

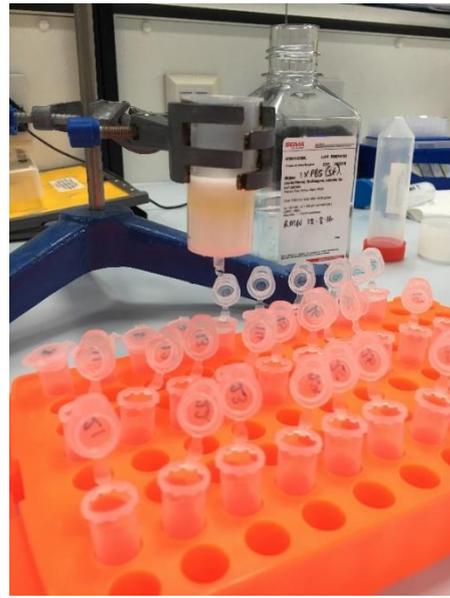
5.3.5.1 Size exclusion chromatography columns

Exo Spin Midi Columns (Cell Guidance Systems) were used to obtain EVs, with no ultracentrifugation performed in this method. These columns were pre-equilibrated with ultra- pure water and 20% ethanol, and were then equilibrated with sterile PBS prior to EV isolation (2x10mL). One millilitre of thawed double-spun plasma was applied when there was no remnant buffer on the top surface of the column and 500µL fractions were then collected (n=24) (Fig 5.1 a-c). Based on the product information sheet and our local laboratory experience, it was established that exosome/EV rich

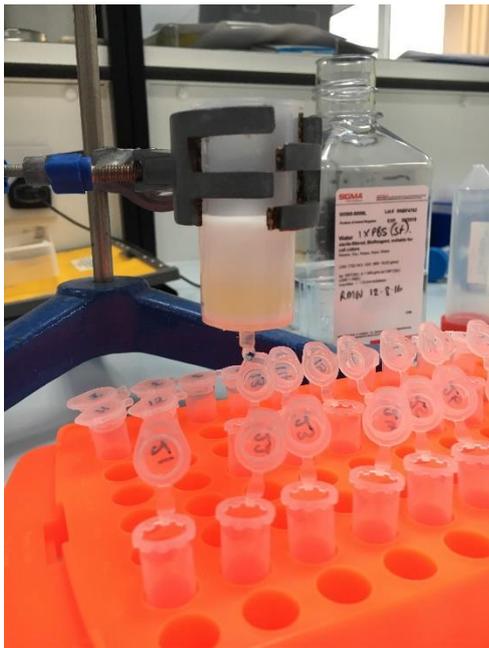
fractions accumulate in fractions 5-10. These 6 fractions were then pooled together giving a total sample volume of 3 mL; EV concentration was then measured by NTA as previously described. Unfortunately, the overall EV/mL plasma concentration was lower than in the samples which were obtained by ultracentrifugation and from a larger volume of plasma. Therefore, for the EV origin and adipocytokine content analysis by TRF immunoassay, only 3×10^9 EVs/well were loaded. It was thus unfortunately not possible to directly compare 'columned' and 'non-columned' EVs since the TRF signal does not have a linear relationship with the number of EVs loaded in the well in our experience.



a)



b)



c)

Fig.5.1: Isolation of EV fractions using Exo-Spin Midi Columns (Cell Guidance Systems) a) Loading of 1mL of plasma b) Collection of early fractions c) Collection of EV-rich fractions.

5.3.6 Plasma adipokine and cytokine concentration

Plasma IL-6, TNF α , adiponectin and FABP4 were measured using the same assays and protocols as described in the Methods chapter (section 2.6).

5.3.7. Other plasma measurements

As part of routine clinical care, individuals also underwent measurement of various markers of metabolic health such as plasma lipids, HbA1c and Thyroid-Stimulating Hormone (TSH) which were performed at the medical biochemistry laboratories at Cardiff and Vale UHB.

Total cholesterol was measured by an enzymatic method, as were triglycerides (glycerol phosphate oxidase). HDL cholesterol concentration was evaluated by a colorimetric method with a selective detergent step to remove the non-HDL cholesterol. The Abbott Architect (Abbott Diagnostics) system was used to perform these analyses. The Friedwald formula was then applied to estimate the LDL cholesterol concentration ($[\text{LDL-cho}] = [\text{Total chol}] - [\text{HDL-cho}] - ([\text{TG}]/2.2)$) [Roberts WC, 1988]. HbA1c was measured by ion-exchange chromatography (Tosoh Bioscience G8 Analyser).

5.3.7 Evaluation of overall wellbeing/health

The EQ-5D-5L tool was used as a standardised measure of health status. This addresses 5 different health aspects: mobility, self-care, usual activities, pain/discomfort and anxiety/depression that can be scored on a 5 level scale or severity [Whynes DK, 2008]. The EQ-5D-5L index values were presented as mean (SD/standard error) for the cohort pre- and post- intervention.

5.3.8 Statistical analysis

Normally distributed data were presented as mean (SD) values, whilst non-normally distributed data were presented as median (range); Normality was tested by the Shapiro-Wilk test. Pre- and post-intervention changes were evaluated using the Student *t* test for normally distributed variables, and the Wilcoxon matched pairs signed rank test for non-normally distributed ones. A *p* value <0.05 was accepted as statistically significant and GraphPad software version 6.0 was used.

5.3.9. Sample size and power calculations

The primary outcome of this study was to evaluate the effects of a multidisciplinary weight loss programme on circulating EVs. As there were no data available on the effects on circulating EVs of such a programme, power calculations were initially based on the recent paper evaluating changes in EVs following bariatric surgery by Campello *et al* [Campello E, 2016].

In this publication, twenty patients with grade III obesity undergoing bariatric surgery had EV measurements taken at time 0, 3 and 12 months' post-bariatric surgery. This study showed a significant decrease of all EV types at 12 months (Annexin V-EVs, endothelial-derived EVs, platelet-derived EVs, leukocyte-derived-EVs, with percentage reductions of $-16 \pm 10\%$, $-30 \pm 23\%$, $-27 \pm 16\%$ and $-35 \pm 18\%$, respectively) with corresponding 41% loss of body weight [Campello E, 2016].

Allowing for up to 10% dropout, it was estimated that recruiting 50 subjects into my study would provide over 80% power to detect a shift of 0.3 times the standard deviation of within-group changes in EV subtypes and concentration at the 5% alpha level using 3-way ANOVA.

However, due to time constraints and insufficient numbers of new patients meeting the inclusion criteria being referred to the service, I was unable to recruit this number of participants within the 6 months' time period.

Disclosure

Most patient recruitment, sample collection, initial processing, storage and analysis was performed by myself, hence I was not blinded whilst performing the analyses.

Dr Katherine Connolly and Mr Cass Whelan assisted in the EV isolation by SEC and the immunophenotyping of that sub-cohort.

5.4 Results

Twenty-one patients were recruited to take part in the study but only 15 completed it due to loss of engagement with the service or failure to attend follow-up appointments. Therefore, for the analysis of the effects of the multidisciplinary weight loss programme on circulating EVs, only 15 paired data sets were analysed. Since there were only 5 male patients recruited and only 2 of them completed the programme, no analysis of gender-driven differences was performed with regard to plasma EVs and adipocytokines in this cohort.

The mean baseline BMI amongst all 21 recruited patients (F=16, M=5), age 49.7(11.2) years was $55.1 \pm 7.19 \text{ kg/m}^2$ and mean weight $152.6 \pm 26.2\text{kg}$.

5.4.1 Baseline and follow-up anthropometric characteristics, glucose, HbA1c and lipids

The baseline and follow-up measurements (n=15) are presented in the table below. There were no significant differences between baseline and 6 months' follow-up in any of the clinical measures studied (Tab. 5.1).

Variable (mean (SD))	Baseline	6 months follow up	P value
Weight (kg)	152.8(23.2)	150.32(23)	0.77
BMI (kg/m ²)	54.92(6.6)	53.7(6.8)	0.62
TSH (mIU/ml)	1.56(1.06)	1.71(0.93)	0.68
HbA1c (mmol/mol)	46.5(18.1)	43.7(13.8)	0.63
Total cholesterol (mmol/L)	4.66(0.92)	4.77(0.97)	0.75
Triglycerides (mmol/mL)	1.95(0.77)	2.04(0.95)	0.77
HDL (mmol/mL)	1.13(0.26)	1.22(0.34)	0.42
LDL (mmol/mL)	2.67(0.81)	2.7(0.98)	0.92

Tab. 5.1: Summary of anthropometric and metabolic variables pre-and post-intervention of the sub-cohort that completed the study (n=15, M=2, F=13, age 48.2 ± 11.4 years).

5.4.2 Measurement of psychological well being

The mean EQ-5D-5L score pre-intervention was 0.255 ±0.41 and post-intervention 0.3±0.221, p=0.71.

5.4.3 Plasma adipocytokines

5.4.3.1. Plasma IL-6

There was no difference between baseline and follow-up plasma IL-6, with mean concentrations of 2.39 ±0.66 pg/mL vs 2.22 ±0.98 pg/mL; p=0.49. The median IL-6 concentration in the healthy cohort from chapter 3 was significantly lower compared to baseline IL-6 in the morbidly obese cohort from this study (1.49(0.42-7.4) vs 2.24(1.17- 3.83), p<0.01). (Fig.5.2)

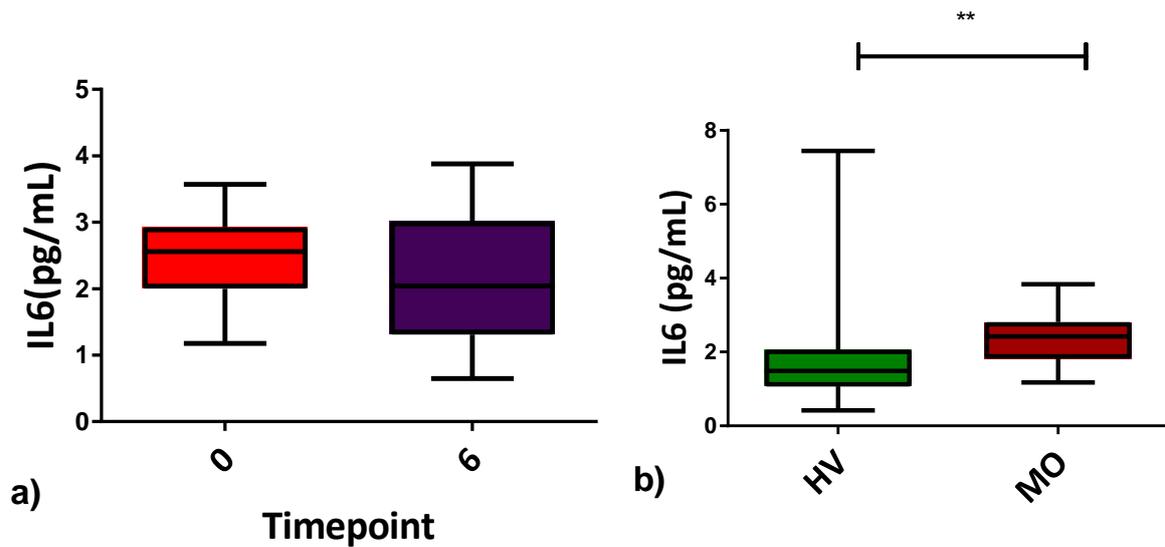


Fig.5.2: Differences in plasma IL-6 concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (HV)(n=40) and morbidly obese (MO) patients (n=21), **=p<0.01.

5.4.3.2 Plasma adiponectin

Plasma adiponectin did not differ between baseline and 6 months (median baseline concentrations 47.4 (16.2-99.15) vs 52.05 (17.85 vs 164) $\mu\text{g}/\text{mL}$, respectively; $p=0.38$). As expected, the median plasma adiponectin in the healthy cohort was significantly higher compared to the morbidly obese one (203.4 (42.75-822.9) vs 47.4 (16.2-103.5) $\mu\text{g}/\text{mL}$, $p<0.0001$). (Fig.5.3)

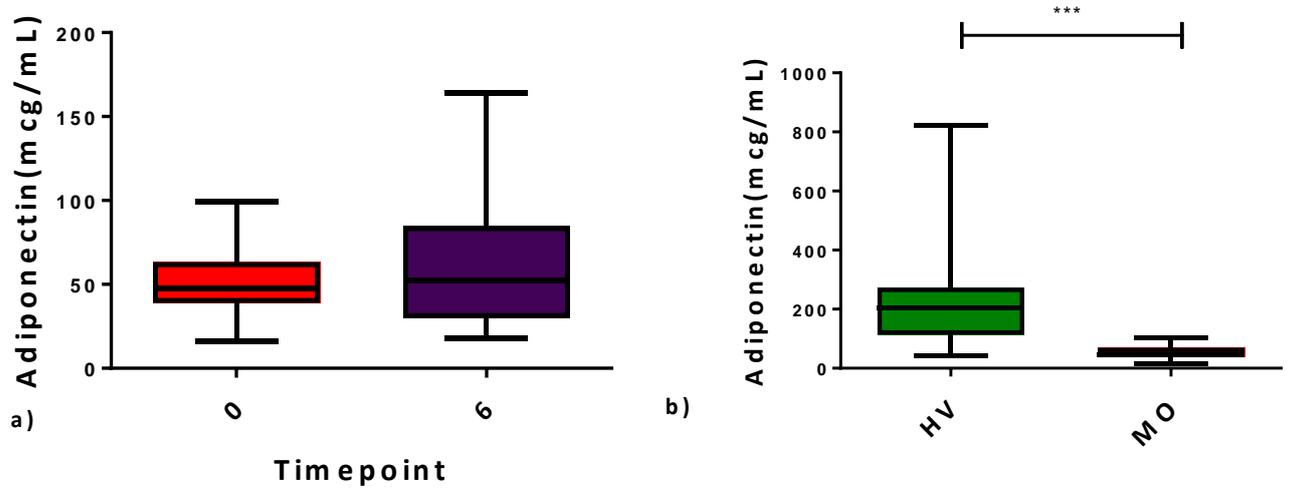


Fig.5.3: Differences in plasma adiponectin concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (n=40) and morbidly obese patients (n=21), *=p<0.005.**

5.4.3.3. Plasma TNF α

The median plasma TNF α concentration was not different pre-and post-intervention (58.13 (29.27-374) vs 45.93 (19/51-314.6) pg/mL, p=0.168). There were also no significant differences between HV and MO cohorts: 47.67 (2-833) vs 53.25 (29.27-374) pg/mL, p=0.45 (Fig.5.4) which was unexpected but is likely due to wide error bars in the measurements.

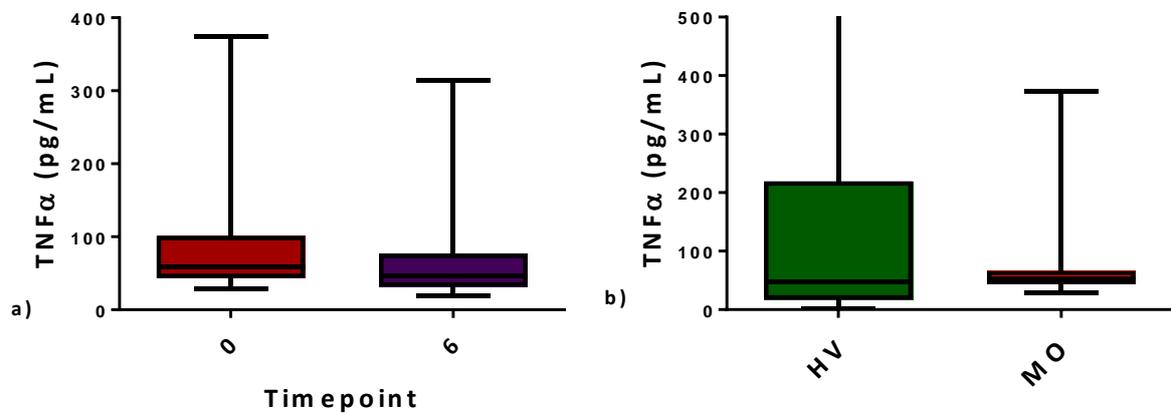


Fig.5.4: Differences in plasma TNF α concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (n=40) and morbidly obese patients (n=21).

5.4.3.4 Plasma FABP4

There were no significant differences in mean plasma FABP4 concentrations at the beginning of the enrolment to the weight management programme and 6 months later (56.74 ± 10.44 vs 51 ± 22.55 ng/mL, $p=0.38$).

The morbidly obese patients had significantly higher mean plasma FABP4 concentrations compared to healthy volunteers (55.44 ± 9.5 vs 19.9 ± 9 ng/mL, $p < 0.0001$).

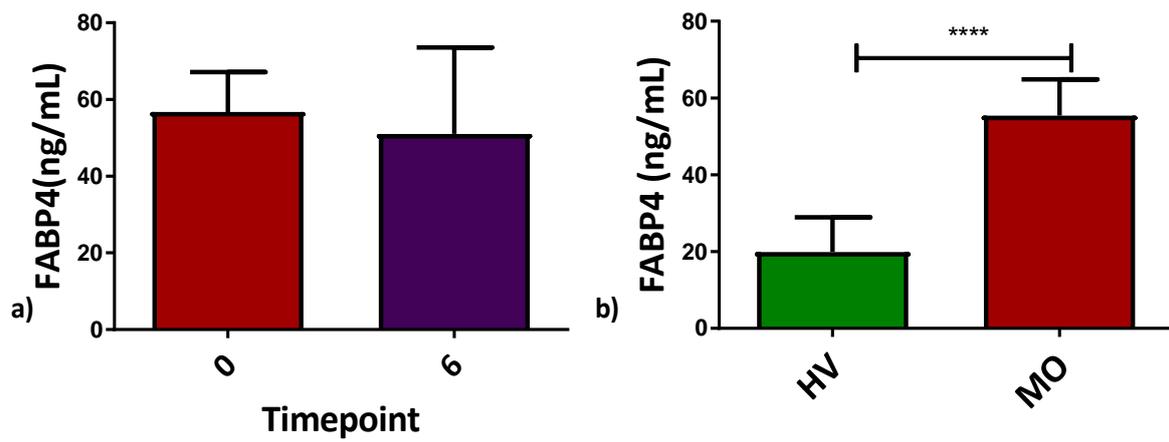


Fig.5.5: Differences in plasma FABP4 concentrations: a) in the morbidly obese cohort at baseline and 6 months' follow-up (n=15) b) between healthy volunteers (n=40) and morbidly obese patients (n=21), **=p<0.0001.**

5.4.4 The effects of lifestyle intervention on circulating plasma EVs concentration, cellular origin and profile.

Participation in the weight loss management programme did not affect total EV concentration in plasma (1.24 ± 0.72 vs $1.1 \pm 0.52 \times 10^{12}/\text{mL}$, $p=0.6$) (Fig. 5.6 a) and the size distribution analysis revealed no differences either in concentration of smaller versus larger EVs subpopulations ($p>0.99$). (Fig. 5.6 b-c)

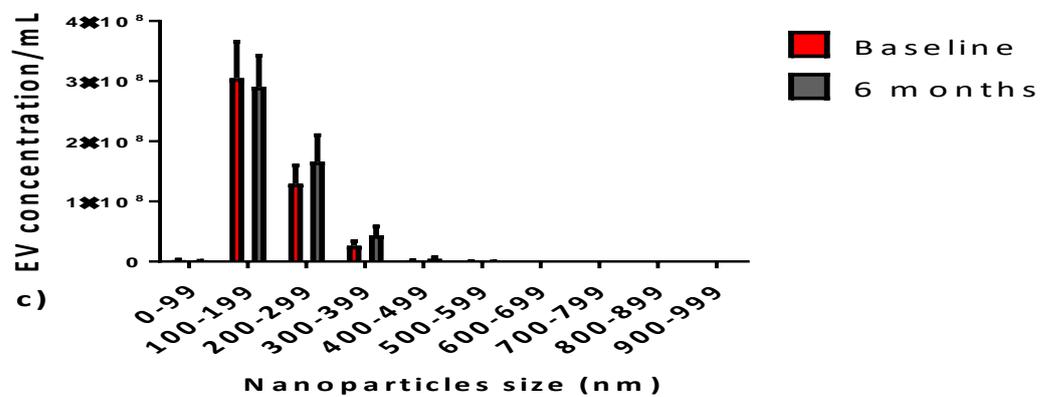
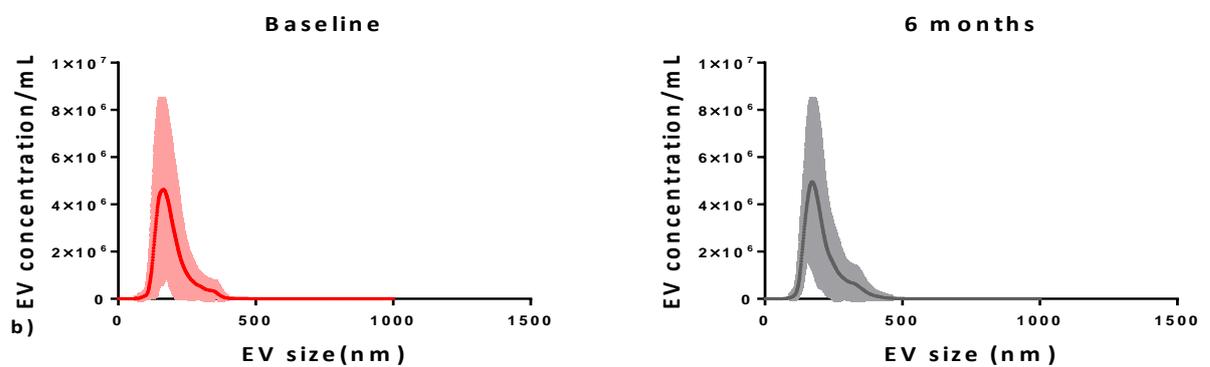
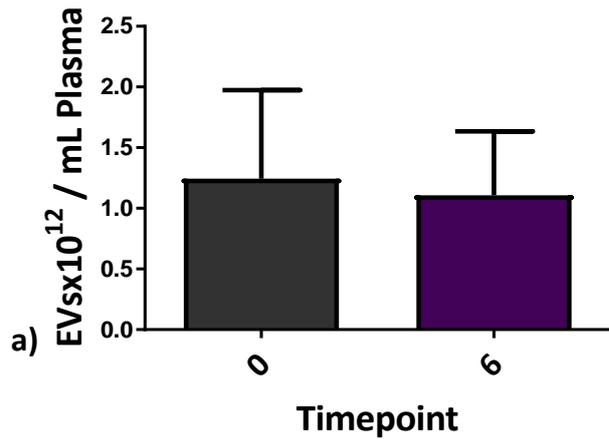


Fig.5.6: a) Mean EV concentration/mL of plasma pre-and post-lifestyle intervention did not differ ($p=0.6$); b) and c) The size distribution analysis by two-way ANOVA did not reveal any differences in EV concentration/mL between the timepoints when analysed 'bin by bin' (b) or grouped in 100nm intervals (c), both $p>0.99$.

Following the lifestyle intervention, there was a significant reduction in the mean platelet-derived (CD41) EV TRF signal 33310(19692) vs 15559 (13803) arbitrary units (a.u), $p=0.01$, but the other cellular origin markers as well as exosomal markers did not differ (median CD11b: 14903(5417-174226) vs 56810(12460- 118259) a.u, $p=0.83$, median CD235a: 6263 (2668-19603) vs 7134 (92-11977) a.u, $p=0.91$, median CD235a 6263(2668-19603) vs 7134 (82-11977), median CD144: 11405(5905- 36748) vs 11547(2593- 34280) a.u, $p=0.55$, median CD63: 40172(17941- 115798) vs 41319(9135- 78858) a.u, $p=0.73$ and median CD9: 49565(17393- 117688) vs 27449(5183- 203980) a.u , $p=0.233$. (Fig. 5.7)

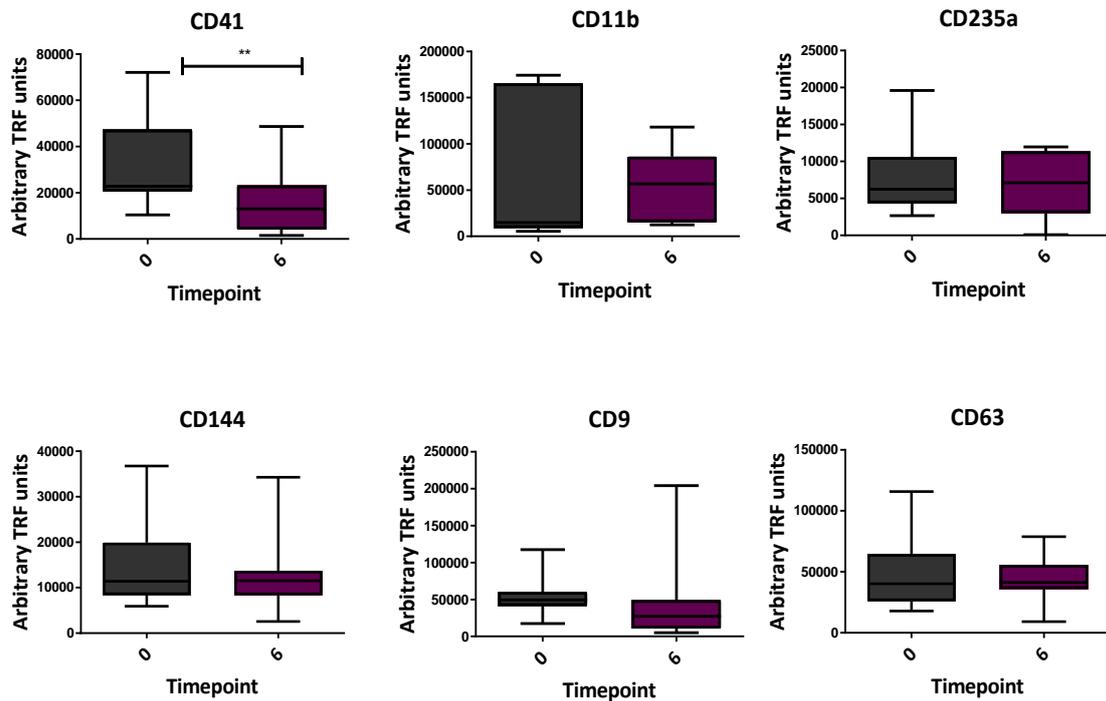


Fig.5.7: Expression of EV cellular origin markers (CD41:platelets, CD11b:monocytes/macrophages, CD235a: erythrocytes, CD144: endothelial cells) and exosomal markers (CD9 and CD63) at baseline and 6 months after lifestyle intervention. (p<0.01)**

With regard to EV adipocytokine content, significant changes were observed in EV-contained FABP4, with median EV FABP4 pre-and post-intervention being 45455 (11637- 77248) vs 20099(6218- 137144) a.u respectively, p=0.0033. (Fig 5.8)

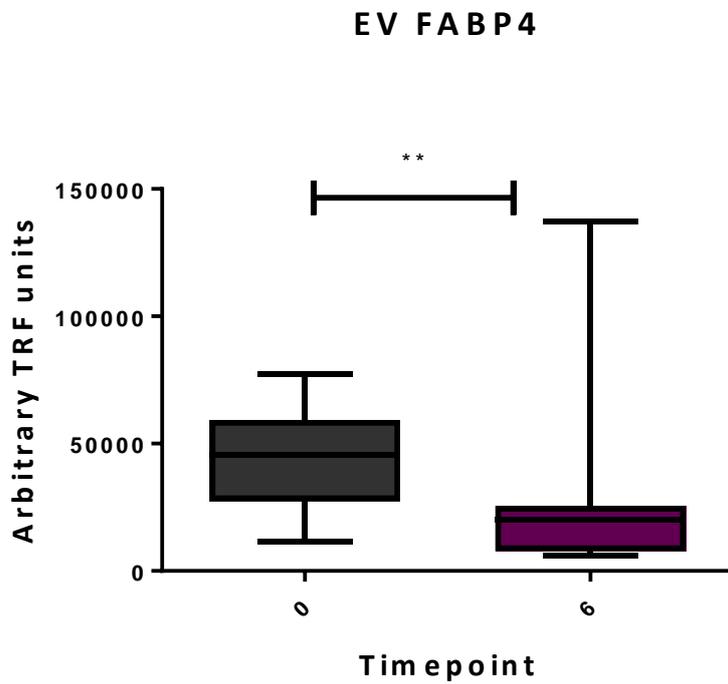


Fig.5.8: Changes in EV-expressed FABP4 at baseline and 6 months following lifestyle intervention. (=p<0.05)**

There were no statistically significant changes observed in other adipocytokines between the baseline and follow-up visits, with median EV TNF α values of 30161(17651- 107710) vs 16218(1346- 40973) a.u, p=0.052; median EV interferon γ 59583(4558- 157275) vs 20149(4529- 142380) a.u, p=0.068; median EV adiponectin 119103(73825- 433938) vs 82753(48390- 276872) a.u, p=0.067 and median PPAR γ 12921(2373- 50183) vs 9278(1855- 87145) a.u, p=0.55. (Fig.5.9)

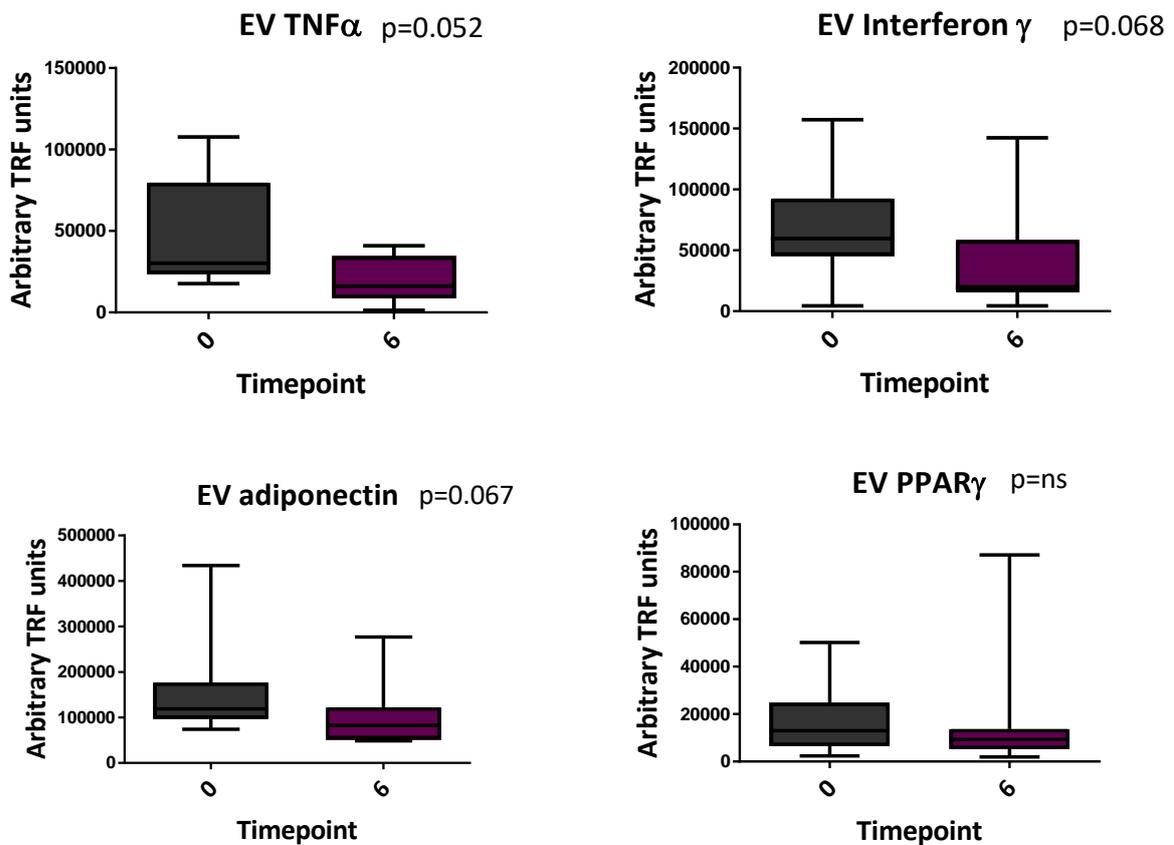


Fig. 5.9. EV expressed TNF α , interferon γ , adiponectin and PPAR γ at baseline and 6 months following lifestyle intervention.

Unfortunately, the assay did not appear to detect signals from EV-contained IL6, with detectable signals above the baseline in only 10 samples overall, hence paired analysis was not possible.

I subsequently undertook an analysis of my data split according to whether patients had type 2 diabetes or not. There were no differences observed in EV concentration or adipokine profile between participants with (n=8) or without T2DM (Tab 5.2) (n=13) which is likely due to the fact there were no BMI/age differences between those cohorts (53.4 ± 5.6 vs 54.3 ± 7.3 kg/m² p= 0.82; 48.6 ± 8 vs 49.2 ± 14 years, p=0.9). This could be explained by the lack of power due to low numbers in each subgroup.

Measurement (median with range)	T2DM (n=8)	Non-diabetic (n=13)	P value
EV concentration/mL	1.08 (0.23-1.66)	0.845 (0.22-7.05)	0.8
CD41	27836 (21203-65046)	22694 (10402-72078)	0.58
CD11b	14729 (7077-174226)	14948 (2111-482013)	>0.99
CD235a	7421 (4652- 19606)	9630 (2668- 24785)	>0.99
CD144	13762(7461-24991)	10771 (3941-58123)	0.599
CD9	49663 (23277-78883)	48348 (17393-79133)	0.77
CD63	41054 (27108-101295)	36459 (17941-115798)	0.38
EV TNF α	29047 (18682-60883)	23973 (15835-107710)	0.57
EV Interferony	59824 (50674-81591)	51509 (4558-157275)	0.24
EV adiponectin	128738 (99576-167990)	118413 (73825-433938)	0.57
EV FABP4	37733 (11637-58466)	37764 (19450-76448)	0.9
EV PPAR γ	22895 (4220-26587)	9174 (2373-69268)	0.53

Tab.5.2: Comparison of EV concentration, cellular origin and adipokine content at baseline between participants with T2DM and those with no diabetes.

5.4.5. Differences in EV profile between morbidly obese subjects and healthy volunteers

The median EV concentration at baseline in the morbidly obese (MO) cohort was significantly higher compared to the healthy volunteers (HV) from chapter 3: 9.4 (2.200- 70.50) vs 4.13 (2.200- 12.15) $\times 10^{12}$ /mL, $p < 0.0001$. (Fig. 5.10)

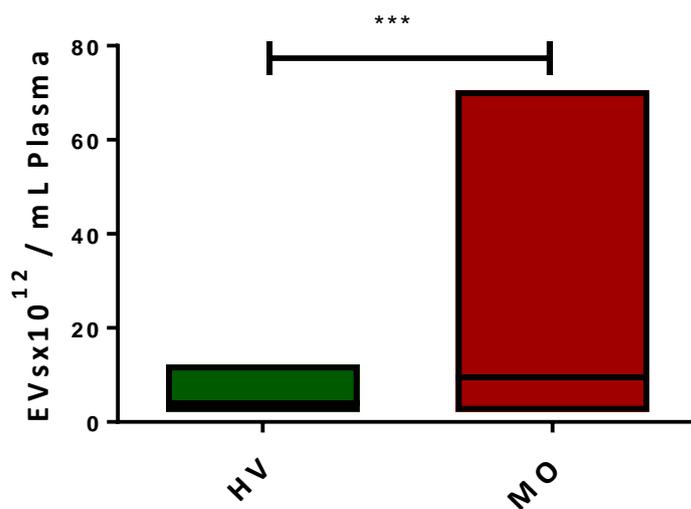


Fig.5.10: Differences in EV concentration/mL of plasma between healthy volunteers (n=49) and morbidly obese individuals (n=21), *=p<0.005.**

The EVs from the MO cohort also had significantly higher median expression of CD41 (platelets), CD235a (erythrocytes) and CD144 (endothelial cells) markers (22926 (10402-72078) vs 12962 (2568-34360) a.u, $p < 0.0001$; 12080 (3941-58123) vs 6076 (320.0-36106) a.u, $p < 0.01$; 9585 (2668-24785) vs 5232 (416.0-25128) a.u, $p < 0.01$). No differences were observed for CD9 (exosomes) or CD11b (monocytes/macrophages) (50874 (17393-117688) vs 33222 (5536-104080) a.u, $p = 0.054$; 14903 (2111-482013) vs 2756 (404.0-112260) a.u, $p = 0.96$). (Fig.5.11)

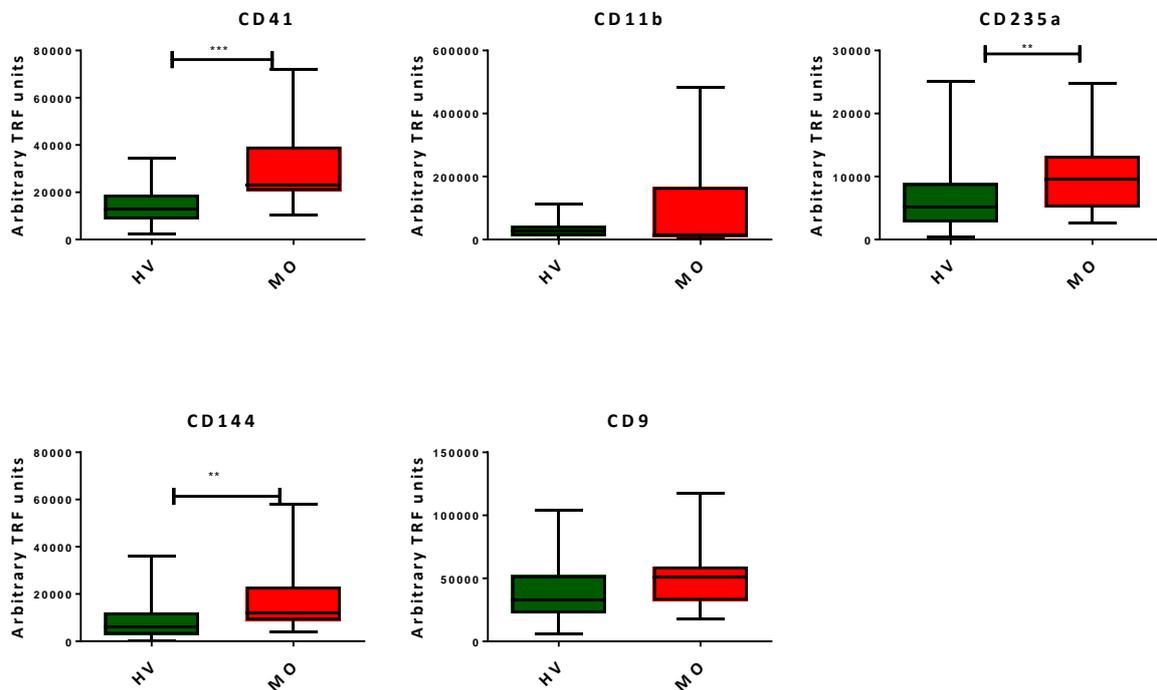


Fig. 5.11: Comparison of median (ranges) expression of markers of EV cellular origin between healthy volunteers (HV, n=49) and morbidly obese subjects (MO, n=21), **= $p < 0.01$, *= $p < 0.005$.**

MO subjects showed significantly higher EV expression of TNF α , interferon γ , adiponectin and FABP4 (27769 (15835-107710) vs 5425 (42.00-35230)a.u; 57028 (4558-157275) vs 37982(1240-103628)a.u; 47214(5264-114310) vs 121859(73825-433938); 38849 (11637-77248) vs 14445 (258.0-57026) a.u, all $p < 0.001$) with no differences for PPAR γ expression (11392(328.0- 33394) vs 18455(2373- 69268)a.u, $p = 0.105$). (Fig.5.12)

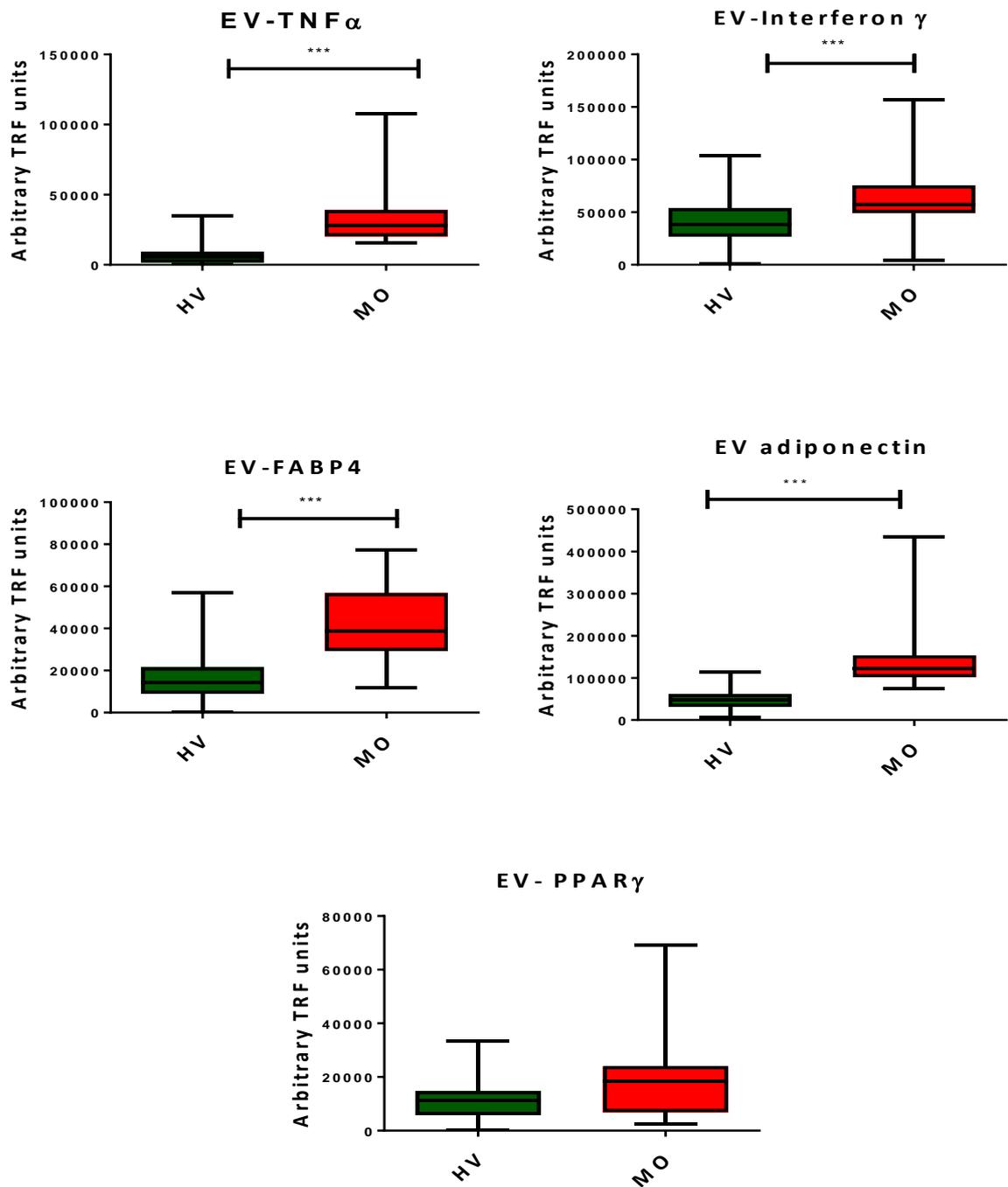


Fig.5.12: Differences (median(range)) in EV-expressed adipocytokines (TNF α , interferon- γ , FABP4 and PPAR γ) between healthy volunteers (n=49) and morbidly obese subjects (n=15), *p<0.005).**

5.4.6 Changes in pre- and post-lifestyle intervention in plasma EV profile isolated by size exclusion chromatography

There were 10 paired surplus plasma samples available to analyse the changes in circulating EV profile pre-and post-intervention using Exospin midi columns for EV isolation. Unfortunately, the yield of EVs was very low in fractions obtained from 2 of these subjects by this method and the TRF signals obtained were thus below the detection threshold of the assay and had to be excluded. Hence only 8 paired samples were included in the final analysis. As the overall EV concentration and number were much lower (sample dilutions required to obtain EV concentration readings on Nanosight were 1:200 rather than 1:10000 for the differential centrifugation method from higher plasma volume), from the total available volume of 6 pooled fractions (3mLs) I was only able to load 3×10^9 EVs/well for the TRF immunoassay. The same antibodies were used in the same concentration as in previous experiments, as described in the Methods chapter. However, given that the relationship between the TRF signal and the EV load is not linear based on our experience, no direct comparison was performed between TRF signals from EVs isolated by differential ultracentrifugation vs those obtained by size exclusion chromatography.

I was however able to compare the profile of main cellular origin of EV families for samples obtained by UC vs SEC (Fig.5.13) which demonstrated that the profile of EV cellular markers distribution was very similar pre and post intervention (with the consistently dominant signal from CD11b) with only subtle differences observed between UC and SEC sample profiles.

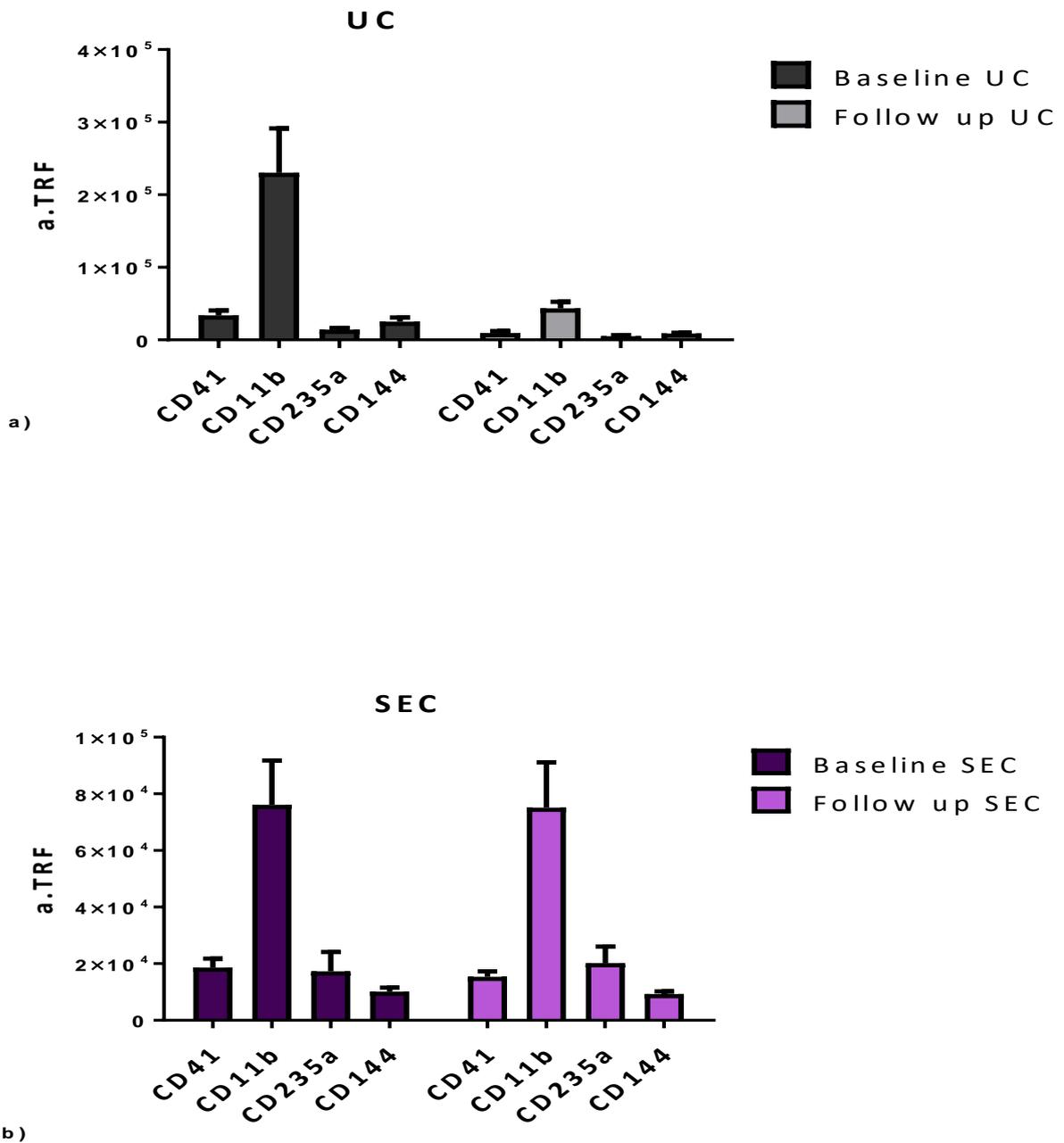


Fig.5.13: Distribution of TRF signal for main EV cellular origin markers (CD41-platelets, CD11b-monocytes/macrophages, CD235a-erythrocytes, Cd144-endothelial cells) in baseline and 6 months follow up EV samples isolated by differential ultracentrifugation (UC) (a) and size exclusion chromatography columns (SEC) (b).

There were no significant differences observed in the EV cellular markers pre- and post-lifestyle intervention with median CD41: 18053 (8817-33688) vs 14150 (9618-27727)a.u, $p=0.62$; median CD11b: 76187(1489- 143564) vs 84320(5704- 137881)a.u, $p=0.87$; median CD235a: 9380(4810- 62707) vs 11389(3853- 44727)a.u, $p=0.93$; median CD144: 9003 (5180-16768) vs 8234 (5934- 13175)a.u, $p=0.56$ and the exosomal markers: median CD9: 20086 (3894-44034) vs 21946 (9368- 36799)a.u, $p=0.99$ and median CD63 56822 (19773-126063) vs 54329 (37965-66993)a.u , $p=0.507$. (Fig.5.14)

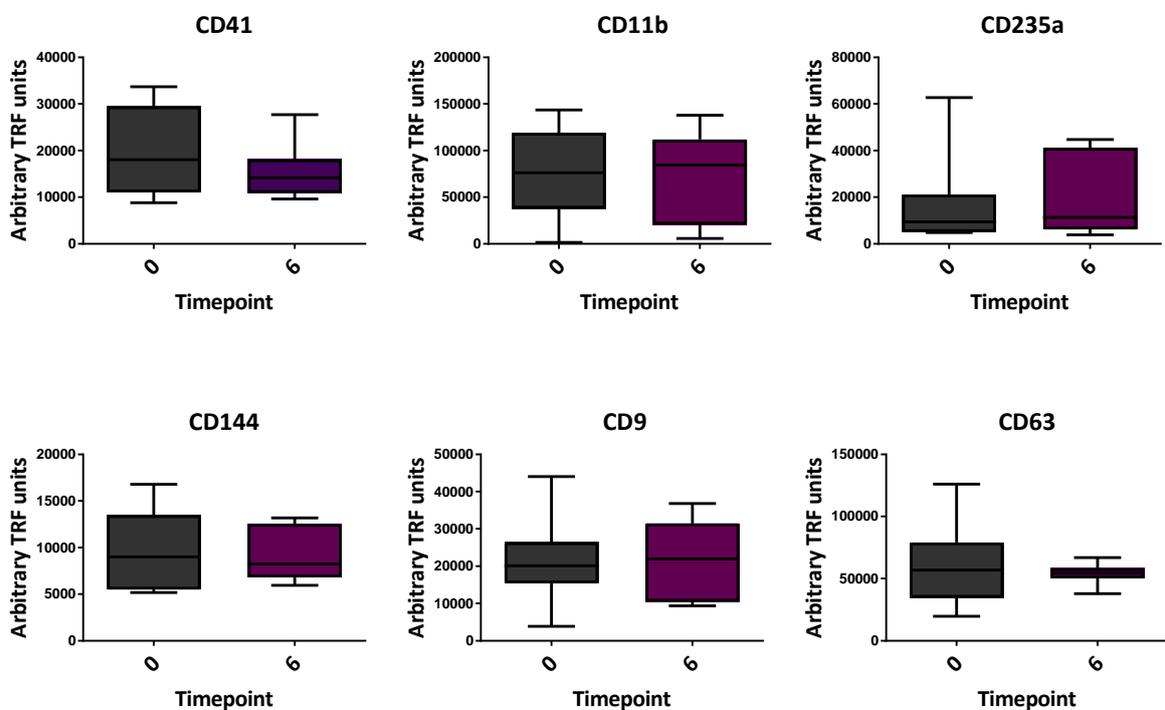


Fig.5.14: Differences in EV cell of origin and exosomal markers pre-and post- lifestyle intervention using Exo Spin columns for EV isolation (n=8).

With regard to EV-expressed adipocytokines there was a difference, of borderline significance, in median EV FABP4 signal [59459(14885- 133456) vs 16344(7231- 32112)a.u, p=0.054] but the other EV adipokines were not different, with median IL6 [11050 (6299-15394) vs 9387(1892-26957) a.u , p=0.11], median TNF α [35006 (17494-59768) vs 19708 (13408-45654) a.u, p=0.13], median Interferon γ [43360 (25860-97858) vs 30567 (16615-65262)a.u , p=0.26], median adiponectin [54147(29578- 181324) vs 40281(14484- 114247)a.u , p=0.46] and median PPAR γ [13990(186.0- 24594) vs 5711(2604- 27923)a.u, p=0.81]. (Fig.5.15)

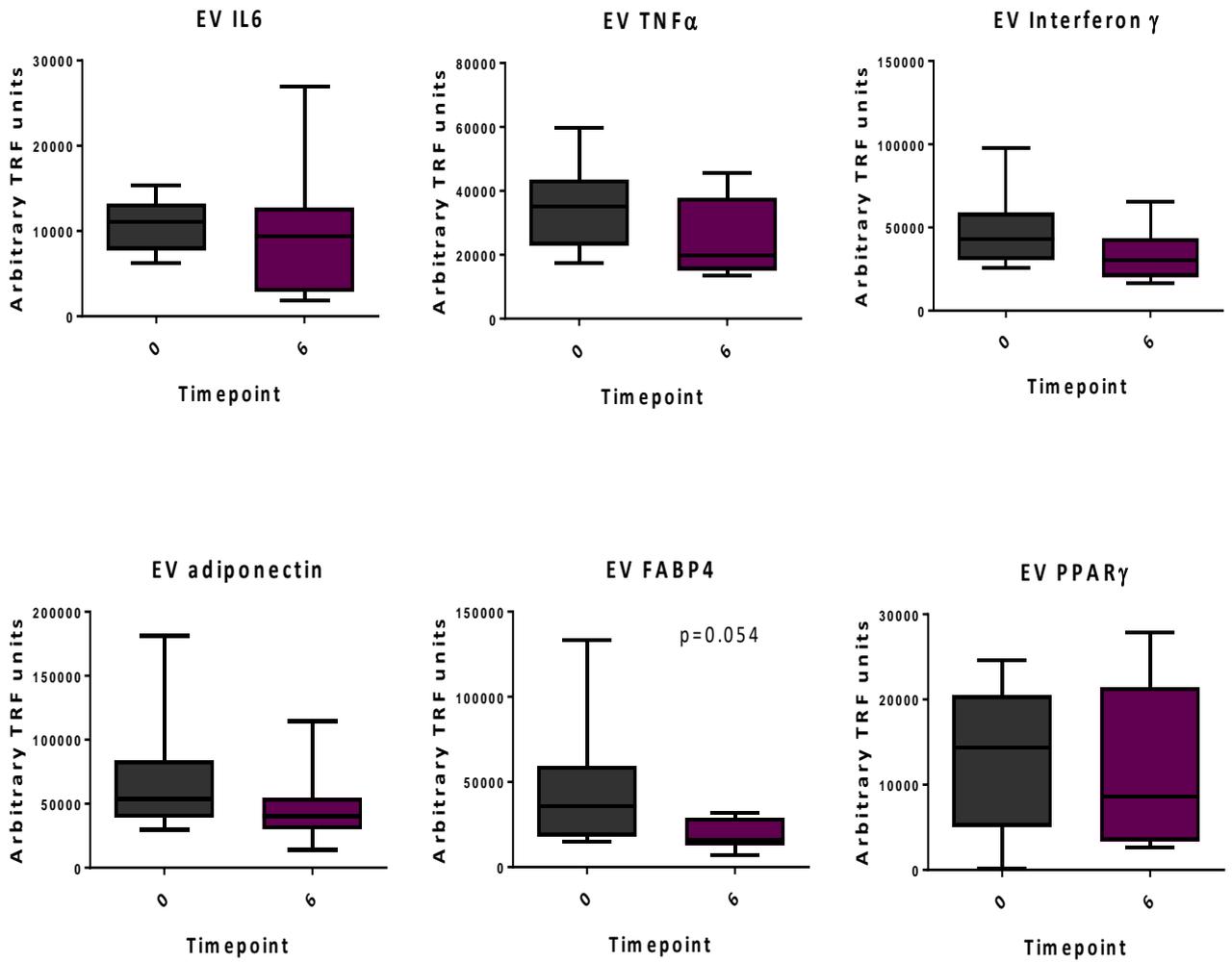


Fig.5.15: Expression of EV adipocytokines pre- and post-lifestyle intervention using Exo Spin columns for EV isolation (n=8).

5.5 Discussion

5.5.1 Lifestyle intervention and circulating EVs

This study showed that even a brief period of lifestyle intervention may have an effect on the circulating EV profile despite no significant effect on weight/BMI, plasma ‘free’ adipocytokines or traditional markers of metabolic health such as lipids or HbA1c. Similar to the bariatric cohort described in chapter 4, EV-FABP4 appears to be the most responsive EV marker. EV-contained TNF α also decreased at 6 months’ follow-up, albeit of borderline statistical significance. Interestingly, I also found a significant decline in TRF signal from platelet-derived EVs following the lifestyle intervention. This was not observed in the sub study using columns for EV isolation but the potential reasons for that will be discussed later in this chapter. Overall, however, there is evidence in my study of lifestyle-induced changes in the circulating EV profile before any other measurable health benefits are seen.

There are only a few studies in the literature that have evaluated the effects of a lifestyle intervention on circulating EVs. One of the reasons for this may be that EV research in the clinical setting is still quite novel. Given the logistics and costs of properly designed lifestyle intervention studies, more traditionally established metabolic markers and health outcomes are of greater interest at present as they can be more easily translated into clinical practice. As discussed in previous chapters, the EV field still suffers from large variations in EV isolation and characterisation techniques. Furthermore, the significant amount of time required for EV isolation would not support their widespread use in large populations. Nonetheless, the small ‘lifestyle modification’ studies that have been conducted to date provide quite a useful insight into the possible role of EVs and add value to the results of my project.

In the most recent paper by Eitan *et al* evaluating the effects of lifestyle intervention, an experimental approach was undertaken aiming to attenuate tumour growth in overweight prostate cancer patients awaiting prostatectomy by limiting daily protein intake, thus decreasing the amount of circulating amino acids [Eithan E, 2017]. Although the trial duration was only 1 month, the authors showed that plasma EVs had increased expression of leptin receptors in the cohort as a whole, as well as L1CAM- (L1 cell adhesion molecule) expressing EVs in the intervention arm compared to the control group. Moreover, there were changes in phosphorylation status of insulin receptor signal transducer protein-1 (IRS1) in L1CAM-expressing EVs in keeping with increased insulin sensitivity. Similar to my study, NTA was used to determine EV concentration and size distribution, with no

changes observed in these parameters post- intervention. The authors also used CD9 as their exosomal marker, again in keeping with my study. They focused mainly on EV content with regard to leptin receptor and insulin signalling protein 1 (IRS1), and suggested that although leptin and insulin levels have been extensively researched in the context of obesity, more focus is needed on receptors and mediators of their action. They proposed that EVs reflect biological processes within their cell of origin and are therefore likely to be very 'plastic' and reflect cellular processes in response to diet quite rapidly. This could open a new avenue of research in evaluating the molecular processes in response to food intake in human subjects. Of interest, the authors also proposed that evaluating EV adipokine expression may be better than evaluating their total soluble plasma concentration, as the plasma-derived proteins represent a 'mixture' derived from different cells; since EVs carry their cell of origin markers, this can help us establish the effects of diet on specific cell populations [Eithan E, 2017]. The observations in this paper support my hypothesis that the dietary intervention in my study possibly led to a decrease in adipocyte-derived EVs in the plasma, as seen by decreased FABP4- and TNF α -expressing EVs.

With regard to other dietary interventions, there are 2 recent studies on the benefits of consumption of natural cocoa on vascular health and the EV profile. Horn *et al* showed that in response to a 30-day period of high dose cocoa flavanol drink ingestion in patients with coronary artery disease, the levels of EVs expressing the endothelial markers CD31⁺/41⁻ and CD144⁺ decreased (by 25 and 23%, respectively), with no such effect seen in the group that consumed low doses [Horn P, 2013]. Since EVs correlated inversely with flow mediated vasodilatation in this study, it was postulated that this reduction in EVs may be important in the associated improvement in endothelial function. A further functional study was also performed evaluating the procoagulant activity of EVs (measured as the formation of activated factor X per time) but this was not affected in response to either low or high flavanol diet [Horn P, 2015].

McFarlin *et al* also showed similar beneficial results of natural cocoa consumption on vascular health. In their cross-over study, young healthy females of various BMIs (normal, overweight, obese) were evaluated, and asked to consume daily either a cocoa-rich product or a placebo for 4 weeks with a 2-week wash-out period. In obese subjects, natural cocoa consumption led to a significant decrease in endothelial-derived EVs (CD42a-/45-/144+) and monocyte-derived ones (CD62L) as evaluated by flow cytometry. Regardless of BMI, there was an 18% increase in plasma HDL concentration and a 60% decrease in endothelial-derived EVs [McFarlin BK, 2015].

Zhang *et al*, in their study on dietary intervention in newly diagnosed type 2 diabetes mellitus patients, compared the effects of 8 week standard dietary advice versus an oat-rich diet on the EV

profile. Interestingly, the oat-rich diet led to a reduction in concentration and proportion of tissue factor- and fibrinogen-expressing platelet-derived EVs and CD 11b (monocyte/macrophage) expressing EVs. In contrast, standard dietary advice only led to a decrease in the fibrinogen-expressing platelet-derived EVs [Zhang X, 2014].

Finally, an Italian study recruited 100 patients from a lipid clinic with CRP >2mg/dL and LDL 100-160mg/dL with no history of cardiovascular disease, and randomised them to receive either a nutraceutical preparation containing red yeast rice, berberine, policosanol, astaxanthin, folic acid and coenzyme Q10 for 3 months or not. They evaluated the effects of such an intervention on endothelial-derived EVs as well as lipid profile and CRP. As in my study, there were no changes observed in BMI, but the group receiving the nutraceutical preparation displayed a significant reduction in endothelial-derived EVs. This reduction was greatest in the subgroup that achieved the biggest decrease in LDL cholesterol and CRP [Pirro M, 2016].

As opposed to dietary supplementation with 'healthy' food products, a study by Eguchi *et al* reported that calorie restriction to 1500kcal/day over a 12 week period in subjects with metabolic syndrome (n=14) led to decrease in BMI, fat mass and overall plasma EV concentration as well as perilipin A-expressing EVs (by Western blotting). They also confirmed an increased plasma EV concentration in obese vs lean individuals and a significant correlation of EV concentration with HOMA-IR (as I noted in chapter 3) [Eguchi A, 2016]. The authors suggested that 'stressed' adipocytes might release a higher EV load into the circulation and that EVs might thus be viewed as markers of adipose tissue stress and hypertrophy, which is linked with the risk of developing insulin resistance and T2DM [Eguchi A, 2016]. In contrast to my study, perilipin A rather than FABP4 was used as the marker of adipocyte-derived EVs. Perilipin A is a protein that is involved in coating of lipid droplets and regulation of lipolysis and is considered to be adipocyte-specific [Brasaemle DL, 2007]. However, in the lean control group evaluated by Eguchi *et al*, this adipokine was only detectable in one subject, limiting its usefulness as a sole marker to distinguish adipose tissue health between lean and obese subjects. Studies evaluating the effects of weight loss on more than one adipocyte EV marker, such as perilipin A and FABP4, are therefore needed. During the conduct of this study I attempted to use perilipin antibody as an additional adipocyte marker for the TRF immunoassay (Perilipin-1 (D1D8) XP, Rabbit mAb~9349, Cell Signalling). However, in contrast to my colleagues who used it for Western Blotting, I was only able to detect EV perilipin signals in 5 out of 21 baseline non-columned EV samples and in view of these unsatisfactory results and the fact I did not have enough EV material to evaluate anti-perilipin antibody from an alternative supplier, further measurements were abandoned.

The above studies suggest that brief dietary changes may lead to acute changes in the EV profile that could potentially be beneficial to vascular and/or overall health, but data are currently lacking on the long-term effects of such interventions. My study may therefore add particular value in view of the longer participation period (6 months). It should be noted, however, that the dietary intervention in the weight loss management programme offered to patients participating in my study focused on healthy eating in general rather than dietary supplementation with certain products or 'superfoods'. Furthermore, a limitation of my study is that it was embedded within routine clinical care, without a careful assessment of adherence to dietary change that a clinical trial might have ensured, although this offers the advantage of closer representation of the changes that might be seen in 'real life'.

Other studies have examined the effects of unhealthy dietary interventions on the circulating EV profile. This was recently reviewed by Vefaiadou *et al.* Although only a handful of studies have examined the effects of a high fat diet on EVs in healthy and diseased subjects, all have shown that consumption of high fat meals (50-100g fat daily) increased circulating EV concentration in both healthy and metabolically-compromised subjects, suggesting that EVs may mediate in part the adverse effects of a high fat meal on the endothelium [Vefaiadou K, 2012]. This is further supported by a rodent study undertaken by Heinrich *et al* which showed that high fat diet-fed obese rats had increased total as well as endothelial-, leukocyte- and platelet-derived EVs. These EVs also had the ability to activate reactive oxygen species formation, and vascular cell adhesion molecule (VCAM)-1 expression in primary rat cardiac endothelial cells [Heinrich F, 2015]. These findings support the view that dietary factors can influence EV release and that EVs may in part mediate the effects of diet on cardiometabolic health.

As previously discussed, the multidisciplinary programme offered to study participants in my project also comprised individually tailored psychological support. Although there were no differences in the health questionnaire scores post-intervention in the group that completed the programme, it should be noted that there is some interest developing into the role of EVs in psychological wellbeing. A study by Williams *et al* considered the relationship between platelet-derived EVs and depressive symptoms in patients hospitalised for the management of acute coronary syndrome (ACS). They found that those with ACS and moderate depressive symptoms had increased plasma TNF α , IL-6, and CRP as well as higher levels of platelet microparticles and higher platelet aggregation [Williams MS, 2014].

5.5.2 Differences in EV profile between healthy volunteers and morbidly obese subjects

The ability to compare the EV profile between the healthy volunteers and the morbidly obese individuals was one of my main study goals whilst performing this work. The results showing a higher total EV concentration and higher platelet- and endothelial-derived EVs in morbidly obese subjects compared to healthy volunteers supported my initial hypothesis. As I found in my chapter 3 study, there was no correlation between EV concentration or EV cellular origin markers and weight/BMI; this suggests that changes in the EV profile are likely driven by other mechanisms within adipose tissue itself rather than weight alone. Although I acknowledge that the healthy volunteers in my chapter 3 study were significantly younger than my morbidly obese population, I did not observe any correlation between age and the analysed EV markers in any of the groups, hence I do not believe that the lack of age-matching significantly affects interpretation of my data.

A number of recent studies have confirmed changes in the EV profile in subjects with obesity or obesity-driven comorbidities such as T2DM, cardiovascular disease and hypertension. This suggests that EVs are not only biomarkers but may also contribute to development of metabolic and cardiovascular complications [Lakhter AJ, 2014]. For instance, endothelial EVs were found to affect the development of arterial stiffness in patients with diabetes [Chen Y, 2012]; in my study the MO cohort had significantly higher CD144 TRF signals compared to HV. (Fig.5.11)

Interestingly, increased total circulating EVs levels have been described in the presence of obesity, irrespective of the presence of metabolic syndrome [Stepanian A, 2013].

The MO cohort in my study also had higher TRF signals from FABP4, TNF α and interferon γ . As I showed previously, these EV-expressed adipocytokines correlate with the exosomal marker CD9 and with one another, which suggests they are co-secreted by adipocytes into EVs in the exosomal range as discussed in chapter 4. Although these EV adipocytokines did not correlate with weight, I believe the significant differences in their expression between healthy and morbidly obese individuals suggest they may be markers of adipose tissue hypertrophy and inflammation.

As shown in many other studies, plasma free adipokines such as FABP4 and IL-6 were also higher in my study in the MO group.

5.5.3. Effects of methodology on EV analysis – quality vs quantity?

Given the growing need within the EV research community to streamline EV isolation in order to facilitate easier application in the clinical setting, I decided to isolate EVs using ExoSpin columns from the surplus plasma in my study. Although differential ultracentrifugation is considered the 'gold standard' isolation method, and one which is still most widely used worldwide [Gardiner C, 2016], it has its limitations. Firstly, it is very time-consuming and costly, and the results may vary depending on the equipment, rotor, speed and time used in the protocol. Some authors postulate that the high centrifugation forces used may lead to damage of some EVs and loss of material. Secondly, the question of sample purity and co-isolation of protein aggregates is often raised. Therefore, a great deal of effort is being put in to establish quicker, more reproducible methods of EV isolation such as ultrafiltration, size exclusion chromatography or precipitation methods. Hence, for my additional sub-study, I decided to use the ready-made commercial columns by Life Biosciences using a protocol that was developed previously by a colleague in a local EV research group and which was shown to deliver reproducible results [Welton J, JEV 2015]. The postulated advantage of this approach was to obtain 'purer' EV samples. My preliminary data, akin to those by Welton *et al* [Welton J, 2015], suggested that the EV-rich fraction comes before the large peak of protein-rich fractions as shown in the Methods chapter (section 2.5.2.2). The hypothesis was that by undertaking EV isolation by 2 different methods this would allow me to understand whether the changes I observed were truly within EVs or possibly due to some free protein contaminants. The additional advantage of this approach was that, hypothetically, it only required a small sample volume (1mL) in contrast to the larger volume of plasma required for ultracentrifugation (5-7mLs). Although my experiments produced reproducible results, I was finding the particle peaks using NTA as expected [Welton J, 2015] and all samples tested positive for CD63 and CD9 on the TRF immunoassay, it became apparent early in the study that EV yield was considerably compromised. As ultracentrifugation is also believed to lead to significant loss of material, I was hoping that the SEC method would allow me to recover more EV material from the limited sample volume I had. However, I faced a similar problem as in chapter 4 where I was not able to load the same amount of EVs per 96 well plate as in my previous 'standard' experiment.

I therefore decided to change the aim of this sub-study. Rather than comparing TRF signals from the same load of 'columned' vs 'non-columned' EV samples for the cellular and adipocytokine markers, I decided to reduce the loaded EV number per well to 3×10^9 / well and simply compare the baseline to follow-up EV profile within this subgroup. However, it turned out that such a low loading number led to readings below the background EV signal for some markers (values lower than wells where EVs only with no primary antibody were added) which possibly indicates that I did not have enough EV

material containing that specific marker to be able to detect it. Such markers included CD235a (erythrocyte) and CD144 (endothelial), which might be expected to show lower expression based on previous studies, and PPAR γ . In some instances, I also found undetectable readings for the other markers. Interestingly, however, I obtained TRF signals for EV IL-6 in all the subjects in my sub-study though I used a different antibody supplier as discussed above. What also can be observed from figures 5.7 and 5.14 is that irrespective of the isolation method used, TRF signals for the exosomal markers CD9 and CD63 are detectable. Furthermore, neither of the isolation methods showed any differences in the pre- and post-intervention signals which means that both methods in this study allowed reproducible EV/exosome isolation despite their pros and cons. From my experience, however, of the current methods available for EV isolation, implementation of SEC would require much more than 1ml of plasma meaning that more columns would need to be used. This would therefore not provide any significant time or cost advantage compared to the ultracentrifugation method.

The main common observation from both isolation techniques is the reduction in EV FABP4 signal, which provides some evidence for a reduction in adipocyte stress/inflammation following a structured healthy lifestyle intervention.

There was no difference in EV TNF α in the 'columned' SEC sub-cohort. In my opinion, this result could be secondary to both the very small sample size (n=8) and the low EV load available for this experiment. It is notable that the standard errors for the TRF adipokine signals in my study were wide, which limits the chances of detecting significant differences after intervention. Similar to previous chapters, I did not observe any correlation between EV-expressed and plasma free adipokines, which argues against a significant effect of free protein aggregates /contaminants as confounders.

The most striking difference between the ultracentrifugation and the SEC was the strongly significant reduction in CD41-expressing EVs 6 months' post-intervention when samples were isolated by ultracentrifugation compared to no effect on platelet-derived EVs when using the Exo Spin columns. At first glance the reduction in CD41 expression may be unexpected as subjects did not have any new anti-platelet medication introduced during the study period. However, other studies of lifestyle intervention have shown an effect on platelet- and endothelial-derived EVs, hence this result may not be entirely surprising. Importantly, all plasma samples were double-spun in the same manner at the time of sample collection, before being subjected to either ultracentrifugation or SEC, hence there should be no differences in platelet contamination between them. A very similar protocol (x2 2500g spins for 15mins) was used by Arraud *et al* who performed electron microscopy of their EV

samples. They did not find any significant platelet contamination and postulated that such an isolation method does not lead to artificial platelet activation that could lead to release of additional platelet EVs [Arraud N, 2014]. Platelet-derived EVs are reported to be between 100-250nm in diameter [Aatonen MT, 2014] so it is perhaps possible that some of the larger platelet EVs were 'lost' whilst passing through the Exospin columns since these primarily aim to extract exosomes which are small in diameter. This, coupled with the low number of samples in this study, could have contributed to the discrepancy between methods.

Study limitations

As discussed in the methods section, it was not possible to recruit the number of participants that I initially intended. There are numerous reasons for this that were outside the control of this study. The new tier 3 weight management services were just being established when this study was being designed, and the services were undergoing some readjustments when the recruitment commenced which unfortunately coincided with a 2-month period when the review of newly referred patients was limited. Secondly, not all patients attending this once-weekly clinic were eligible for the study given their numerous comorbidities and/or complex psychological issues associated with their obesity. Finally, the drop-out rate in the study/clinic attendance was 30%, which likely reflected either a lack of motivation and/or lack of rapid effects on body weight and appearance that many of the study participants were hoping for.

The outcomes achieved by the subjects I recruited and followed up therefore does not truly reflect the overall outcomes achieved by patients attending weight loss management service at UHL between 2015-2017. According to the Annual Report data the median weight loss for patients who completed 6 months at the tier 3 service was -4.4kg (1.1-30.6) and for those who completed 12 months: -7.3kg (1.3-33.1), n=48 and n=33, respectively (Data provided by Dr D Datta, Lead Physician). This is in keeping with published outcomes from a UK weight loss management centre with a similar set up (Aintree University Hospital, Liverpool) where 39% of patients were able to lose $\geq 5\%$ of initial body weight at 12 months follow up [James M, 2008]. If I were able to follow up my cohort of patients for 12 months, I would possibly observe better outcomes in terms of weight loss as well. However, this was not possible due to time restrictions.

A second limitation of my study was that the 'intervention' was not well defined but was individually tailored to suit a participant's needs. There were also no healthy food supplements/'superfoods' provided in contrast to other studies. However, a strength of this study was the very frequent (almost weekly) involvement of the local dietician teams that encouraged the patients to make healthier dietary choices which possibly may have contributed to the observed EV changes.

Lastly, I did not undertake any experiments on EV function in my study. It would be of great interest to evaluate the effect of the changes in the EV profile in functional experiments such as recently developed methods in our lab looking at leukocyte adhesion or leukocyte migration. Due to time constraints and limited EV sample I was unable to undertake such experiments. Additional anthropometric characterisation, such as analysis of body fat percentage and/or distribution, would also have been preferable, but was not undertaken in this study which was conducted in a routine clinical setting.

Study strengths

The strengths of my study lie in the standardisation of the EV isolation and characterisation protocols I adopted for this work, which allows for good reproducibility and inter-study comparison. Compared to other lifestyle studies examining changes in EVs, my study benefits from the longer duration and greater depth of characterisation with respect to EV cellular origin and adipokine expression. Finally, the cohort I recruited, in contrast to other studies, was significantly overweight with associated comorbidities, whereas most other studies recruited either healthy individuals or overweight patients with few comorbidities. To my knowledge, no other lifestyle intervention study has examined EV function in such a morbidly obese population that would otherwise qualify for bariatric surgery.

5.6 Conclusions

This study demonstrates that lifestyle intervention aimed at healthy eating and gradual weight loss can lead to a change in circulating plasma EV profile with reduction in FABP4 and TNF α expressing EVs which speculatively may originate from adipose tissue. These changes were observed before any visible improvements in body weight or traditional metabolic health markers were seen, which supports the view that EVs may represent early biomarkers of metabolic and adipose tissue health. The results of my study also confirm previous work showing differences between the EV profile of healthy subjects and morbidly obese individuals.

Finally, the application of two different EV isolation methods was evaluated; whilst the use of SEC for EV isolation may improve sample purity, it reduces EV yield and thus may have reduced value when analysis of multiple markers is required.

Chapter 6

Evaluation of *ex vivo/in vitro* human adipocyte cultures for adipocyte-derived EV characterisation

Perspective

Following the work undertaken on characterisation of plasma-derived EVs from various clinical cohorts, with a particular focus on obese subjects, I wanted to further characterise adipocyte-derived EVs specifically obtained from human adipose tissue.

This work was undertaken under an ongoing project conducted at the University Hospital of Wales which focused on characterisation of inflammatory properties of adipose tissue derived from various fat depots. The work was supported by an interdisciplinary collaboration with surgical Consultants at the University Hospital of Wales in Cardiff: Miss Rachel Hargest, Mr Michael Stechman and Mr David Scott-Coombes.

This is a small pilot study performed alongside experiments that were simultaneously being undertaken by colleagues from my research group on characterisation and function of adipocyte-derived EVs.

6.1 Introduction

EVs originating from human adipose tissue appear to be a very interesting population of EVs given the numerous roles of adipose tissue [Trayhurn P, 2001]. However, comparatively few studies have analysed their characteristics and function. To date, most data are based on rodent studies or commercial lines of adipose-derived mesenchymal stem cells (Ad-MSC) or 3T3-L1 cells, which includes our own research group's experience [Connolly K, 2015].

Human adipose tissue culture itself is very challenging, with numerous pitfalls that have to be taken into consideration such as sample purity (since adipose tissue explants contain a mixture of other cells), and slow growth and differentiation rates which makes repeat experiments quite difficult. In addition, access to the material/donors is limited as adipose tissue samples are usually harvested whilst planned medical procedures are being undertaken. Consequently, there is no option of obtaining more material from the same donor should additional experiments be required or in case of sample loss and/or contamination. Therefore, experiments involving adipocyte cultures require careful planning with respect to methodological strategy. Firstly, consideration should be given as to whether experiments should be undertaken on adipose tissue explants or preadipocytes, as the former contain many other cells such as fibroblasts, immune cells and endothelial cells [Fried SK, 2001]. On the other hand, 'organ' culture is reportedly superior when evaluating the role of adipocytes within adipose tissue and long-term changes in gene expression [Carswell KA, 2015]. Moreover, differences between various adipose tissue depots have been described with regards to their morphology, molecular characteristics and biological function [Frayn KN, 2000, Fruhbeck G, 2008]. Increased visceral adiposity is linked to the development of dyslipidaemia, insulin resistance and subsequently type 2 diabetes [Despres JP, 2006]. Compared to subcutaneous depots, visceral sites express more genes encoding proinflammatory proteins [Dussere E, 2000]. Given the emerging role of EVs as biological paracrine and endocrine messengers, characterisation of EVs derived from various human fat depots under different conditions would provide valuable insight into their role in the regulation of numerous biological processes.

6.2 Aims

Despite the challenges in working with primary adipocyte cultures, I wanted to explore the possibility of utilising human-derived visceral and subcutaneous adipose tissue samples in my EV research. This could help characterise ADEV content and function and allow comparison with the results of my experiments on plasma-derived EVs.

Secondly, I wanted to compare the concentration and content of EVs harvested from subcutaneous and visceral fat depots and finally, to establish a methodology that could be reproduced in further experiments.

6.3 Methods

6.3.1 Adipose tissue harvesting

Visceral and subcutaneous adipose tissue samples were collected from patients undergoing elective laparoscopic or open surgery at the University Hospital of Wales in Cardiff. Additionally, commercial primary human visceral preadipocytes obtained from deceased donors were used (PT-5005, Lonza, UK).

6.3.2 Ethical approval

The study at the University Hospital of Wales and Cardiff University was performed under Research Ethics Committee approval reference 06WSE03/3. Study participants provided informed written consent and were coded using a unique study number. No tissue/cells were stored, as the explants were processed the same day. The work on Lonza cells was conducted at Cardiff Metropolitan University.

6.3.3. General methods

6.3.3.1 Cell culture

In order to obtain preadipocytes from adipose tissue explants, I used established protocols developed by other colleagues working with Professor Marian Ludgate, which are described in the Methods chapter section 2.8.1 where the content of control and differentiation media and experimental timeframes are also detailed. The same culture conditions were applied to the commercial cells obtained from Lonza, UK. Differentiation was evaluated by Oil Red O staining (Methods chapter, section 2.8.6).

6.3.3.2 Adipocyte-derived EV isolation

The methodological approach to adipocyte-derived EV isolation is described in the Methods chapter (section 2.8.3). One hundred millilitres of serum-free medium were collected at day 0 and day 21 of differentiation.

6.3.3.3 Characterisation of ADEVs

The methodology for the evaluation of ADEV concentration and content is described in the Methods chapter (sections 2.8.4 and 2.8.5). FUNRich software (Functional Enrichment analysis) based on

integrated genomic and proteomic database systems was used to analyse the dot assays with respect to the biological and molecular relevance of the expressed proteins [Pathan M, 2015].

6.4 Results

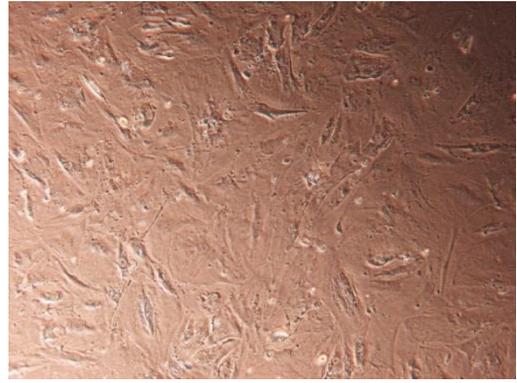
6.4.1 Subcutaneous and visceral preadipocyte culture: methodological observations

As previously discussed, I observed that the culture of human-derived visceral and subcutaneous preadipocytes was very challenging and time-consuming. Based on the work of my colleagues on 3T3 cells [Connolly K, 2015], I was aware I needed to passage my cells until I was able to seed them into 4 x T175 cm² tissue culture flasks (Cellstar, Greiner Bio-One, Germany) that would allow me to collect a total of 100 mL of serum-free medium at day 0 and 21 for EV isolation. That amount of medium was adequate to provide sufficient EV yield from 3T3 cells for further characterisation; in practice this was difficult to replicate using human cells. I faced significant problems with the deceleration of cell growth after passage 2 which made the whole experiment very time-consuming: typically, it would take around 6-8 weeks until the required confluence at passage 3 was achieved for the differentiation to commence. Given the low differentiation rates, I was also culturing the cells in the differentiation medium for 21 days rather than the standard 14 used for 3T3 cells which extended the experimental timescales further. Such a long incubation was also associated with a high rate of fungal infections, which led to the need to repeat the process from a new donor on a number of occasions and required me to start adding an antifungal agent prophylactically to the medium. I also decided to switch from using T175 cm² flasks (n=4) per sample to using x10 T75 cm² flasks hoping that this would allow the cells to reach confluence faster in order for me to obtain a total volume of 100 mL of medium for EV isolation.

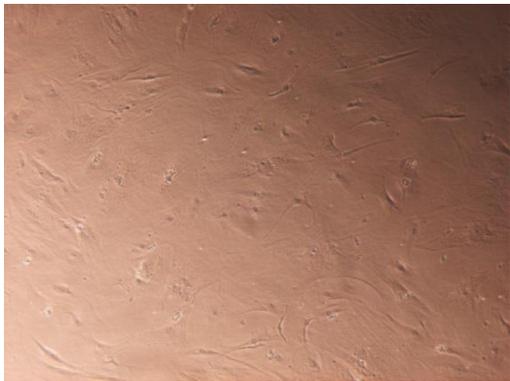
Figures 6.1. and 6.2 illustrate the morphology of the cultured visceral and subcutaneous adipocytes at different stages of their growth and differentiation.



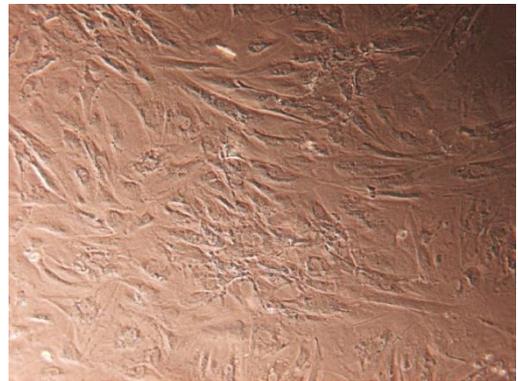
a)



c)

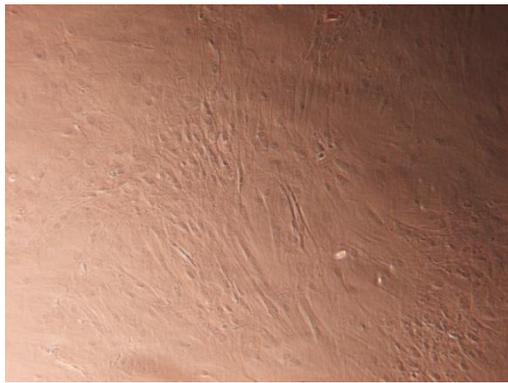


b)

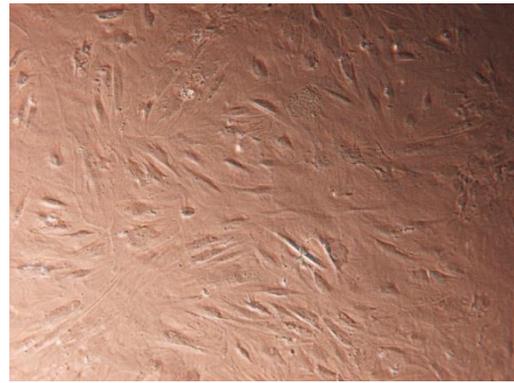


d)

Fig.6.1 Visceral preadipocyte cultures at day 0 (a,b) and day 21 (c,d) of differentiation.



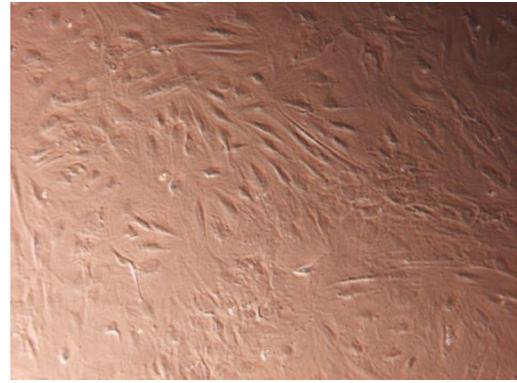
a)



c)



b)



d)

Fig. 6.2: Subcutaneous preadipocyte cultures at day 0 (a,b) and day 21 of differentiation (c,d).

Differentiation rates were not as high as those observed by our group in 3T3 experiments; however, there was an increase in signal when Oil Red O absorbance was evaluated (for this part of the experimental work cells were grown in separate 6 well plates). (Fig.6.3)

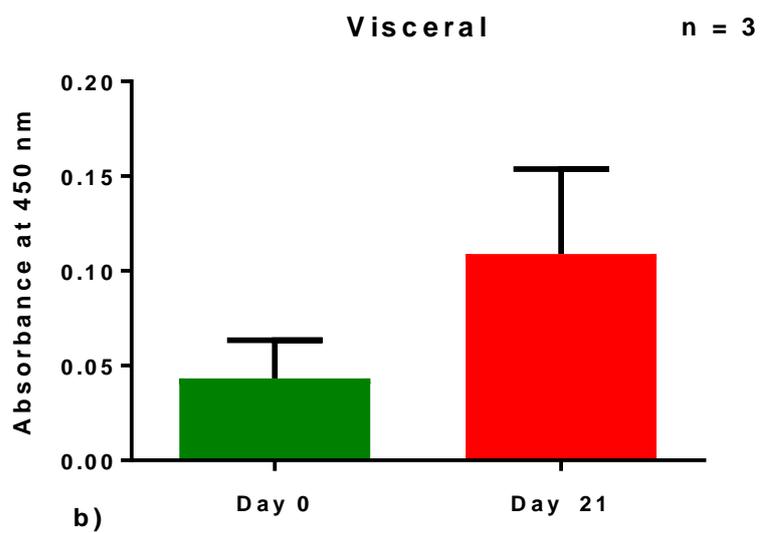
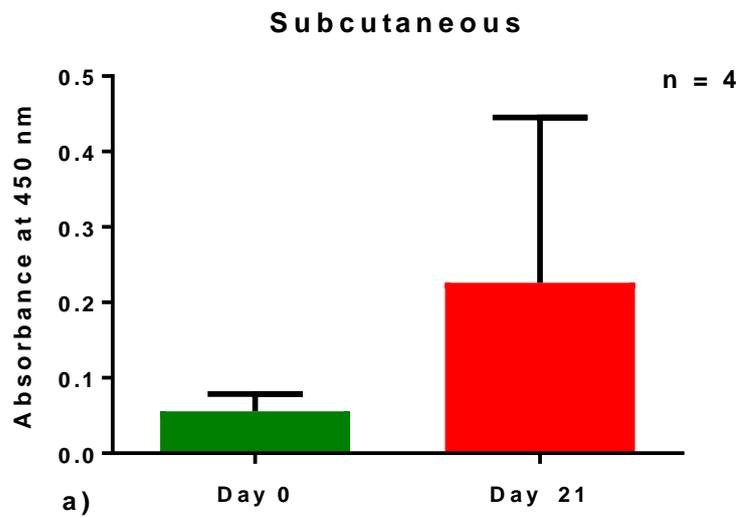
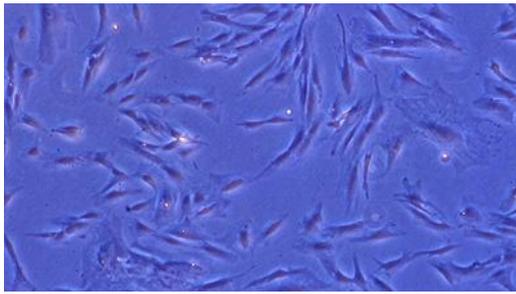


Fig.6.3: Oil Red O absorbance of subcutaneous (a) and visceral (b) adipocyte cultures measured at day 0 and day 21.

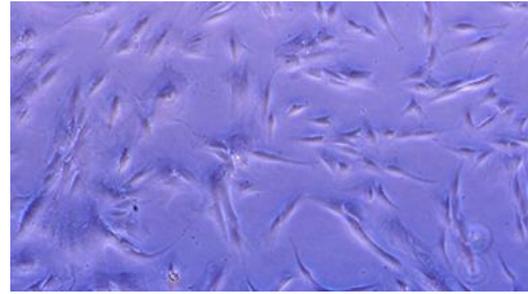
6.4.2 The culture of commercial visceral preadipocytes – methodological observations

In view of the above challenges, I sought to explore alternative methods of culturing human preadipocytes and decided to evaluate the visceral preadipocytes provided by Lonza UK which were obtained from deceased donors. I obtained cells from 3 different donors: a healthy one with no history of diabetes, a donor with a history of T2DM and a donor with a history of T2DM and heart disease. The results were initially very encouraging as following the first passage of the cells from a healthy donor, cells grew very rapidly, with further passages required at 3 days intervals as their confluence was reaching >70%. This enabled me to complete the experiment, from cell seeding through to differentiation, in less than 4 weeks. The cells from the diabetic donor grew somewhat slower but I was still able to complete the experimental process within 4 weeks. The cells from the donor with T2DM and cardiovascular disease grew very slowly, however, making the culture process time-consuming and providing a low EV yield. Unfortunately, the first experiment was difficult to replicate as those cells that were aliquoted post-passage 1 from the initial lot, and stored in liquid nitrogen, showed very slow growth once thawed and seeded again which made it challenging to replicate experiments. I contacted the supplier who stated that they should not be used post-passage 1; thus, given my study design, these cells did not appear suitable for long-term use as this would require purchase of a new vial for each replicate to ensure that the cellular biology remained stable.

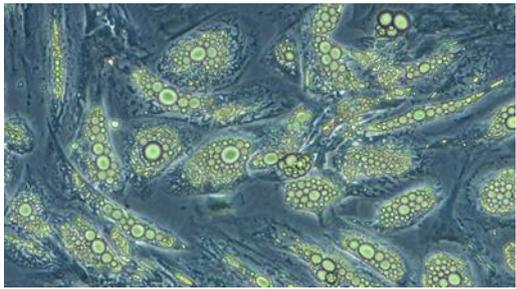
Figure 6.4 shows the Lonza cells' morphology at day 0 and day 21.



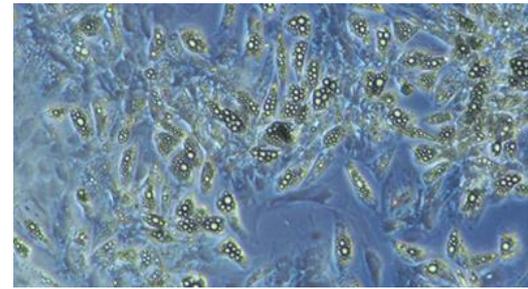
a)



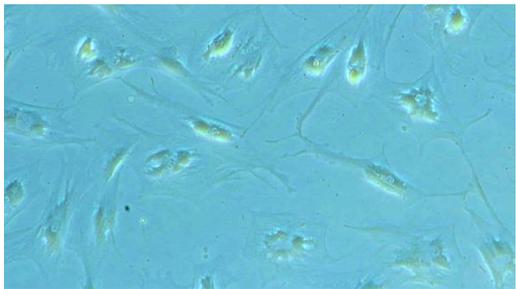
b)



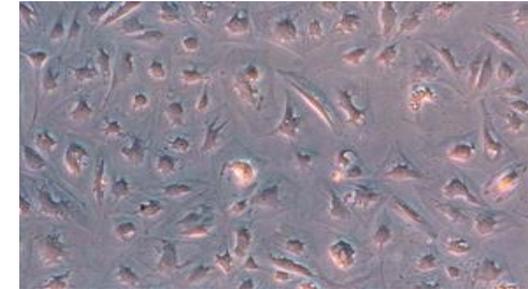
c)



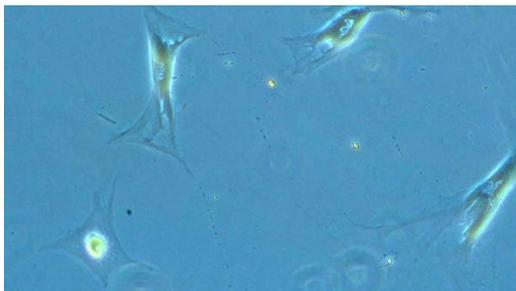
d)



e)



f)



g)



h)

Fig.6.4: a-b Visceral commercial (Lonza) preadipocytes culture from a healthy (control) donor at day 0 (a-b) and day 21 (c-d); from the diabetic donor: day 0 (e) and day 21 (f) and from the donor with diabetes and heart disease: day 0 (g), day 21 (h).

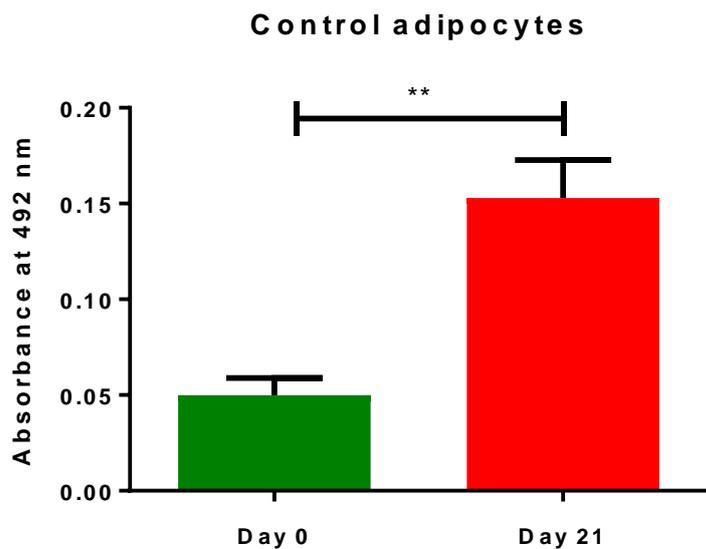
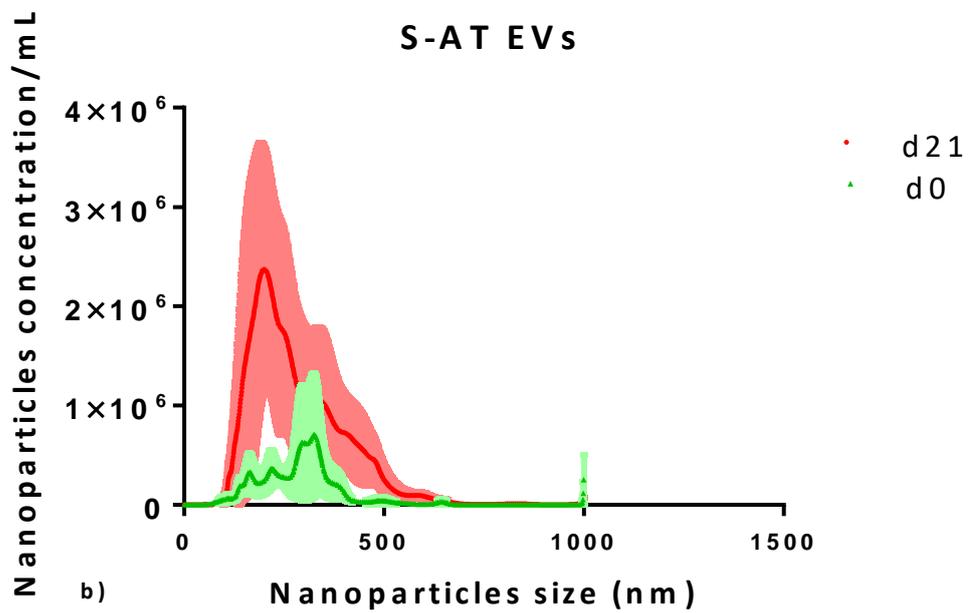
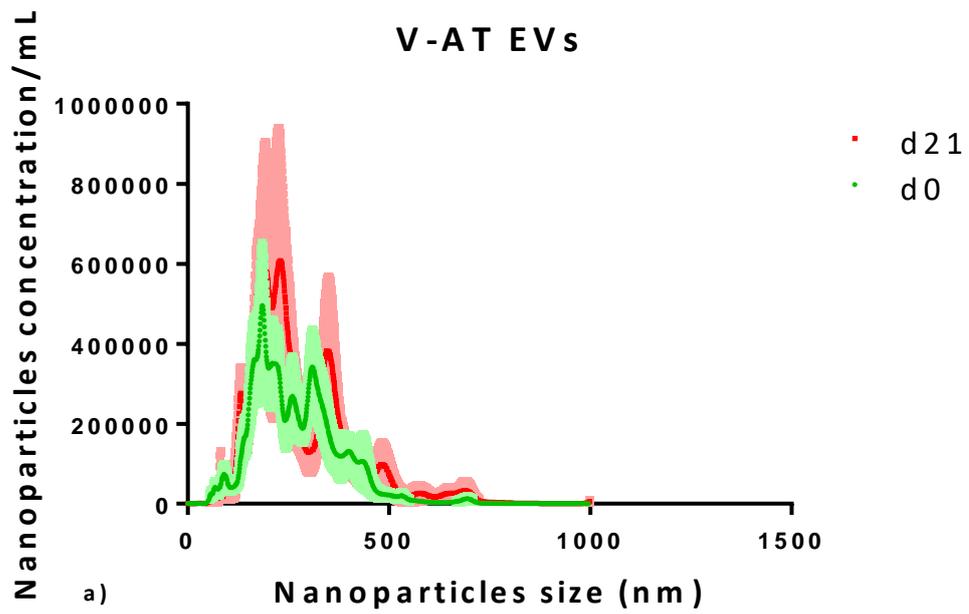


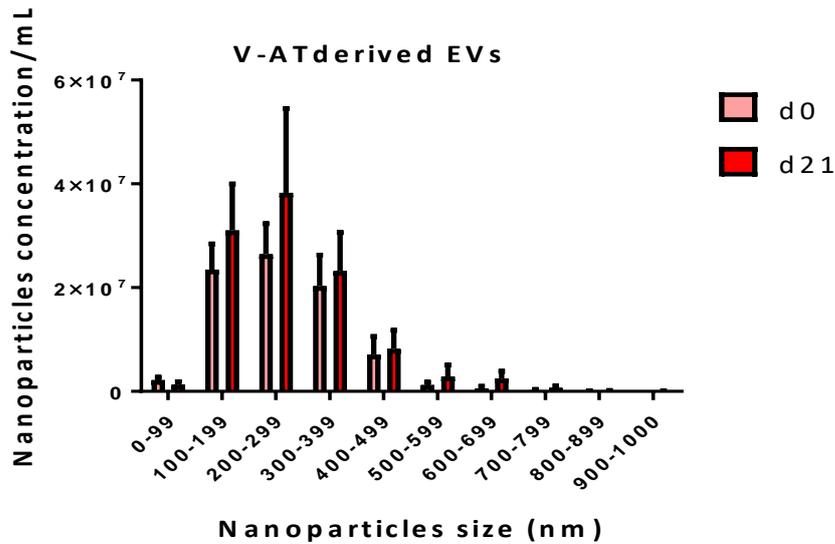
Fig.6.5: Oil Red O absorbance signals for healthy (control) adipocytes at day 0 and day 21.

6.4.3 ADEVs concentration and size

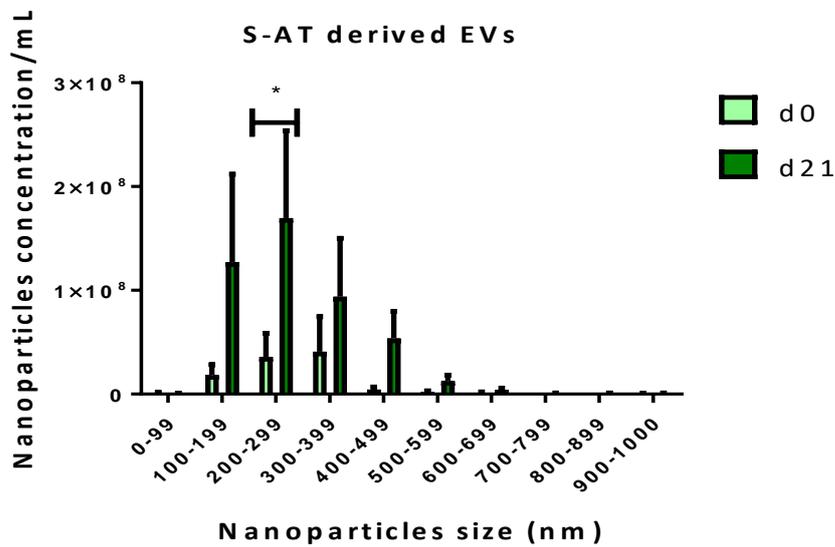
The EV concentration obtained from various donors was low and very variable and did not seem to follow any particular pattern i.e. no differences pre- vs post-differentiation.

As there was only one repetition of the Lonza cells' experiment, no statistical analysis was undertaken. The size distribution of EV is illustrated in Figure 6.6 for ADEVs from visceral and subcutaneous adipocyte tissue extracts and in Figure 6.7 for ADEVs from the commercial cell lines





c)



d)

Fig.6.6: Size distribution general (a-b) and in 100nm intervals (c-d) of EVs obtained from visceral and subcutaneous adipose tissue explants at day 0 and day 21 of differentiation (n=5). There was a significant increase in EVs measuring 200-299nm at day 21 which was observed in EVs derived from subcutaneous adipose tissue only (p<0.05). V-AT- visceral adipose tissue, S-AT- subcutaneous adipose tissue.

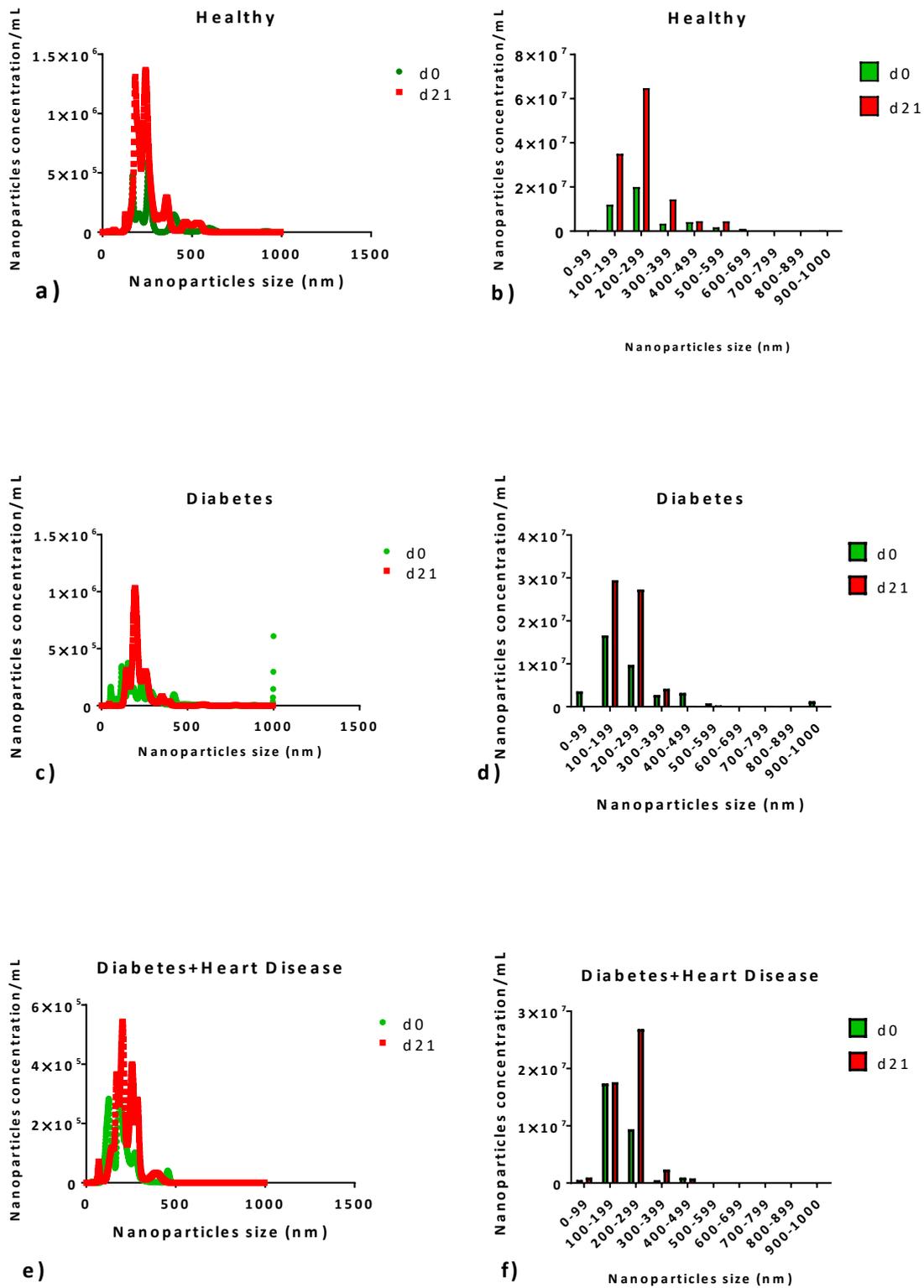
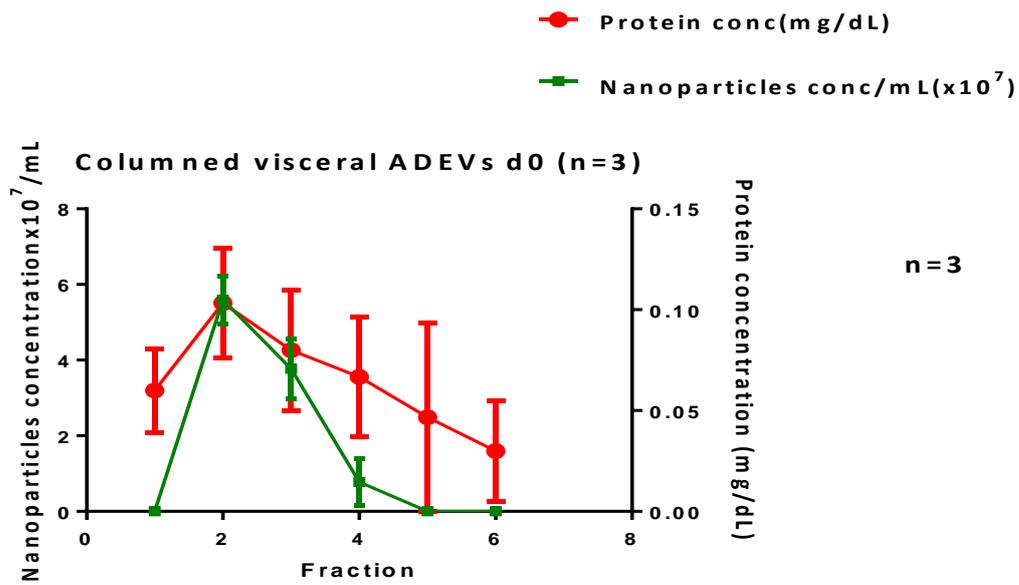


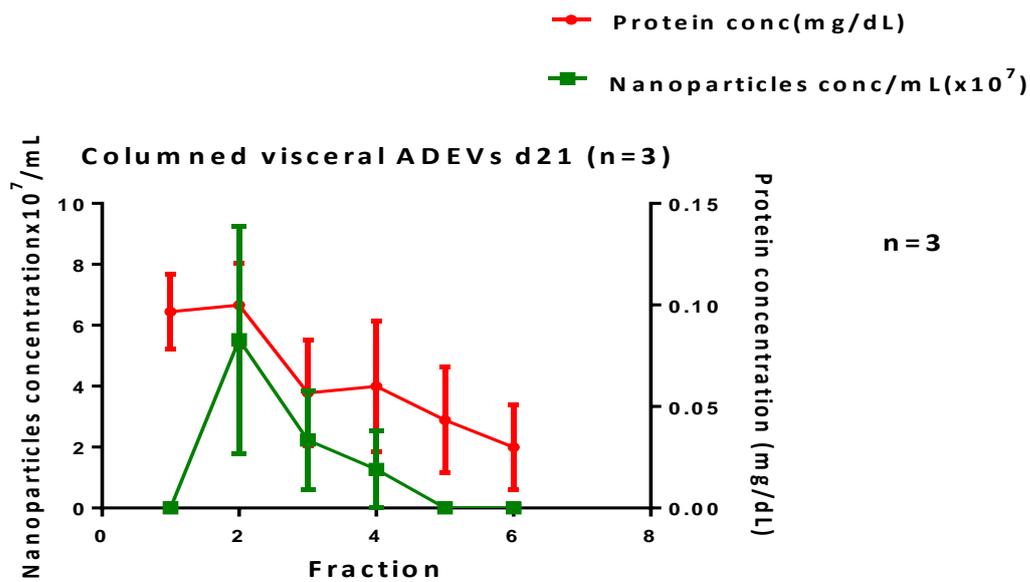
Fig.6.7: Overall size distribution (a,c,e) and in 100nm intervals (b,d,f) at day 0 and day 21 of EVs obtained from culture of commercial visceral preadipocytes from various donors: healthy(control) a-b, diabetic c-d and diabetic with heart disease e-f.

6.4.4 The use of SEC columns

Centrifugation is one of the most commonly used methods to isolate EVs in manuscripts published on ADEVs to date (Table 6.1). Given the ISEV focus on sample purity [Witwer K, 2013], I decided to employ the ExoSpin midi columns (Cell Guidance Systems UK, EXO-01) to purify my samples further in an attempt to ensure the adipokines I was detecting with the proteome profile assay truly represents what is contained on or within the EVs. However, similar to the plasma work described in chapter 5, use of these columns led to significant EV loss from samples where the initial concentration was already quite low. As mentioned previously (Methods chapter 2.8.3.2), 6 x 100 μ L fractions were collected each time and NTA revealed that the EV-rich samples were typically in fractions 2 and 3. The protein concentration was also evaluated in a sample of these fractions for validation purposes. (Fig.6.8) In contrast to the plasma EV column experiments, the nanoparticles were present in 1 to 2 fractions only, with the other fractions hardly showing any particles as detectable by Nanosight. This suggests that not much free/contaminating particles were present in the stock ADEVs in PBS solution prior to the sample being columned.



a)



b)

Fig.6.8. Analysis of protein concentration (mg/mL) by Nanodrop and nanoparticle concentration/mL by NTA of 6 fractions obtained from EXOspin columns revealed a peak of EVs in fraction 2 and 3 in (a) EVs isolated from visceral preadipocyte medium at day 0, and (b) EVs isolated from visceral adipocyte medium at day 21, (n=3).

However, I noted a reduction in the concentration of the already very low yield EVs when using the SEC method compared to EV concentration obtained by differential ultracentrifugation, perhaps because some of the EVs might have remained adherent to the column beads and were therefore lost in passage. Figure 6.9 illustrates the differences in visceral ADEV concentration from 3 different donors pre- and post-SEC column.

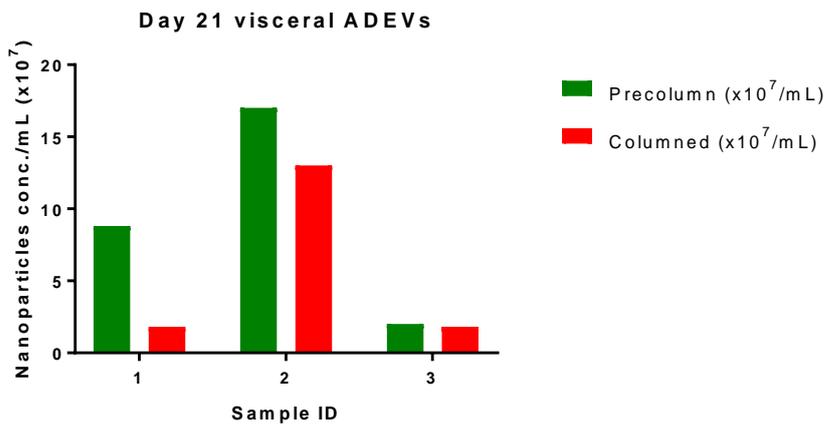
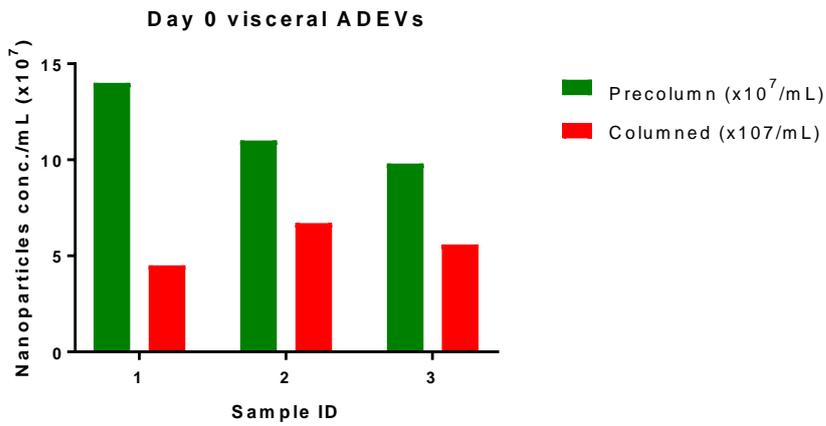


Fig. 6.9. Differences in mean EV concentration pre- and post-SEC column at day 0 and day 21 of differentiation for 3 different visceral ADEVs samples. 1-3 represent paired samples from 3 different donors. The lack of difference observed for donor 3 at d21 is likely due to the very low initial and post-column concentration which could have affected the Nanosight accuracy/detection threshold.

6.4.5 Evaluation of ADEV content

As the EV yield was very low, I was not able to use the TRF immunoassay method we developed for EV protein characterisation, as described in the Methods chapter (2.5.5.2). This led me to search for alternative ways to evaluate ADEV adipokine content, and subsequently the use of a commercial dot assay kit (Human Adipokine Proteome Profiler Array Kit (R&D Systems, UK, ARY024)). These benefit from only requiring a small amount of sample and the ability to assess multiple proteins simultaneously as described (Methods 2.8.5). However, a drawback of this method is that it is more of a screening tool; quantitation is limited, as with other membrane based methods such as Western blotting.

Figure 6.10 presents the heat map for an array of adipokines expressed in visceral and subcutaneous ADEVs obtained at day 0 and day 21 based on mean pixel density signals for a given protein from all donors (visceral (n=4), subcutaneous (n=4)). The Spearman correlation analysis revealed strong correlation between expressed markers in ADEVs from the same depot at different stages of differentiations (visceral d0 vs visceral d21 $r^2=0.73$, subcutaneous d0 vs subcutaneous day 21 $r^2=0.35$) as well as strong correlations with ADEVs from different fat depots but at the same stages of differentiation (visceral d0 vs subcutaneous d0 $r^2=0.56$ and visceral d21 vs subcutaneous d21 $r^2=0.68$, all $p<0.0001$). (Fig.6.11)

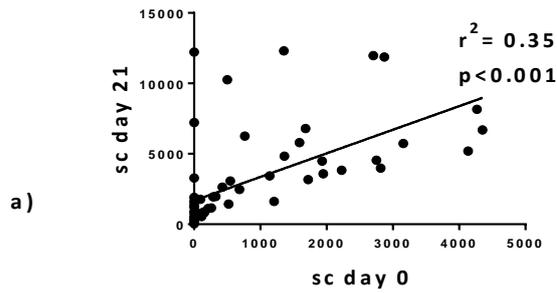
As mentioned previously, I used the FUNRich software (V3) to measure expression of adipocytokines in EV samples against their unique gene expression IDs and analyse the adipocytokine profile expressed by ADEVs with respect to the biological and molecular processes they are involved in. (Fig. 6.12-14)

	mean visc d0	mean visc d21	mean sc day 0	mean sc day 21
(A5-A6)(Adiponectin/Acrp30)	916.686	2483.395	0	34.608
(A7-A8)(Angiopoietin-1)	4106.724	8315.9125	286.4425	1939.252
(A9-A10)(Angiopoietin-2)	6666.63	10410.17	1949.44	3577.71
(A11-A12)(Angiopoietin-like 2)	7925.544	12376.94	1362.6525	4835.428
(A13-A14)(Angiopoietin-like 3)	3098.32	4321.9325	113.3	541.91
(A15-A16)(BAFF/BLYS/TNFSF13B)	3995.608	5243.0475	260.1575	1160.262
(A17-A18)(BMP-4)	4651.046	7598.4025	1139.8975	3431.668
(A19-A20)(Cathepsin D)	11440.544	14492.8675	2703.98	11967.398
(B1-B2)(Cathepsin L)	6715.584	8190.6825	0	7216.804
(B3-B4)(Cathepsin S)	8271.8	13093.2725	3157.805	5725.784
(B5-B6)(Chemerin)	1865.496	4107.3425	0	19.422
(B7-B8)(Complement Factor D)	1943.874	5718.1425	0	875.518
(B9-B10)(C-Reactive Protein/CRP)	1869.324	3457.6225	0	70.502
(B11-B12)(DPPIV/CD26)	17289.172	23196.7	2870.7725	11871.666
(B13-B14)(Endocan)	10699.702	17285.5975	4267.3	8157.658
(B15-B16)(EN-RAGE)	6606.512	11355.645	1931.7675	4483.086
(B17-B18)(Fetuin B)	2396.488	3560.07	142.745	868.8
(B19-B20)(FGF basic)	1683.584	2960.77	155.125	824.582
(B21-B22)(FGF-19)	5862.916	7842.4025	2750.11	4548.384
(B23-B24)(Fibrinogen)	10269.498	11454.2525	4134.4925	5199.07
(C1-C2)(Growth Hormone)	1521.384	4009.0925	0	101.278
(C3-C4)(HGF)	2247.296	4392.605	0	319.718
(C5-C6)(ICAM-1/CD54)	5681.502	11255.13	0	3282.94
(C7-C8)(IGFBP-2)	1292.386	3360.3675	0	36.092
(C9-C10)(IGFBP-3)	5100.628	13438.22	426.42	2636.62
(C11-C12)(IGFBP-4)	8278.396	14976.34	1682.4325	6795.642
(C13-C14)(IGFBP-6)	6427.426	12818.7225	1590.85	5794.454
(C15-C16)(IGFBP-rp1/IGFBP-7)	1131.49	3358.2175	0	1334.554
(C17-C18)(IL-1beta/IL-1F2)	3113.656	3889.715	30.1475	855.248
(C19-C20)(IL-6)	2487.77	5310.54	520.185	1432.91
(C21-C22)(CXCL8/IL-8)	8273.116	6758.8025	2226.085	3832.822
(C23-C24)(IL-10)	5284.918	7543.2825	2815.7	3976.198
(D1-D2)(IL-11)	1218.66	4176.8425	0	400.61
(D3-D4)(LAP (TGF-beta1))	1397.558	2677.5525	0	229.562
(D5-D6)(Leptin)	1809.478	4350.2525	0	1901.782
(D7-D8)(LIF)	1547.254	3481.5375	0	12.666
(D9-D10)(Lipocalin-2/NGAL)	8428.766	5977.005	212.76	1112.782
(D11-D12)(CCL2/MCP-1)	1754.962	3732.38	0	112.542
(D13-D14)(M-CSF)	3053.416	7115.855	325.965	1967.682
(D15-D16)(MIF)	8372.328	13807.1875	502.1	10258.572
(D17-D18)(Myeloperoxidase)	7401.32	2985.415	0	365.886
(D19-D20)(Nidogen-1/Entactin)	2927.818	5354.3625	545.095	3070.368
(D21-D22)(Oncostatin M (OSM))	4288.012	5492.59	1720.395	3162.696
(D23-D24)(Pappalysin-1/PAPP-A)	8153.992	11573.7	4350.3175	6693.628
(E1-E2)(PBEF/Visfatin)	2405.294	4612.54	0	1605.448
(E3-E4)(Pentraxin-3/TSG-14)	2715.178	10554.2225	0	12214.292
(E5-E6)(Pref-1/DLK-1/FA1)	3130.356	6113.6875	0	1216.872
(E7-E8)(Proprotein Convertase 9/PCSK9)	1569.202	3571.8025	0	88.368
(E9-E10)(RAGE)	2865.786	4778.1425	0	517.22
(E11-E12)(CCL5/RANTES)	1670.46	3320.4525	0	0
(E13-E14)(Resistin)	5121.848	7789.06	685.3925	2474.43
(E15-E16)(Serpin A8/AGT)	1855.15	4558.38	5.26	763.194
(E17-E18)(Serpin A12)	2843.108	3307.17	0	356.708
(E19-E20)(Serpin E1/PAI-1)	4364.484	12061.9375	764.895	6259.136
(E21-E22)(TIMP-1)	7619.158	9606.905	1354.405	12306.852
(E23-E24)(TIMP-3)	3181.908	3785.9875	1206.96	1619.648
(F5-F6)(TNF-alpha)	2811.504	8286.99	95.16	1770.114
(F7-F8)(VEGF)	876.318	2833.015	0	160.04

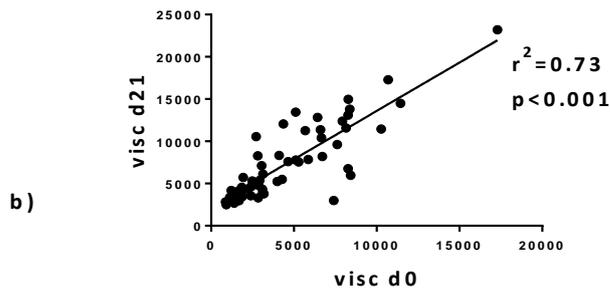
Fig.6.10: Heatmap of adipokines expression presented as mean pixel density for subcutaneous and visceral ADEVs at day 0 and d21 (n=4).

Red represents highest expressing and green lowest expressing proteins. Heat map analysis was conducted within each adipose tissue type (V-AT or S-AT).

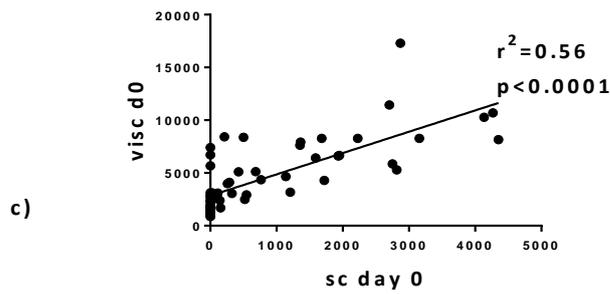
Subcutaneous EVs adipokine profile day 0 vs day 21



Visceral ADEVs adipokine profile d0 vs d21



Subcutaneous versus visceral ADEVs day 0 adipokine profile



Subcutaneous versus visceral ADEVs day 21 adipokine profile

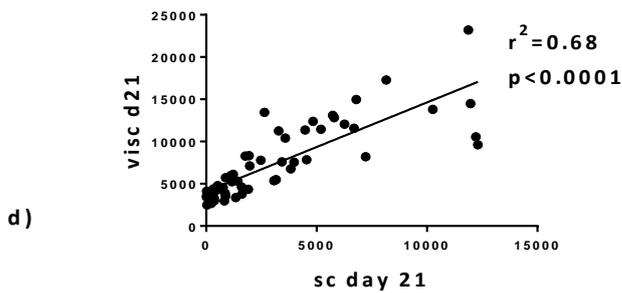


Fig.6.11: Spearman correlation analysis of 58 ADEV expressed adipokines at day 0 and day 21: a) subcutaneous ADEVs day 0 vs day 21; b) visceral ADEVs day 0 vs d21; c) subcutaneous vs visceral EVs at day 0; d) subcutaneous versus visceral EVs day 21.

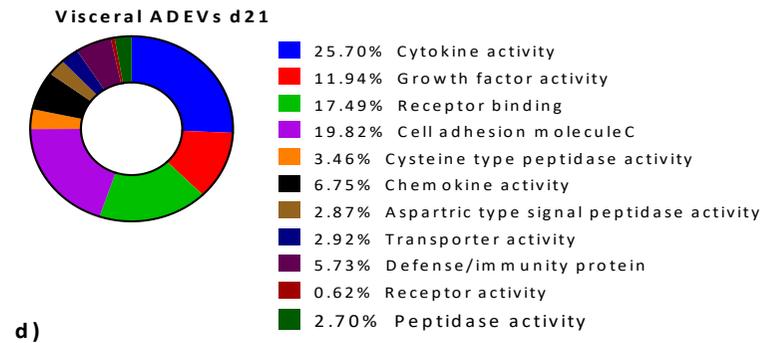
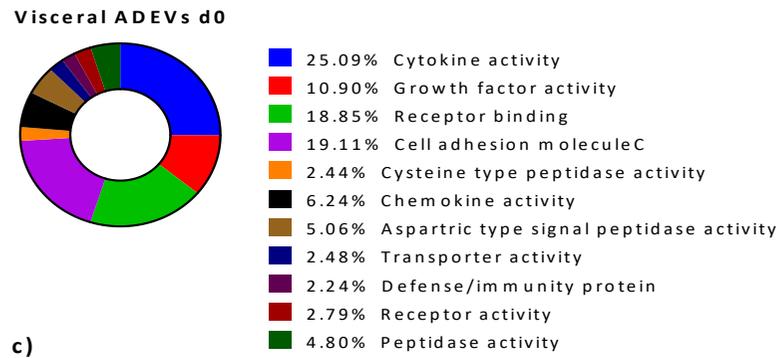
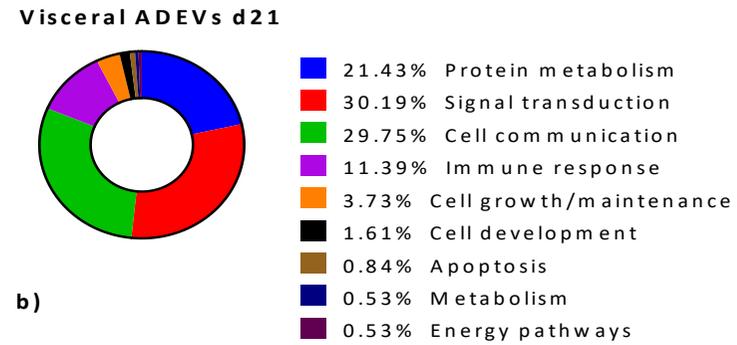
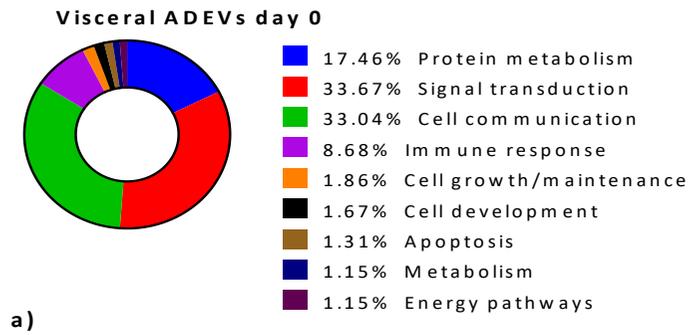


Fig.6.12: Functional enrichment analysis by FunRich of visceral ADEV-expressed proteins' involvement in various biological processes (a-b) and molecular pathways (c-d); a,c-day 0 , b,d- d21 of differentiation (n=5).

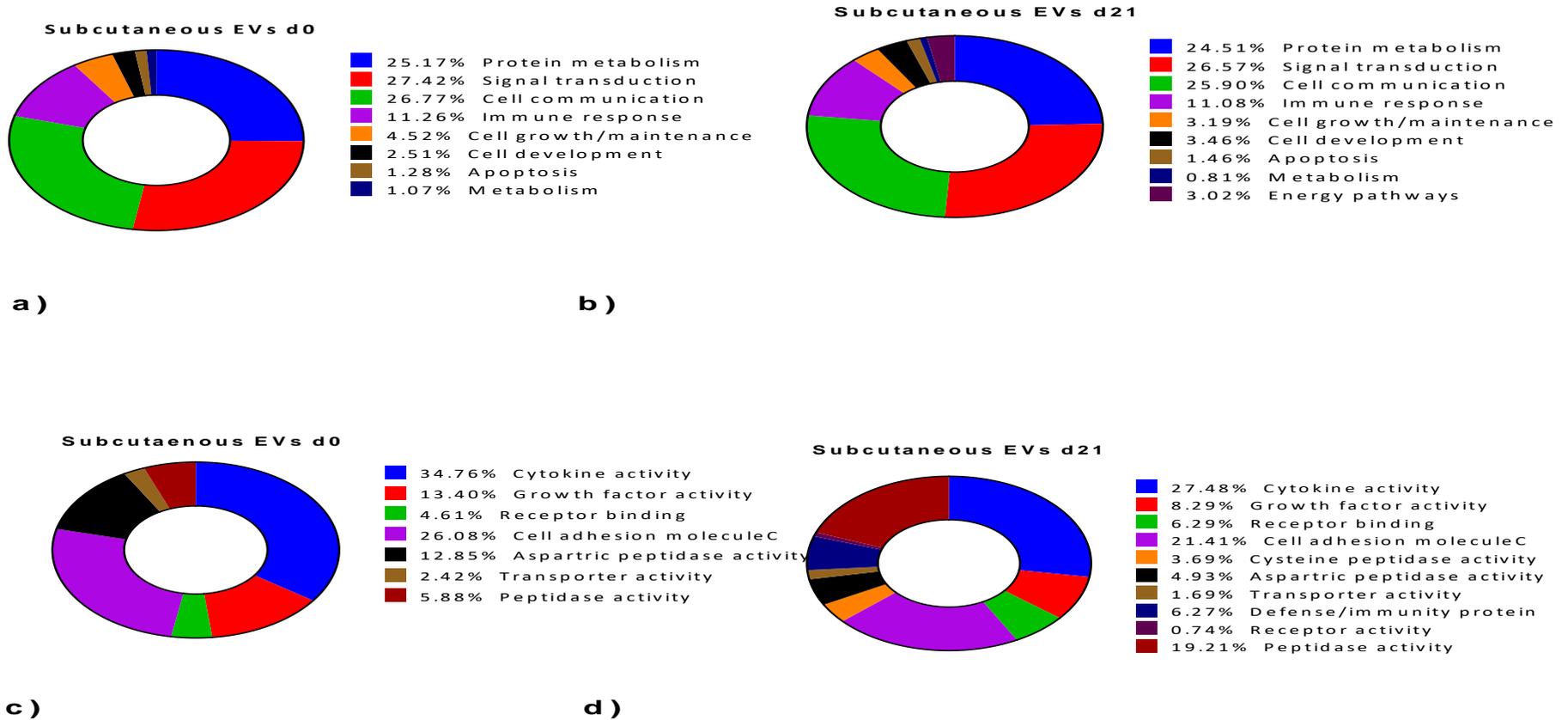
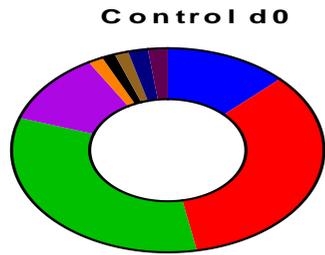
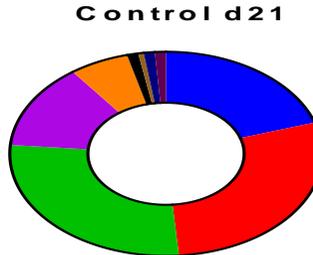


Fig.6.13: Functional enrichment analysis by FunRich of subcutaneous ADEV-expressed proteins' involvement in various biological processes (a-b) and molecular pathways (c-d); a,c-day 0 , b,d- d21 of differentiation (n=4). Interestingly, there are some differences in the distribution and number of the molecular pathways subcutaneous EVs expressed adipokines are involved in at day 0 versus day 21. This could be due the fact that many of the adipokines within S-AT EVs at day 0 were below the detection threshold/absent using the Proteome Profiler Assay.



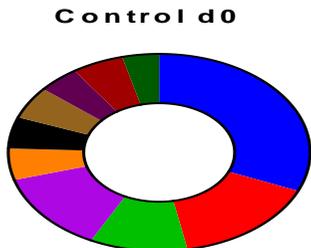
- 12.72% Protein metabolism
- 34.30% Signal transduction
- 33.12% Cell communication
- 11.41% Immune response
- 1.55% Cell growth/maintenance
- 1.37% Cell development
- 1.43% Apoptosis
- 2.05% Metabolism
- 2.05% Energy pathways

a)



- 20.03% Protein metabolism
- 28.65% Signal transduction
- 27.70% Cell communication
- 13.44% Immune response
- 6.15% Cell growth/maintenance
- 1.17% Cell development
- 0.57% Apoptosis
- 1.14% Metabolism
- 1.14% Energy pathways

b)



- 31.38% Cytokine activity
- 15.62% Growth factor activity
- 10.46% Receptor binding
- 13.04% Cell adhesion moleculeC
- 5.15% Cysteine peptidase activity
- 5.15% Chemokine activity
- 5.31% Aspartric peptidase activity
- 4.43% Defense/immunity protein
- 5.52% Receptor activity
- 3.94% peptidase activity

Total=65.96

c)



- 26.31% Cytokine activity
- 13.31% Growth factor activity
- 9.88% Receptor binding
- 13.91% Cell adhesion moleculeC
- 8.02% Cysteine peptidase activity
- 0.56% Chemokine activity
- 3.18% Aspartric peptidase activity
- 6.45% Transporter activity
- 11.05% Defense/immunity protein
- 1.38% Receptor activity
- 5.97% peptidase activity

d)

Fig.6.14: Functional enrichment analysis by FunRich of Lonza control non-diabetic ADEV-expressed proteins' involvement in various biological processes (a-b) and molecular pathways (c-d); a,c- day 0 ; b,d- d21 of differentiation (n=1).

6.5 Discussion

6.5.1. Characteristics and function of ADEVs

Most manuscripts evaluating ADEVs are based on 3T3 cells, Ad-Msc or rodent models and the availability of studies on adipose tissue derived from living human donors is still limited. As shown above, the culture of primary adipocytes from human donors and harvesting of the sufficient amount of EVs required for further analysis is very challenging and time-consuming. However, given that the mechanisms regulating adipose tissue homeostasis and dysfunction are different between humans and rodents [Muniyappa R, 2009], further information about the characteristics and function of human ADEVs is needed. The studies available to date already provide some insight into the potentially important pluripotent function of this EV population which needs to be further explored.

Han *et al* recently published a study in nude mouse models, reporting that exosomes derived from hypoxia-preconditioned mesenchymal stem cells promote neovascularisation and fat graft survival [Han YD, 2018]. Furthermore, Mleczko *et al* evaluated the effects of EVs derived from 3T3 cells, Simpson-Golabi-Behmel Syndrome (SGBS) cells as well as plasma-derived EVs from lean and obese individuals on insulin-mediated glucose uptake. They found that adipocytes exposed to EVs derived from hypoxic adipocytes show a 25% decrease in insulin-stimulated glucose uptake [Mleczko J, 2018]. A dose-dependent decline in AKT S473 phosphorylation was also observed in those cells compared to those that were left untreated or exposed to ADEVs from control/normoxic adipocytes. The effects of plasma-derived EVs from healthy lean and obese donors on the SGBS human adipocyte cell line was also examined and revealed that insulin-stimulated glucose uptake was reduced in adipocytes treated with EVs from obese women [Mleczko J, 2018]. Interestingly, the study did not report any differences in plasma-derived EV concentration between healthy lean and obese subjects, which is in keeping with my findings from chapter 3 (3.4.3.1).

The choice of SGBS cells as opposed to human adipose tissue explants allowed replicate experiments to be performed without the problems of donor-to-donor variability. SGBS is an X-linked condition associated with pre- and postnatal macrosomia, characteristic facial features and numerous congenital malformations [Gollabi, 2011]. The SGBS cell line originated from an adipose tissue specimen of one SGBS patient and has proven very useful in studying adipocyte biology such as differentiation, apoptosis, lipolysis, glucose uptake and regulation of adipokine expression [Fischer-Posovszky P, 2008]. Interestingly, these unique cells which are neither transformed nor immortalised

can proliferate for up to 50 generations, retaining their adipogenic differentiation potential (>90% differentiation rate in 14 days) and, in fact, differentiating quite easily in PPAR γ agonist supplemented and serum/albumin depleted medium. They resemble human subcutaneous adipocytes with regard to their function and gene expression pattern [Wabitsch M, 2001] and can be grown in 96 or 12 well plates, making them an attractive source to study ADEV biology. However, a limitation is they do not represent a genetically normal healthy adipose tissue.

Only a small handful of ADEV studies have been undertaken using adipose tissue derived from human donors. At the time of writing, two important manuscripts have been published by Krankendonk *et al* recently. The authors used ADEVs from adipose tissue explants and SGBS preadipocytes to stimulate primary monocyte differentiation into human adipose tissue macrophages (ATM). Adiponectin positive ADEVs and those originating from visceral rather than subcutaneous fat depot were found to have particularly strong effects on monocyte to macrophage transformation [Krankendonk ME, 2014]. In addition to differential centrifugation, sucrose gradient ultracentrifugation was used in this study to generate EV-containing fractions, as confirmed by specific EV markers (CD9 and CD63), and adipocyte proteins such as adiponectin and FABP4. Further separation of these EV populations by flow cytometry revealed that ADEVs were enriched in adiponectin. Moreover, they had the ability to induce higher mRNA expression of IL6, TNF α and MCP-1 in the adipose tissue macrophages (ATM). They also showed that ATMs pre-treated with EVs from subcutaneous and visceral fat depots could inhibit insulin signalling in adipocytes (HSBG cells) [Krankendonk ME, 2014].

This study also used a multiplex immunoassay to demonstrate that ADEVs contain a mixture of various adipokines. Interestingly, there was no correlation between the number of subcutaneous and visceral ADEVs and BMI ($r=0.01$ and $r=0.16$, respectively, $p=ns$) which is similar to my observations on the plasma-derived EVs expressing FABP4 which did not correlate with BMI/waist circumference as opposed to 'free' plasma concentration of that adipokine (Chapter 3, section 3.5.3.5).

Another recent important study by this group described the effects of EVs from human adipose tissue on insulin signalling in liver and muscle cells in order to evaluate if EVs contribute to the development of insulin resistance [Krankendonk ME, 2014]. *Ex vivo* explants of subcutaneous and visceral fat tissue were used to stimulate *in vitro* hepatocyte and myocyte cultures and insulin-induced Akt-phosphorylation, expression of gluconeogenic genes (Glucose6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK)) was also assessed. As before, ADEVs were measured by flow cytometry and their adipokine content was established by a multiplex assay. It was

demonstrated that ADEVs inhibited insulin-induced Akt phosphorylation in hepatocytes but the effect on myocytes was inconclusive, since in general there was significant individual variation in the measurements reported [Krankendonk ME, 2014]. Hepatic Akt phosphorylation also correlated negatively with G6P-expression, and omental ADEVs had higher IL-6, MCP-1 and MIF (macrophage migration inhibitory factor) concentration. The authors hypothesised that it is the adipokine content of ADEVs that affects either positively or negatively their effect on the development of insulin resistance, with a potential role of MIF in this process [Krankendonk ME, 2014].

I observed interesting differences in the methodological approach in these manuscripts compared to my work. The authors incubated the entire adipose tissue explant in medium and collected the supernatant after 24 hours, centrifuged this at 500g and stored the sample for EV isolation. EVs isolated by differential centrifugation were later used 'fresh' for the functional experiments. The use of the whole tissue explant rather than, as in my project, collagenase digestion followed by preadipocyte culture in flasks, appears to enable much faster EV collection. On the other hand, one may argue that the EVs present in such a supernatant are not purely obtained from adipocytes but rather a mixture of other cells such as macrophages, red cells or endothelial cells.

Further functional studies on human ADEVs were published by Koeck *et al* and showed that fluorescently labelled exosomes derived from visceral adipose tissue integrate into cultured hepatic cell lines and deposit in the perinuclear area. This induces dysregulation of the TGF- β pathway which could indicate that ADEVs play a role in the development of NAFLD (non-alcoholic fatty liver disease), a well-recognised complication of obesity and T2DM [Koeck E, 2014].

Park *et al* isolated visceral ADEVs (by ExoQuick-TC, System Biosciences) and incubated them with mature myotubes obtained from lean and obese individuals. ADEVs suppressed Akt phosphorylation in the myocyte culture but had no effect on insulin-stimulated glycogen synthesis and glucose oxidation [Park S, 2016].

Finally, Ferrante *et al* isolated ADEVs from human subcutaneous and visceral adipose tissue samples from lean and obese adolescents and profiled exosomal miRNA. They used small pieces of adipose tissue explants which were cultured in 12 well plates and FBS- free medium which was then harvested, centrifuged to remove cells and debris and finally EVs were isolated by a commercial precipitation assay. They found 55 mature miRNAs differentially expressed between lean and obese subjects, with TGF- β and Wnt/ β -catenin as the main pathways targeted by those miRNAs [Ferrante SC, 2015]. TGF- β and Wnt/ β -catenin are involved in the development of chronic systemic inflammation and fibrotic disease [Henderson WR, 2010, Akhmetshina A, 2012]. Obese visceral ADEVs were shown to be taken up by lung epithelial cell lines (A459) and to affect, after 24 hours

incubation, the recipient cells' gene expression of the activin receptor type-2B (ACVR2B), which is involved in TGF β signalling and was downregulated in the presence of obese ADEVs [Ferrante SC, 2015]. The authors used a flow cytometry bead-based technique, which used CD63 as an exosomal marker, and demonstrated that the visceral adipose tissue explants mainly shed EVs of adipose rather than macrophage origin, since both lean and obese ADEVs were positive for FABP4 with very low signals for the macrophage marker CD14, and no signal for the endothelial marker CD31.

Further interesting insights into the role of ADEVs come from studies on various cell lines and are summarised in Table 6.

Source	Isolation technique	Results	Reference
Ad-MSC	1	EVs had inhibitory effect on T cells differentiation and activation and reduced proliferation and IFN- γ release	Blazquez <i>et al</i>
Ad-MSC	5	Treatment with exosomes in graft-versus-host disease patients leads to symptoms reduction	Ludwig <i>et al</i>
Rat adipocytes(primary)	2	509 proteins identified within ADEVs with differential expression of 200 proteins between obese diabetic and non-diabetic rats	Lee <i>et al</i>
Ad-MSC	1	Comparison between MSC and EVs showed enrichment of 4 miRNAs, 255 mRNAs, and 277 proteins enriched in EVs	Eirin <i>et al</i>
Ad-MSC	1	Exosomes promote migration and upregulation of cancer-related signalling pathways in human breast adenocarcinoma cell line (MCF-7)	Lin <i>et al</i>
3T3-L1 cells	1	Higher EV concentration pre-adipogenesis with differences in EV protein expression pre- and post-adipogenesis	Connolly <i>et al</i>
Mice visceral adipose tissue	3	Visceral ADEVs from obese mice induce differentiation of monocytes to macrophages and development of insulin resistance in lean mice	Deng <i>et al</i>
Ad-MSC	1	EVs promote migration and upregulation of cancer-related signalling pathways in human breast adenocarcinoma cell line (MCF7)	Lin <i>et al</i>
3T3-L1	4	EVs reduced the aggregation of mutant Huntington protein (mHtt), reduced mitochondrial dysfunction and prolonged cells survival	Lee <i>et al</i>
3T3-L1, primary rat adipocytes; plasma	1	Perilipin A is enriched in ADEVs, especially from obese donors and its expression reduces with low calorie diet intervention	Eguchi <i>et al</i>
3T3-F442A (preadipocytes cell line); mice adipose tissue	3	EVs promote tumor cells migration through fatty acid oxidation	Lazar <i>et al</i>
Ad-MSC	2	EVs reduced the neuronal cells apoptosis and increased remyelination and activation of neuroglial precursors	Farinazzo <i>et al</i>
Ad-stromal cells	4	EVs protected motor neuron-like cells (NSC-34) from oxidative damage and improved their survival	Bonafede <i>et al</i>
Ad-MSC	4	Exosomal miR-122 improved the sensitivity to chemotherapeutic agents in hepatocellular carcinoma cells	Lou <i>et al</i>
3T3-L1	2	The hypoxic ADEVs had different expression and increased secretion of proteins compared to normal ADEVs	Sano <i>et al</i>

*Isolation methods: 1- centrifugation, 2- centrifugation and filtration, 3- Centrifugation and density gradient, 4- Commercial kit, 5- not specified

Tab.6.1: Summary of manuscripts on ADEVs in murine and rodent models to date in chronological order (adapted from Jayabalan N, Front Endocrinol (Lausanne) 2017; 8: 239).

6.5.2 Methodological pitfalls, limitations and future considerations

Although my study did not provide lots of data in terms of detailed EV characterisation, despite multiple methodological difficulties, it equipped me and my research group with a useful insight into practical considerations that can be applied in further studies on ADEVs. Regrettably, because my main research focus was on the plasma-derived EVs, I did not have the opportunity to further explore alternative methodological ideas and the functional use of EVs derived from human adipose tissue nor the miRNA content of those EVs. I believe that efforts at optimising the harvesting of EVs from human adipose tissue samples derived from elective surgery is worth doing, as this provides the phenotypically closest model of human ADEVs. Although rodent and cell line experiments may seem easier and quicker to conduct, conclusions from these studies cannot be directly translated to human biology. Therefore, the use of adipose tissue explants from subcutaneous and visceral fat depots should continue in our group's further experiments but in hindsight I would consider using whole fat explants as described by Krankendonk *et al*, rather than the labour-, time- and resource-intensive method of preadipocyte culture in T25 cm² and T75 cm² flasks. The authors showed that EVs that are not adipocyte-derived from these adipose explants are in the minority [Krankendonk ME, 2014]. Having access to the post-operative material from elective surgery and being able to collect the EV-containing medium for experiments within 24 hours of obtaining the samples, would make it easier and faster to run various functional experiments and the EV yield could perhaps be higher. This might then potentially enable us to re-explore the use of SEC columns as the problem with sample/EV loss may not be so limiting then. The issue of sample purity could also be further addressed by evaluating the samples by magnetic bead experiments aiming to pull out EVs expressing antigens such as FABP4 and CD9/CD63, as used in recent studies[Ferrante SC, 2015]. A similar protocol is currently being developed by my colleagues: Dr K Connolly and Miss D Mathew [Connolly KD, 2018].

Even if contamination by other EV populations from adipose tissue explants was observed, this might nevertheless be interesting, especially in comparing ADEV differences between subcutaneous and visceral depots or even various types of visceral depot such as omental, perinephric or perihepatic fat. The methodology could also perhaps be used to perform miRNA analyses. This is lacking in my work due to an inability to obtain enough biological material, but has become a very important area within the field of EV research. Moreover, the explants could also then be collagenase-digested and grown to obtain preadipocyte samples for corresponding protein and mRNA analysis from the preadipocyte cells themselves.

As discussed, the work performed on Lonza preadipocytes appeared encouraging at first but given the slow and unpredictable growth and differentiation rate of cells past P1 and the fact the cells originate from elderly deceased donors, these drawbacks would limit their value in future, larger scale experiments. However, the use of the SGBS cell line as described by other authors [Krankendonk ME, 2014, Mleczko J, 2018] appears to be an interesting and time-saving alternative in functional EV studies.

On the positive side, my work has enabled us to establish a reproducible proteome profiler protocol which should prove useful for analysis of EV adipokine content in future studies and will enable inter-study comparison of results for our group. Somewhat surprisingly, I observed that there were striking similarities in the adipokine profiles expressed within EVs derived from subcutaneous and visceral fat depots and also between day 0 and day 21, as shown by the heat map and the Spearman correlation analysis (Fig. 6.10 and 6.11) and the functional enrichment analysis looking into the biological processes and the molecular pathways these adipokines are involved in (Fig.6.12-6.14). The similarities in profiles between day 0 and day 21 could, in my opinion, be explained by slow growth and differentiation rates of the cells I harvested from living donors. (Fig.6.1-6.3). The only subset of EVs that appear to show differences in their adipokine profiles at day 0 vs day 21 were S-AT derived EVs, although as can be seen from the heat map many of the adipokines were below the detection threshold of the Proteome Profiler Assay which in itself is more of a screening rather than quantitative tool. Therefore, as one may expect, these could be true differences, but no firm conclusions can be made based on the pixel density of the spots alone.

It was interesting to observe however, that overall signals from V-AT derived EVs appeared to be higher and most of the adipokines were detectable within the V-AT EVs using this assay as opposed to the S-AT EVs. Despite that, as mentioned above, there was strong correlation between the adipokine profiles of EVs from those 2 adipose tissue types. As both types of adipose tissue were cultured in the same media and conditions, it would perhaps be interesting to compare S-AT and V-AT derived EVs harvested from cells exposed to hypoxia/TNF α to mimic the local milieu changes occurring in adipose tissue in obesity. Concomitant data on adipokine profiles obtained from corresponding cell lysates would add further depth to this data set and I am hoping this will be pursued by my colleagues who continue the work on characterisation and function of ADEVs.

Finally, the use of NTA for evaluation of ADEV size distribution I applied in this study also enables direct comparison with other research groups since NTA is a well standardised and broadly used methodology in EV research.

6.6 Conclusions

The evaluation of ADEVs and their potential use in functional studies remain an important area to be addressed in future studies by our group. However, development in the methodology for both the adipocyte culture as well as EV harvesting is required to enable a quicker pace of such experiments, better reproducibility and decreased risk of EV material loss during the isolation processes. The methodology used in this chapter turned out not to be suitable for large scale experiments but alternative methods could be explored based on the more recent literature. However, the proteome profiler assay appears to be promising as a screening tool for ADEV protein content. In hindsight, I do not think that the use of SEC columns added much in terms of sample purity, as I harvested EV from FCS-free medium and was losing lots of EVs from an already low starting concentration. This may have affected my final results on the dot assay.

Chapter 7

Conclusions and future directions

7.1 Relevance of research undertaken

In the last 2 decades there has been a tremendous rise in interest in the biology of extracellular vesicles, which is reflected both by the number of scientific publications on the subject (>75000 to date) [PubMed, 2018] and the establishment of the International Society of Extracellular Vesicles in 2011. It is no longer a matter of debate that EVs are carriers of important biological information between cells, and EVs have thus been proposed as potential biomarkers of many pathological processes. However, the exact mechanisms controlling their secretion and content are still not clear and more research is needed to characterise their role in physiological conditions. Their potential use in clinical practice could be applied in various ways from disease and/or treatment response biomarkers to drug delivery vectors, but progress is currently hindered by significant variation in isolation techniques, difficulties in separating/selectively isolating EV populations of interest, and the fact that current methods are too expensive and time-consuming to be used routinely in translational medicine.

In view of the current obesity pandemic and the recognition of adipose tissue as an active endocrine organ, EV research has also begun to examine the role and characteristics of EVs in obesity and their potential contribution to obesity-driven comorbidities.

My study therefore sought to explore how obesity affects the circulating EV profile and how they change in response to treatment. Secondly, current EV isolation and characterisation techniques were applied and reviewed with regard to their future applicability.

7.2 Contribution of my findings to the field of EV research

Although the work I performed is largely descriptive in nature, given the EV field is a novel area in biomedical sciences, I believe my work contributed significantly and provides new knowledge on plasma EV characteristics.

Firstly, I have shown that the circulating plasma EV concentration, cellular origin and adipokine content in metabolically healthy volunteers is unaffected by their BMI and waist circumference. Similar observations were recently reported by other researchers [Mleczko J, 2018, Krankendonk ME, 2014, Zhang X, 2014]. However, I observed that the EV profile in healthy men appears to be more pro-inflammatory compared to healthy women, which is also accompanied by lower plasma 'free' adiponectin concentration in male subjects and higher plasma 'free' IL6. Intriguingly, these gender differences in EV profile were not observed in subjects who developed comorbidities secondary to obesity such as type 2 diabetes mellitus. Many previous studies evaluated EV characteristics in cohorts with established metabolic syndrome or obesity-associated comorbidities

[Lakhter AJ, 2015, Krankendonk ME, 2014]. There are not many studies focusing on EV characterisation in the healthy state, although it is believed that EVs play an important role in cellular cross-talk and maintaining physiological homeostasis in health as well [Samuelson I, 2018]. If EVs are to be used in future as disease biomarkers, not only is a more standardised and quicker method for their isolation and characterisation needed but also a normal 'references range' of EV characteristics will need to be defined.

In my study, the TRF signal from EV-expressed adipokines did not correlate with the free plasma concentration evaluated by commercial ELISA assays, which suggests that mechanisms involved in adipokine packaging into EVs differ from those regulating secretion of 'free' adipokines into the circulation. This is further supported by my observation that EV adipokines such as FABP4, adiponectin, TNF α and interferon- γ correlated with the expression of the exosomal marker CD9, suggesting this specific subpopulation of EVs is the main EV carrier of these molecules and might therefore be of greater interest with respect to future potential study in clinical practice.

Although no EV profile differences were demonstrated between 'healthy lean' and 'healthy overweight and obese' individuals, I observed that "free" plasma FABP4 concentration differs significantly between these subgroups before differences in traditional metabolic health markers, such as HOMA-IR, are observed. Plasma FABP4 also correlated very strongly with BMI in all the cohorts I evaluated. This adds further to a growing body of evidence that plasma FABP4 is an adipokine which is likely to emerge as an important and sensitive marker of cardiometabolic health [Hotamisligil GS, 2015, Furuhashi M, 2015]. Given the ease of its measurement it could be easily incorporated into various cardiometabolic risk stratification models [von Eynatten M, 2012, Xu A, 2007].

Circulating adiponectin, which has emerged as an important regulator of various metabolic processes [Hotta K, 2000, Arita Y, 1999, Hivert MF, 2008], appears to be involved in an interplay with plasma EV dynamics since I observed a strong negative correlation between plasma adiponectin and plasma EV concentration, as well as with endothelial- and platelet-derived EVs, FABP4-, IL6 and interferon- γ expressing EVs. I cannot easily explain this result but speculate that, since adiponectin is perceived as the 'guardian' of metabolic homeostasis, there might be some mechanisms by which this adipokine regulates EV secretion and/or content that are yet to be elucidated (as discussed in chapter 3).

There are not many studies evaluating the changes in circulating plasma EVs in response to surgical or conservative/lifestyle management of morbid obesity. The work I conducted shows that bariatric surgery is associated with a transient rise in plasma- and EV-associated FABP4, likely reflecting

increased lipolysis and alterations in adipose tissue homeostasis, and that dietary and lifestyle changes affect EV profile in severely obese subjects, with lower signals observed from platelet-derived EVs as well as FABP4- and TNF α -expressing EVs before significant weight loss is achieved. These results suggest that plasma EVs appear to be very plastic and dynamic in response to both invasive therapies and more conservative approaches to weight loss such as diet. This was apparent in recent studies examining the effects of diet on circulating EVs. [Zhang X, 2014, Eithan E, 2017, Horn P, 2015] Intriguingly, the changes in the EV concentration and profile were not as significant post-bariatric surgery as I initially predicted, despite significant weight loss achieved by all participants, whilst changes in EVs were observed prior to any weight loss in the conservative management group. On the one hand this could be attributed to the small sample size in the study, or the fact that the plasma sample for EV isolation in the bariatric surgery cohort was stored for significantly longer than in the other cohorts prior to ultracentrifugation, which could have affected the expression of various markers I analysed. On the other hand, the subjects from the surgical cohort, despite a mean 6 months total weight loss of 20% (121.6 ± 26 kg from 151.3 ± 31 kg), still had an average BMI of 44 ± 10.2 kg/m² (from 54 ± 12.6 kg/m²) which classifies them as severely obese [WHO, 1995]. Perhaps, once a certain degree of metabolic derangement is achieved, the 'baseline' EV profile remains quite static, and more dynamic EV differences would be observed if samples were obtained soon after surgery (24-72hours). Though the subjects in the conservative management cohort suffered from severe obesity as well (mean BMI 54.92 ± 6.6 kg/m²), they did not have as many metabolic comorbidities and therefore did not qualify for bariatric surgery as per the DUBASCO score criteria (chapter 4.3.1). This is perhaps the reason why their EVs profile were more 'plastic' in response to simple lifestyle intervention. Further work on such clinical cohorts using the most up to date EV isolation methods, functional assays and EV RNA analysis is required to explore these observations further.

7.3 Suggestions for further research

I believe the research I conducted can be developed in several ways and some aspects of this work are already being continued by my colleagues from our research group at Cardiff Metropolitan University and Cardiff University.

7.3.1 Developments in plasma EV isolation and characterisation in obese cohorts

The recent developments in EV isolation methodology need to be addressed in further studies. I briefly attempted this in chapter 5 by using SEC commercial columns. Since many researchers now employ size exclusion chromatography methods for plasma EV isolation, it would be interesting to characterise EVs from the cohorts I described using this isolation methodology and a similar array of

antibodies for EV origin and content characterisation. However, our group's current TRF immunoassay methodology requires quite a significant plasma volume to obtain sufficient EV number. This would be associated with increased time and cost of running the experiments if more than one Exospin Column was required to obtain enough EV material from one donor to replicate some of the TRF experiments and/or perform other assays. It may therefore be necessary either to consider other novel isolation methods of EVs such as anion exchange chromatography [Heath N, 2018] or filtration microfluidic devices [Liang LG, 2017] and/or alternative methods of analysing EV content such as microarrays/dot assays as the screening tool for proteins of interest. These could potentially be complemented by the development of local protocols for EV RNA expression analysis based on the recent literature [Crossland R, 2016, Maatescu B, 2017], which would require less sample volume and might be a good base for future functional EV studies. Such an altered approach to the analysis of various obese clinical cohorts could provide more insight into a possible explanation of the results I described and could also be used to validate my findings.

7.3.2 Development of Adipocyte-derived EV isolation and characterisation

The adipocyte-derived EV work was quite limited due to the length of time the preadipocyte cultures required and the very low final concentration of isolated EV material. Therefore, I do not think in its current form performing these experiments is sustainable. One option would be perhaps employment of commercially available human preadipocytes such as the Lonza cells I used, but these would incur significant cost in the context of EV experiments, especially as they are not recommended for use beyond passage 1. Therefore, as discussed in chapter 6, evaluation of EV isolation from the whole adipose tissue explant media [Krankendonk ME, 2014] could be trialled instead alongside immunoaffinity based methods for EV isolation targeting the exosomal markers and adipokines to ensure that the material obtained represents ADEVs rather than EVs from contaminating cells [Ferrante SC, 2015]. As discussed above, EV RNA studies on material obtained from the adipocyte culture medium in parallel with PCR analysis of the adipose tissue culture itself would also be of great value, especially in the context of potential functional experiments that are being developed by our group and which I discuss below.

7.3.3. Functional studies: why do these matter?

As stated above, the research I present in my thesis is of a descriptive nature and understandably, more interest is directed nowadays towards the potential biological role of EVs and how that could be applied in clinical practice rather than the analysis of their characteristics alone. EVs are already being explored as potential vaccine or chemotherapy delivery agents [Fais S, 2016] and the potential

for such modified EVs to be used to affect target cells biology could completely change our approach to modern pharmacotherapy.

I was therefore hoping to incorporate some functional experiments into my research on plasma EVs from lean and morbidly obese subjects. This was unfortunately not successful, but I think the hypotheses are still worth exploring with the developments in EV methodology. Firstly, I was hoping to evaluate the potential chemotactic properties of plasma-derived EVs on peripheral blood neutrophils isolated from healthy donors. The scientific question I wanted to answer was whether EVs isolated from obese subjects (both “healthy obese” and those with comorbidities) would have stronger chemoattractant properties than the EVs from healthy lean donors and how the neutrophils would react to EVs obtained directly from the adipose tissue explants (visceral and subcutaneous). In order to attempt these experiments, I used 3D μ -slide chemotaxis plates (80326, Ibidi GmBh, Germany) where the chemoattractant and control medium are injected to 2 chambers separated by a column where neutrophils resuspended in gel are injected and incubated and chemotaxis can be measured in real time by video-microscopy and cell tracking. In theory, video recording should allow to visualise the movements of the neutrophils towards the chemoattractant which can be measured and compared to controls. Unfortunately, I was faced with a lot of technical issues associated with this method, such as leakage of neutrophils into the main chambers or lack of neutrophil movement towards control chemoattractant (CCL-19 (chemokine ligand 19) or FMLP(N-formyl-L-methionyl-L-leucyl-phenylalanine)) which I used in various concentrations. Despite repeated trials and attempting various modifications which both myself and my colleagues evaluated, the control experiments were not successful and due to time restriction associated with recruitment of clinical cohorts, processing and analysis of my other samples, I was not able to continue to work on developing this functional experiment. In hindsight, perhaps a different chemotaxis assay could be explored to test my hypothesis, such as the Boyden chambers or micro-chemotaxis chambers [Taylor L, 2018], especially since recent investigations show potential chemotactic properties of EVs. Akbar *et al* showed that labelled endothelial EVs, when injected into tail vein in mice, left the systemic circulation and accumulated in the spleen and mobilised splenic monocytes. Moreover, *in vitro* exposure of THP-1 and RAW264.7 cells to endothelial cell-derived EVs increased their chemotaxis to MCP-1 [Akbar N, 2017]. Similarly, Eguchi *et al* showed *in vitro* and in mice models that adipocyte-derived EVs induce migration of monocytes and primary macrophages, and transplanting EVs from plasma of ob/ob mice into the wild-type mice induces monocyte activation in the circulation and inflammatory macrophage infiltration [Eguchi A, 2015].

My colleague, Dr Rebecca Wadey, has recently developed a leukocyte adhesion assay with the aim of establishing if peripheral blood leukocytes (isolated from healthy donors in the same way as my

chemotaxis experiments) would show increased adhesion to primary endothelial cells (isolated from human umbilical cords) when pre-incubated with EVs isolated from 3T3 cells [unpublished data].

Once this methodology was established and validated, which was towards the end of my experimental work, I attempted this experiment on 2 occasions using surplus thawed EVs from healthy and obese individuals from my study cohorts. Although I did not observe any differences in adhesion of the leukocytes preincubated with EVs from lean versus morbidly obese donors, I believe repeated experimental work using this new assay should be attempted. I suspect the failure of my experiment might have been due to the fact that I used frozen EVs stored in -80°C for several months as they were surplus material from previous experiments; this could have affected their biological properties. Therefore, repeating these experiments by using freshly isolated EVs, ideally purified also by SEC columns and stored for a maximum of 24-72 hours at 4°C could add a new perspective to the work on biological properties of EVs in obesity. Furthermore, the potential effects on gene expression in the endothelial cells exposed to EVs from various sources (plasma/ adipocyte-derived) and donors (healthy vs morbidly obese) could be evaluated to complement recently published functional ADEV studies [Mleczko J, 2018, Ferrante SC 2015, Koeck ES, 2014].

The potential pitfall of experimental design to use fresh EVs would however be the fact this work would be very time consuming using current isolation methodologies and therefore logistically it would be difficult to run these experiments on a large number of samples, unless several researchers worked simultaneously with responsibility for various steps of the isolation, characterisation and functional assay processes. Therefore, as discussed above, alternative isolation techniques may have to be considered.

7.4 Conclusions

In summary, the recent developments in EV research appear to have opened a new chapter in our understanding of many pathological and biological processes. Further progress in understanding EV biology is likely to lead to significant developments in personalised medicine and modern nanopharmacological therapies in years to come.

The work I conducted has contributed to our knowledge on EV characteristics in the context of obesity and it formed a useful basis for my current and future research group colleagues who will hopefully pursue some of the hypotheses and ideas further, thus enabling us to gain a more holistic insight into the characteristics and role of EVs in obesity.

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Appendix

Cardiff and Vale University Health Board

Level 3

Specialist Weight Management Service

Obesity Pathway

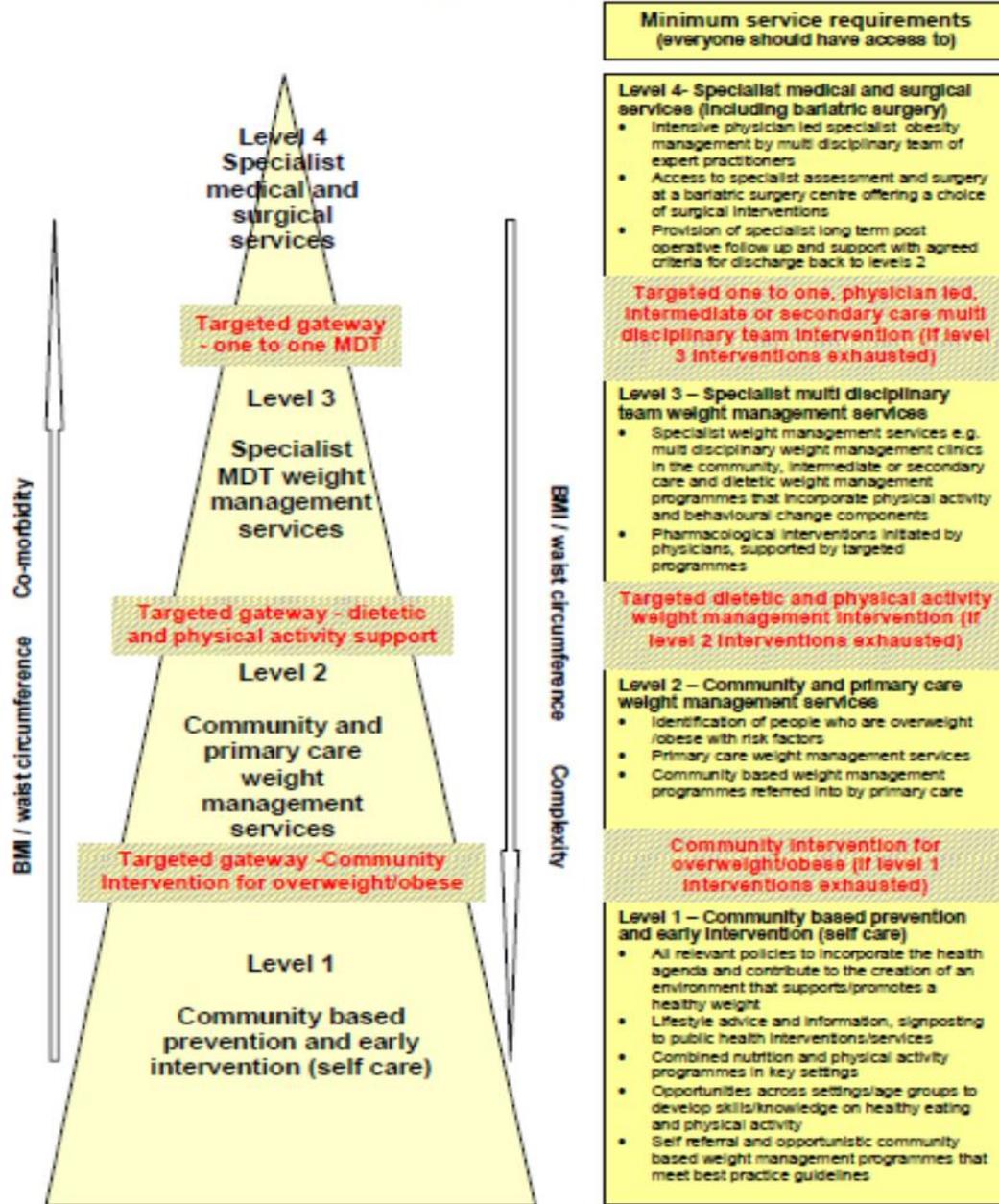


Fig.1 All Wales Obesity Pathway

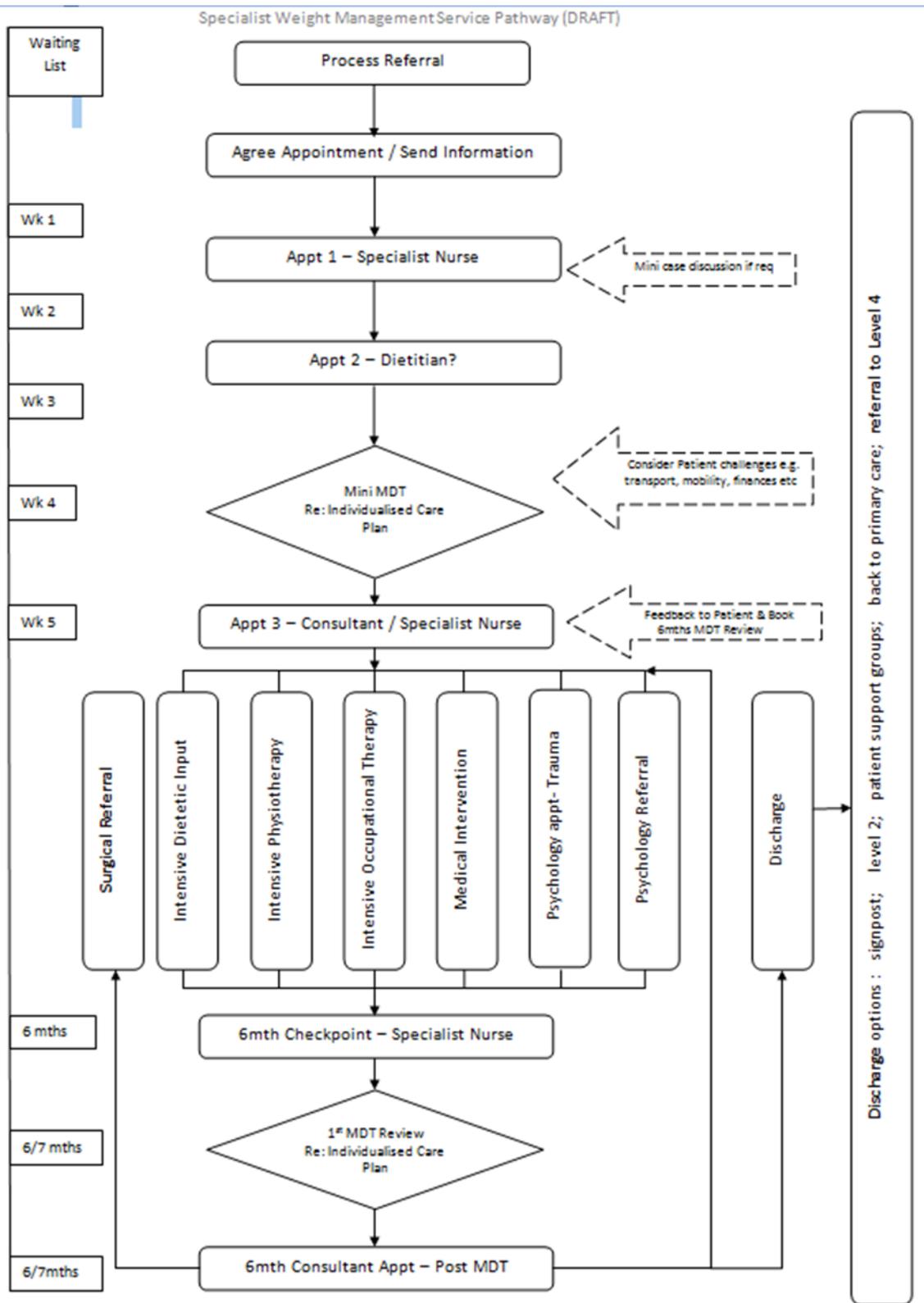


Fig. 2: Level 3 SWMS Initial assessment and intervention pathways

Roles of Multidisciplinary Team

Dietitian

The Specialist Dietitian delivers two clinics each week in Llandough Hospital out-patients and the initial six months part of the intervention is more intensive with frequent 1:1 appointments. Longer term the frequency of appointments are reduced to reflect the person's growing confidence with making changes.

Motivational Interviewing is used as the foundation for the Dietetic intervention and at the initial meeting the focus is on engaging the person in the dietetic service as well as the Level 3 service as a whole. The patient led model focuses on the behaviours and habits that the person feels they need to change in order to successfully lose weight long-term. In a collaborative approach the patient and Dietitian work together on nutrition education, where necessary, including portion sizes, calorie requirements, reading food labels, meal plans etc, as well as working on the emotional relationship with food.

For many people within this service they do not attend the first appointment ready to make changes and time is spent working with them on building their confidence to make changes, deciding if they want to attempt lifestyle change and supporting them with lapses. Motivation fluctuates throughout the 18 months within the service and patients are supported with this in order to evoke their own motivation to change and work through the challenges.

The Dietitian also provides long-term dietetic monitoring for patients who have undergone bariatric surgery.

Doctor

The medical consultant is allocated 1.5 sessions (5.6 hours per week) for the service. All referrals are reviewed by the consultant and MDT and triaged. All new patients are seen in a weekly MDT clinic at University Hospital Llandough and are reviewed every 3-4 months. Medical issues related to obesity are investigated and managed with medication changes made as appropriate, together with appropriate liaison with the patient's GP, secondary care consultants and the bariatric surgical centre in Swansea. Education sessions, focusing on the process of appropriate referral and management, together with advice on use and withdrawal of the weight management drug Orlistat have been delivered to both GP clusters and individual practices. Offers of in-practice education have been provided across primary care in the UHB.

Nurse

The clinical nurse specialist supports patients with complex needs throughout their weight loss journey. Once referrals are triaged into the service, the CNS meets with each patient, to complete a detailed evidenced based physical, emotional, and psychological assessment. This is an early intervention approach, providing awareness raising, psycho-education and signposting for patients. Working from a trauma informed perspective the consultation is based on a person-centred approach, to ensure that patient's feel heard, supported, and involved during the appointment. This appointment is a critical component in the service pathway which facilitates not only the ability to gather key patient information but is a useful opportunity to gauge patient expectation and their commitment and ability to further engage well. This assessment is discussed in the weekly MDT to formulate an individualised plan for the patient and agree the best pathway of care and service provision.

The CNS role is key to the overall service, acting as an efficient conduit between professional disciplines and as a relationship builder for the patient and their families in accessing and benefiting from the healthcare and service provided. The patient is therefore encouraged to claim ownership of their health and to develop a trusting therapeutic relationship not only with the CNS at each of their six-month appointments, but the wider MDT.

Occupational Therapist

The role of Occupational Therapy within the Specialist Weight Management Service is to focus on the occupational needs of the client group. Obesity and its co-morbidities, together with attitudinal barriers, stigma and discrimination has an effect on occupational choice and participation, potentially leading to occupational deprivation and marginalisation.

The service is delivered primarily through domiciliary visits, allowing assessment of the person's daily occupations (the practical and purposeful activities that allow people to live independently and have a sense of identity. This could be essential day-to-day tasks such as self-care, work or leisure) within their own home and local environment.

Reasons for referral have been varied. These have included assessment of function in the home, provision of equipment to increase independence, identification of barriers to setting, maintaining and achieving goals, encouragement to develop positive routines, support to explore opportunities to increase social contact and support to maintain their work role. Interventions focus on building rapport and trust, supporting behaviour change in the context of occupational participation, liaising with other organisations and services, provision of equipment.

Physiotherapist

The role of the physiotherapist working in the weight management service is primarily to support people to work towards meaningful life goals in which weight management, physical activity and movement have an important part to play. While the importance of being physically active is well recognised, in reality people often experience difficulties in doing so. Physiotherapists provide valuable input and expertise for service users who are struggling or unable to progress their activity levels with the support from their social networks or health and wellbeing services available in the community. A treatment plan for a service user may comprise of:

- personalised lifestyle advice, taking into account individual attitudes, beliefs, circumstances, cultural and social preferences, and readiness to change prescription, supervision, and progression of appropriate physical activity to increase muscle strength, flexibility, and endurance, and sustain energy output to enhance and maintain weight loss under safe and controlled condition
- management of associated conditions such as arthritis, persistent pain, and other musculoskeletal or long-term conditions, such as COPD or heart disease
- co-ordination of comprehensive and sustainable programmes of management in collaboration with service users, other health and social care professionals, and community services.

Assessments and treatment may be delivered individually or with groups. The physiotherapist role includes the support of other members of the team in the multi-disciplinary management of obesity helping to optimise clinical outcomes and patient experience. The physiotherapist will participate in regular supervision and ensure that their practice is up to date and informed by current guidance and available research evidence.

The physiotherapy component of the weight management service has had a therapist in post since January 2016. The service has been running from Whitchurch physiotherapy outpatient department with 15 hours of clinical and administration time per week. This has been reduced to 11.5 hours per week over the last 6 months secondary to maternity leave.

Outcome measures specific to pain, function, fear of movement and quality of life are collected and repeated at 3,6,9 and 12 months.

Psychologist

Due to the limited access to clinical psychology services (0.4 wte) the service has adopted a direct clinical service and consultation model (working through others). This has enabled psychologically informed care from the point of entry into the service. In addition to direct clinical care the psychologist offers clinical supervision and support for other team members including the level 2 weight management service, through monthly psychological consultations. These cover issues such as risk, trauma, mental health difficulties, mental health services, signposting, attachment, transference and a range of psychological models of care. The consultation group is currently being evaluated and appears to be well received amongst colleagues.

Service Co-ordinator

The role of Weight Management Service co-ordinator for the Level 3 service has proven to be complex much like its patient base. Working within a multi-disciplinary team, offering patients a consistent point of contact to assist with appointment making and accessibility challenges, has proven to be beneficial for the patients since service inception. This also contributes to good team communication by centralising information on how well people are engaging with the service in order to proactively identify any issues and necessary action required on the behalf of the clinical team. The role encompasses traditional clinic co-ordination elements however, it crosses inter-departmental functionality normally seen separated within the larger Medical Records department.

This specific role also includes daily secretarial, administrative and information support to the MDT team members on an ad hoc basis, the joint co-ordination of MDT meetings, minute taking and

assisting and liaising with patient data collation, sharing and reporting on a regular basis. The co-ordinator works closely with the specialist nurse as a conduit for satellite team members to proactively centralise patient treatment information, the majority of which involves a significant amount of manual intensity in the absence of a suitable IT solution. To collect and monitor service activity at each stage of the patient pathway, the co-ordinator also actively creates and assists with a number of databases, worksheets, process flow diagrams and data capture forms used by the clinical team members and management team.

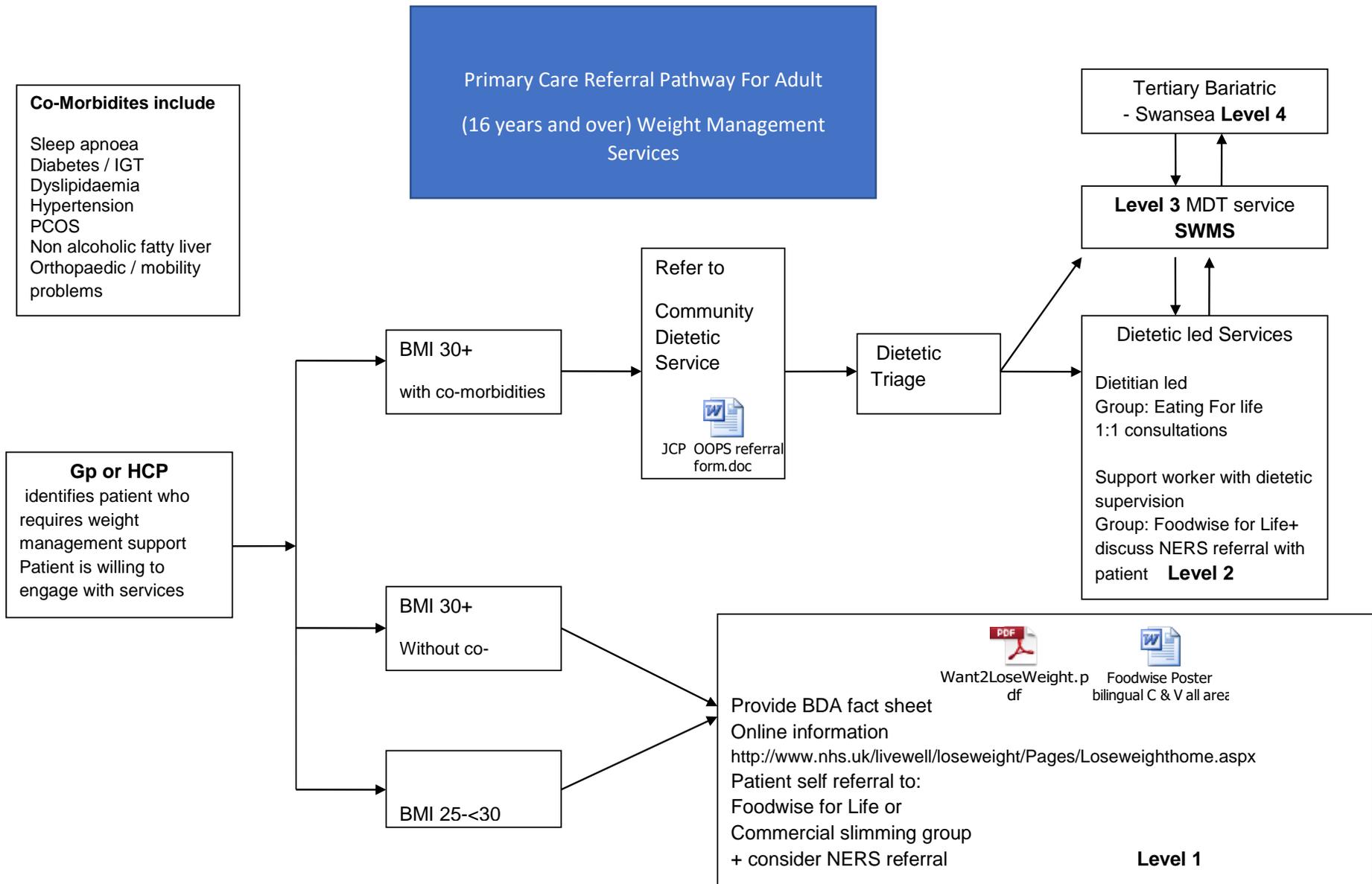


Fig.3: Primary Care Pathway Referral for Adult