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<th>The glycosylation signature of human urinary extracellular vesicles</th>
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<tr>
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<td>Krueger, Anja</td>
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THE GLYCOSYLATION SIGNATURE OF
HUMAN URINARY EXTRACELLULAR VESICLES

A thesis submitted to the National University of Ireland in fulfilment of the
requirements of the degree of

Doctor of Philosophy

By
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Thesis Supervisor: Professor Matthew D. Griffin
Co-supervisor: Professor Lokesh Joshi

December 2015
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Abstract

Cells of virtually all organs and tissues are known to release large numbers of subcellular particles called extracellular vesicles (EVs) into bodily fluids such as blood and urine. There has been a growing interest in isolation of urine EVs (uEVs) as a source of diagnostic and prognostic tests for kidney disease. While the protein and nucleic acid composition of human uEVs has been extensively characterised, their content of carbohydrates (glycans) is less well studied. The overarching goal of this thesis was to define the normal surface glycan profile of human uEVs and determine whether it is altered in kidney diseases.

Initially, an ultra-centrifugation (UC)-based method was shown to be superior to a centrifugal concentration method for isolating high-quality uEVs from healthy human urine. The resulting uEVs were then analysed for binding of carbohydrate-specific proteins (lectins) using two techniques – flow cytometry and lectin microarray (LM). It was shown that uEVs express a broad, complex array of different glycans on their surfaces. Next, a new method was developed to sub-divide uEVs into density-based fractions while eliminating contamination by non-vesicle protein. Using LM, electron microscopy and nanoparticle tracking, density-based uEV fractions were shown to vary in size and to have specific differences in surface glycosylation. The surface glycosylation profiles of uEV fractions from healthy adults were then compared to those from patients with the kidney condition glomerulonephritis (GN). It was found that mid- and high-density uEV fractions of GN patients were larger and had altered binding of five specific lectins.

Novel contributions of the thesis include: (a) Methodologies for uEV isolation and analysis of EV glycosylation. (b) Detailed surface glycan profiles of healthy human uEVs and of density-based uEV sub-fractions. (c) Evidence for kidney disease-specific alterations to uEV surface glycan content that may be of value for future urine biomarker development.
Acknowledgements
This Ph.D. thesis was supported by HRB funding and was run as a cooperative project between the Regenerative Medicine Institute and the Glycoscience Group at the National University of Ireland, Galway under the supervision of Prof. Matthew D. Griffin, Prof. Lokesh Joshi and Dr. Jared Q. Gerlach.

The PhD fellowship was funded by a project grant from the Health Research Board of Ireland (grant number HRA_HSR/2010/63). Elements of the research were also supported by grants from Science Foundation Ireland to the Primary Supervisor (grant number 09/SRC/B1794) and to the Co-supervisor (grant numbers 08/SRC/B1393 and 07/SC/B1250) and by a grant to collaborators at Mayo Clinic, USA from the National Institutes of Health NIDDK (grant number 5RC1DK086161-02). Research infrastructure was additionally supported by the Higher Education Authority of Ireland and by the European Regional Development Fund. Additional, financial contributions to this work were given directly or indirectly by AGRC as well as the College of Medicine, Nursing and Health Sciences at National University Ireland Galway.

The work described resulted in one published paper (second authorship) as well as one manuscript in progress (first authorship). Several poster presentations at relevant conferences as well as one public talk at the European Renal Cell Study Group (ERCSG) in February 2015.

Major contributions to this work were based on the input of the entire Glycoscience team including Dr. Michelle Kilcoyne, Dr. Stephen Cumminingham, Dr. Marian Kane, Dr. Mike Cairns, Dr. Iain Shaw in particular Dr. Jared Q. Gerlach and Prof. Joshi Loke as well as other inspiring group members. Excellent primary supervision was given by Matthew D. Griffin and Dr. Jared Q. Gerlach over the past five years.

Support from the microscopy and flow cytometry core facilities of the National Center for Biomedical Engineering Science (NCBES) was provided by Prof. Peter Dockery, Dr. Eadaoin Timmins, Dr. Pierce Lalor, Prof. Rhodri Ceredig and Dr. Shirley Hanley. Cooperation partners in regards to nanoparticle analysis were Dr. Adriele Prina-Mellow and Ciaran McGuire of the Nanomedicine Group, Institute of Molecular Medicine, School of Medicine, Trinity Centre for Health Sciences, St. James’s Hospital, Dublin, Ireland as well as Advanced Materials and BioEngineering Research (AMBER) and Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN), Trinity College Dublin, Dublin, Ireland. Cooperation partners in regard to urine extracellular vesicles samples from ADPKD patients and age-matched controls were Dr. Marie Hogan and Dr. Chris Ward of the Division of Nephrology and Hypertension, Mayo Clinic, Rochester, Minnesota, USA.

Final acknowledgements are associated with my supportive GRC members who gave advice along the way: Prof. Rhodri Ceredig, Prof. Thomas Ritter as well as Dr. Maria Tuohy.
### Abbreviations

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<tr>
<td>Ø</td>
<td>Average</td>
</tr>
<tr>
<td>°C</td>
<td>Degree celsius</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>ACR</td>
<td>Albumin to creatinine ratio</td>
</tr>
<tr>
<td>ADPKD</td>
<td>Autosomal dominant polycystic kidney disease</td>
</tr>
<tr>
<td>AF555</td>
<td>Alexa fluorophor 555 dye</td>
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<tr>
<td>AF647</td>
<td>Alexa fluorophor 647 dye</td>
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<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>ALIX</td>
<td>ALG-2-interacting protein X</td>
</tr>
<tr>
<td>AMBER</td>
<td>Advanced Materials and BioEngineering Research</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
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<td>AQP-1</td>
<td>Aquaporin-1</td>
</tr>
<tr>
<td>AQP-2</td>
<td>Aquaporin-2</td>
</tr>
<tr>
<td>B16-F10</td>
<td>Melanoma cell line</td>
</tr>
<tr>
<td>BACE1</td>
<td>Beta-secretase transgenic knockout mice</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>bIgG</td>
<td>Bovine serum immunoglobulin G</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BS</td>
<td>Bartter’s Syndrome</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CF</td>
<td>Centrifugal filtration</td>
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<td>CHAPS</td>
<td>3-(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CM-DIL</td>
<td>Cell tracker dye acts within the cell</td>
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<tr>
<td>CRANN</td>
<td>Center for Research on Adaptive Nanostructures and Nanodevices</td>
</tr>
<tr>
<td>CT</td>
<td>Connecting tubule</td>
</tr>
<tr>
<td>CT-scan</td>
<td>Computed tomography scan</td>
</tr>
<tr>
<td>D₂O</td>
<td>Heavy water</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetic mellitus</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>e.g.</td>
<td>For example</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-barr virus</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemical luminescent</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER associated degradation</td>
</tr>
<tr>
<td>ERGIC</td>
<td>Cargo associated receptor</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complex Required for Transport</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>EtN-PO₄</td>
<td>Ethanolamine phosphate</td>
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EV  Extracellular vesicles
FCM  Flow cytometry
Fet  Calf serum bovine fetuin
FITC  Fluorescein isothiocyanate
FSC  Forward scatter
FSGS  Focal Segmental Glomerular Sclerosis
FTLA  Finite Track Length Adjustment
GalCer  Galactosylceramide
GFR  Glomerular filtration rate
GlCer  Glucosylceramide
GMV  Glomerular membrane vesicle
GN  Glomerulonephritis
GPI  Glycosylphosphatidylinositol
GS  Gitelman’s Syndrome
H9  T cell line
HD  Haemodialysis
hESC  Human embryonic stem cells
HIV  Human immunodeficiency virus
HKUPP  Human Kidney and Urine Proteome Project
hMSC  Human mesenchymal stem cells
HRP  Horseradish peroxidase
Hrs  Hours
HAS  Human serum albumin
HSP70  Heat shock protein 70
HSP90  Heat shock protein 90
HUVEC  Human umbilical vein endothelial cell
IC  Intercalating cell
Ig  Immunoglobulin
IgAN  IgA Nephropathy
ILV  Intraluminal vesicles
kDa  Kilodalton
KDIGO  Kidney Disease: Improving Global Outcomes
KIM-1  Kidney injury molecule-1
KTx  Kidney transplantation
kV  Kilovolt
Lac  Lactose
LacNAc  Lactosamine
LAMP-1  Lysosomal-associated membrane protein-1
LAMP-2  Lysosomal-associated membrane protein-2
LC  Light chain
LM  Lectin microarray
LN  Lupus Nephritis
LOX  Human melanoma cell line
M  Molar
m²  Square meter
MCD  Minimal Change Disease
MCF-7  Breast cancer cell line
MDA  Human breast adenocarcinoma cell line
<table>
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<tr>
<td>MDRD</td>
<td>Modification of Diet in Renal Disease Study related equation</td>
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<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>Ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>MN</td>
<td>Membranous Nephropathy</td>
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<td>MNT-1</td>
<td>Human melanocytic cells</td>
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<td>MOPS</td>
<td>3-(N-morpholino)propansulfonic acid</td>
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<tr>
<td>MPGN</td>
<td>Membrano-Proliferative Glomerulonephritis</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
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<td>MWCO</td>
<td>Molecular weight cut-off</td>
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<td>NGAL</td>
<td>Neutrophil gelatinase-associated lipocalin</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>Nm</td>
<td>Nanometer</td>
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<td>NS</td>
<td>Nephrotic Syndrome</td>
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<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
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<td>OST</td>
<td>Oligosaccharyltransferase</td>
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<tr>
<td>P1800</td>
<td>Pellet at 1800 xg</td>
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<td>P2500</td>
<td>Pellet at 2500 xg</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PC</td>
<td>Principle cells</td>
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<tr>
<td>PC-5</td>
<td>Prostate cancer cell line</td>
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<tr>
<td>PD</td>
<td>Peritoneal dialysis</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PE-Cy7</td>
<td>Phycoerythrin- Cyanin 7</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PKH26</td>
<td>Lipophilic membrane cell tracker dye</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
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<tr>
<td>PNGase</td>
<td>Peptide N-glycanase</td>
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<tr>
<td>PODXL</td>
<td>Podocalyxin-like protein</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>ROMK1</td>
<td>K+ channel in the kidney</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SIGLEC</td>
<td>Sialic acid immunoglobulin-type lectin</td>
</tr>
<tr>
<td>SkMe1-5</td>
<td>Human skin cancer cells</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>SN</td>
<td>Supernatant</td>
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<tr>
<td>SNAP</td>
<td>Soluble NSF attachment protein</td>
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<tr>
<td>SNAPE</td>
<td>Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor</td>
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<td>Sp-DilC18</td>
<td>Lipophilic carbocyanine dye</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TBMD</td>
<td>Thin Basement Membrane Disease</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS/BSA</td>
<td>Bovine serum albumin in tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween 0.025% (v/v) or 0.05% (v/v)</td>
</tr>
<tr>
<td>TCEP-HCl</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>THP</td>
<td>Tamm-Horsfall Protein</td>
</tr>
<tr>
<td>TRPV-5</td>
<td>Transient receptor potential vanilloid type 5</td>
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<tr>
<td>TSG101</td>
<td>Tumor susceptibility gene 101 protein</td>
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<tr>
<td>UC</td>
<td>Ultracentrifugation</td>
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<td>uEV</td>
<td>Urinary extracellular vesicles</td>
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<td>USRDS</td>
<td>United States Renal Data System</td>
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<td>UT-A1</td>
<td>Urea transporter A1</td>
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<td>UTI</td>
<td>Urinary tract infection</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Xg</td>
<td>Number of times the gravitational force</td>
</tr>
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Carbohydrate formula and symbols

Symbols:

- Man
- Glc
- GlcNAc
- Gal
- Fuc
- GalNAc
- Neu5Ac
- Lac
- LacNAc

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Chapter One

Introduction

*Extracellular Vesicles and Glycosylation: Their Biological Significance and Potential for Clinical Application in the Field of Kidney Disease*
1 Introduction

1.1 Extracellular vesicle subtypes and their biological properties

1.1.1 Extracellular vesicle subtypes

Extracellular vesicles (EVs) prepared by most isolation techniques primarily comprise vesicles of endosomal origin (“exosomes”) and outer membrane-derived vesicles (“membrane shed vesicles”, “ectosomes” or “microvesicles”) generated by direct budding from the cell surface (figure 1-1) (Harding, Heuser, and Stahl 1983; Heijnen et al. 1999; Muralidharan-Chari et al. 2009; Zitvogel et al. 1998). Exosomes have upper and lower diameter ranges reported between 30-40 nm and 100-130 nm and a floating density of 1.13-1.19 g/cm$^3$. They are spherical in their natural state but are frequently described as “cup shaped” due to artefactual changes which occur due to sample processing for transmission electron microscopy (TEM) (Conde-Vancells et al. 2010; Johnstone et al. 1987; Graca Raposo et al. 1996; Théry, Ostrowski, and Segura 2009; Théry et al. 2006a). Proteins, genetic material, carbohydrates and lipids are components of these nanovesicles (Batista et al. 2011; Miranda et al. 2014; Pisitkun, Shen, and Knepper 2004; Saraswat et al. 2015; Théry et al. 2001; Valadi et al. 2007). Certain biomolecules are reported to be specifically associated with exosomes including proteins of endosomal origin such as ALG-2-interacting protein X (ALIX), Lysosomal-associated membrane protein-1 (LAMP-1) and Lysosomal-associated membrane protein-2 (LAMP-2) as well as high cholesterol content as shown by Möbius et al. (Möbius et al. 2003; Graca Raposo et al. 1996; Théry et al. 2001). As discussed below, recent EV research indicates that the protein contents of exosomes and membrane shed vesicles overlap significantly (Colombo, Raposo, and Théry 2014).

Membrane shed vesicle are considered to be more heterogenous in nature than exosomes (Muralidharan-Chari et al. 2009). These vesicles are also round and are described as having a diameter range of 200 to 1000 nm although membrane shed vesicles of diameter as low as 50 nm have been reported (Booth et al. 2006; Hara et al. 2010; Marzesco et al. 2005). In general, membrane shed vesicles and exosomes cannot currently be fully differentiated. Nonetheless, they are reported to mainly float at a higher density than exosomes (>1.23 g/cm$^3$) and to have a distinct lipid composition.
A third category of EV, referred to as “apoptotic bodies” or “apoptotic blebs”, is also well recognised. These irregularly shaped particles, which have a reported size range between 500 nm and 2000 nm, are released by outward blebbing of the surface membrane of cells undergoing apoptotic programmed cell death resulting in negatively charged phosphatidyl serine on the outer leaflet (Akers et al. 2013; Crescitelli et al. 2013; Théry, Ostrowski, and Segura 2009). The constituents of apoptotic bodies may include elements of the cytosol, endoplasmic reticulum and nucleus (histones, condensed chromatin) (Cline and Radic 2004; Dieker and Muller 2009). For example, a comparison of protein content in EVs and apoptotic blebs (>800 nm) isolated from thymic T cells showed higher amounts of total protein content but lower protein variability in apoptotic blebs compared to exosomes (Turiak et al. 2011). Finally, an additional sub-type of EV (which may be of specific relevance to the range of EVs present in urine) are microvillus-derived vesicles which are reported to bud from the tip of microvilli present on specialised epithelial cells such as those of the kidney. The reported size range of these less well characterised EVs is 50 – 1000 nm (Hara et al. 2010; Walmsley et al. 2010).
1.1.2 Extracellular vesicle biogenesis

Generation of EVs is incompletely understood. However, for exosomes in particular, a central role of the Endosomal Sorting Complex Required for Transport (ESCRT) has been established through the application of knock-down and other experimental strategies while ongoing research is identifying other protein sorting mechanisms (Bobrie et al. 2012; Kowal, Tkach, and Théry 2014; Roucourt et al. 2015).

Membrane shed vesicle: Membrane vesicles are shed by most cells. The formation of membrane buds into the extracellular space was demonstrated by Muralidharan-Chari et al. in 2009 using the human melanoma cell line, LOX (Muralidharan-Chari et al. 2009). Earlier, outwards budding of cell membranes was associated with flipping of phosphatidyl serine to the outer membrane leaflet. Usually the cell membrane contains a natural ratio of lipids in the inner and outer membrane which is regulated by the enzymes flippase, floppase and scramblase. Elevated intracellular calcium is associated with inhibition of flippase and activation of scramblase as well as floppase. Thus the normally occurring asymmetry of the cell membrane is disturbed leading to deformation/protuberance in the membrane (Cocucci and Meldolesi 2015; Hugel et al. 2005). Most recently, a release mechanism was suggested by Cocucci and Meldolesi involving the interaction of tumor susceptibility gene 101 protein (TSG101) and ESCRT-III on the intracellular cellular membrane. Budding was proposed to occur after organisation of conical spiral including filaments of ESCRT-III while Vsp4 was suggested to be involved in scission. Outwards budding of membrane vesicles was observed to be regulated by the ARF6-GTP complex and final scission involved phosphorylated myosin that may induce downstream contraction caused by the actin-cytoskeleton (Akers et al. 2013; Cocucci and Meldolesi 2015; Muralidharan-Chari et al. 2009).
Exosomes:

Figure 1-2: Illustration of the biogenesis of exosomes including the central role of the ESCRT in sorting exosome biomolecular content. Non-ESCRT-dependent loading pathways and downstream secretory and degradative pathways are also illustrated. Adapted from Morelli and Robbins Nat Rev Immunol. 2014 Mar;14(3):195-208.

As shown, exosome biogenesis begins with inward-budding of the surface membrane thereby forming an early endosome within the cell which may incorporate some cell surface proteins, (figure 1-2). Within this early endosome, the precursors to exosomes, intraluminal vesicles (ILVs), are generated resulting in a multivesicular body (MVB). The intraluminal contents of the MVB may then be processed in several ways including regulated transport to the cell surface and secreted as exosomes into the extracellular environment (Harding, Heuser, and Stahl 1983; Pan et al. 1985). Distinct sorting mechanisms for incorporation of biomolecules into ILVs of the multivesicular endosome are now recognized (Villarroya-Beltri et al. 2014).
**ESCRT:** The best explored sorting machinery is the ESCRT complex which comprises approximately thirty proteins distributed into four individual complexes named ESCRT-0 to III and is dependent on ubiquitination of proteins prior to sorting (Hurley 2015; Kowal, Tkach, and Théry 2014). Briefly, ESCRT-0 in the membrane of the endosome specifically recruits ubiquitinated proteins and interacts with Hrs protein a member of ESCRT-I. Complex formation between ESCRT-I and ESCRT-II leads to active budding of endosomal membrane into the inner compartment (Villarroya-Beltri et al. 2014). Excision of budding membrane to form ILVs is modulated by ESCRT-III. Vsp4, an AAA-ATPase, is thought to generate the energy required for ILV scission (Hurley and Hanson 2010; Wollert et al. 2009).

**Tetraspanins:** Tetraspanins are highly enriched in exosomes as was demonstrated in 1998 by Escola et al. with exosomes derived from B cells (Escola et al. 1998). Possible interacting partners for exosomal tetraspanin 8 from rat pancreatic adenocarcinoma cells were identified by Rana et al. and included integrin chains as well as other tetraspanins. Tetraspanin 8 overexpression in these cells resulted in release of EVs that were enriched in tetraspanin 8 itself as well as CD49d and other proteins that appeared to be selectively sorted (Nazarenko et al. 2010; Rana et al. 2012). Similar observations in Epstein-Barr virus (EBV)-transfected B cell lines indicated co-localization of the tetraspanin protein CD63 and LMP-1 in late endosomal compartments and in released EVs, suggesting targeted sorting of these proteins into exosomes. This process was also shown to be ESCRT-independent (Verweij et al. 2011). Additionally, lymphoblast-derived EVs from CD81 wild-type and knockout mice were investigated for CD81-associated proteins using mass spectrometry. The comparison showed that lack of CD81 was associated with depletion of 25 other exosomal proteins (Perez-Hernandez et al. 2013). Finally, van Niel et al. demonstrated that CD63 is associated with pigment-specific type I integral membrane protein (PMEL) in EVs from human melanocytic (MNT-1) cells in an ESCRT-independent manner (Van Niel et al. 2011). All of these studies provide evidence for an important and specific role of tetraspanin proteins in exosome biogenesis and content.

**Ceramide:** ESCRT-independent sorting was demonstrated by Trajkovic et al. in 2008 in a mouse oligodendroglial cell line. Co-localization studies showed that proteolipids co-localized with LAMP-1 and glycosylphosphatidylinositol (GPI)-anchored proteins in raft micro-domains and a specific role for ceramide in ILV generation was identified. Sphingophosphomyelinase and sphingophosphomyelinase 2 are enzymes for generation of
ceramide (Trajkovic et al. 2008) and additional studies have provided evidence that inhibition of sphingomyelinases is associated with decreased exosome release (Kosaka et al. 2010; Yuyama et al. 2012). Spingosine1-phosphate receptor was also shown to be associated with ESCRT-independent release of flotillin+/CD63+/CD81+ exosomes (Kajimoto et al. 2013).

**Sumoylation:** This post-translational modification was recently linked with miRNA delivery into exosomes. The binding protein hnRNPA2N1 was found to be sumoylated and knock-down experiments indicated a lower miRNA content in EVs compared to control cells (Villarroya-Beltri et al. 2013). Sumoylation-mediated sorting into EVs has been reported to be ESCRT-dependent and may represent an alternative to ubiquitin-dependent ESCRT sorting (Kunadt et al. 2015).

**Glycosylation:** Sk-MeI-5 cells and their released EVs are surrounded by a sugar coat. EWI-2 is a glycoprotein that was found in EVs of Sk-MeI-5 cells. After removal of individual complex N-glycan glycosylation sites within the protein by mutation of asparagine residues to glutamine, the cell surface expression levels of EWI-2 remained unchanged and enrichment in EVs was reduced. This particular study provides some evidence that protein glycosylation plays a role in their sorting into EVs (Y. Liang et al. 2014).

Exosomes are still commonly viewed as an homogeneous EV subtype based on size and floating density. It is becoming clear, however, that there is significant heterogeneity in protein, glycoprotein, lipid and RNA contents of exosomes that is based on a diversity of biogenesis pathways (Colombo et al. 2013).

### 1.1.3 Extracellular vesicle release

Release of EVs by individual cell types is a highly variable and regulated process. Thus, tumor cells have been described as constantly releasing EVs, while in immature dendritic cells, B cells and macrophages stimulation or antigen exposure promotes release of overall or specific EV types (Anand et al. 2010; Buschow et al. 2009; Saunderson et al. 2008; D. D. Taylor and Gercel-Taylor 2005). In some cell types, stimulation of specific cell surface receptors such as purinergic receptors (monocytes, neutrophils) and thrombin receptor (platelets) has been linked with increased EV release (Heijnen et al. 1999; MacKenzie et al. 2001). Similarly, the release of membrane vesicles in microglial cells was associated with an increase in intracellular calcium and with signaling of phospholipase C by activation of
receptors associated with serotonin ligand (Glebov et al. 2015). Furthermore, hypoxia and a range of other cellular stressors (shear and oxidative) are also reported to modulate EV release (Hedlund et al. 2011; Nomura et al. 2000; T. Wang et al. 2014).

**Exosome release:** The release of exosomes from MVBs has been primarily studied in cultured cells under “resting conditions” (Colombo, Raposo, and Théry 2014). In eukaryotic and prokaryotic cells, members of the Rab family of GTPases have been consistently shown to play important roles throughout the process of ILV formation, endosome trafficking and MVB fusion with the plasma membrane. Among the more than 60 members, RAB2B, RAB5A, RAB7, RAB9A, RAB11, RAB27A, RAB27B and RAB35 have been linked to EV intracellular transport and release through knock-down and over-expression in a variety of cultured cell types (Baietti et al. 2012; Colombo, Raposo, and Théry 2014; C. Hsu et al. 2010; Ostrowski et al. 2010; Savina, Vidal, and Colombo 2002). The finer details of how these individual GTPases regulate trafficking of exosomes and other EV subtypes is beyond the scope of this introduction. However, it is important to emphasize that experimental evidence has revealed a high level of complexity suggesting that EV released from different MVBs are regulated by different RAB proteins and that RAB family members may be used differentially by individual cell types for EV transport. **Figure 1-3**, which is adapted from the recent extensive review by Colombo et al. provides a simplified summary of some of the key insights regarding the role of Rab proteins in EV transport and release (Colombo, Raposo, and Théry 2014; Robbins and Morelli 2014).
Figure 1-3: Proposed roles of individual Rab proteins in the intracellular transport and release of exosomes carrying specific selected protein cargoes. Potential roles for ESCRT complexes and the GTP-ARF6 complex in formation and release of membrane shed vesicles is also illustrated. Adapted from Colombo M, Raposo G and Théry C. Annu Rev Cell Dev Biol. 2014;30:255-89.

Also known to be involved in EV membrane fusion and release are proteins of soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptor (SNARE) family. For example, Gross et al. reported that release of Wnt-containing exosomes by Drosophila and human cells is dependent on the R-SNARE Ykt6 (Gross et al. 2012). Overall, the role of SNARE proteins and other components of membrane fusion complexes in EV release has not been extensively investigated to date (Robbins and Morelli 2014).

Membrane shed vesicle release: Triggered release of membrane shed vesicles may be induced in diverse cell types by stimuli that result in a rise in intracellular calcium and actin cytoskeleton re-modeling and may be regulated by ARF6 (figure 1-3). Increased intracellular calcium levels are involved in inhibiting relevant enzymes leading to disruption of lipid asymmetry in cell membranes (Muralidharan-Chari et al. 2009).
1.1.4 Uptake and functions of EVs in health and disease

It is now well accepted that EVs released from diverse cell types may be taken up by and potentially influence the phenotype and function of other cells. The mechanisms and biological consequences of this concept are far reaching and may be highly relevant to a wide variety of diseases. Several EV uptake mechanisms have been described with individual studies implicating protein-protein and/or protein-carbohydrate interactions in vesicle uptake by recipient cells (Barrès et al. 2010; Bruno et al. 2009; Escrevente et al. 2011; Franzen et al. 2014; Nazarenko et al. 2010; Rana et al. 2012). For example, in PC12 cells, EVs were observed to adhere to the cell surface and to be internalised by endocytosis or membrane fusion (Tian et al. 2013, 2014). Other internalisation mechanisms including pino- and macropinocytosis have also been described for EV uptake in culture systems (D. Feng et al. 2010; Tian et al. 2014). Alternatively, EVs derived from malignant T24 urinary carcinoma cells were transferred to recipient non-malignant urinary papilloma cells via nanotubes following endocytotic uptake (Ogorevc et al. 2014). The uptake of EVs from pancreatic adenocarcinoma cells was demonstrated to involve the interaction of tetraspanin 8 with integrin chains and CD9 on recipient cells. In rat aortic endothelial cells, however, EV uptake was linked to tetraspanin 8 interactions with cell surface receptors CD54 or CD106. Cell selectivity (in vitro) and organ specific (in vivo) uptake of tetraspin 8-positive EVs was demonstrated in this work (Rana et al. 2012).

The main physiological role for EVs is cell-to-cell communication (Valadi et al. 2007). A very wide range of cell types can release EVs with the potential to influence cells in their immediate microenvironment in autocrine and paracrine manner as well as cells at distant sites via dissemination in the blood stream and other biological fluids (Lai et al. 2010; Luga et al. 2012; Salido-Guadarrama et al. 2014).

As described in greater details below, transport of functionally active biomolecules such as miRNAs, mRNAs, receptors and enzymes by EVs has been repeatedly demonstrated along with plausible changes to key cellular functions (Hakulinen et al. 2008; Pan et al. 1985; Valadi et al. 2007). To date, literature on EV function in health and disease has focused on several specific fields including immune response, tumorgenesis, tissue repair/regeneration and direct clinical applications such as biomarker discovery and therapeutic delivery (EL Andaloussi et al. 2013; Azmi, Bao, and Sarkar 2013; Bruno and Camussi 2013; Erdbrugger and Le 2015; Robbins and Morelli 2014).
In the case of immune response, a significant role for EVs was initially appreciated through the demonstration of major histocompatibility complex (MHC) I and/or II-containing EVs released from antigen presenting cells such as DCs and B cells (Denzer et al. 2000; Mallegol et al. 2007). Functionality of B cell-derived MHC II+ EVs was demonstrated through direct stimulation of T cells (Graca Raposo et al. 1996). Many other studies have since been published which confirm diverse roles for EVs in regulating immune responses.

In tumorgenesis also, important functional effects of EVs have been demonstrated (Azmi, Bao, and Sarkar 2013; Brinton et al. 2014; Simona et al. 2013). A variety of tumour cells release high levels of EVs including breast, colon and pancreatic cancer cell lines. These EVs contain immunologically relevant proteins such as MHC I, MHC II, FasL, TRAIL, NKG2D ligand and TGF-β as well as diverse miRNAs (Mincheva-Nilsson and Baranov 2014; Simona et al. 2013; X. Zhang et al. 2015). Tumour cell EV-bound FasL was shown to induce apoptosis in CD8+T cells leading to immune suppression (Kim et al. 2005; Wieckowski et al. 2009). Tumour-derived EVs may also be involved in expansion of regulatory T cells (T_{reg}) (Szajnik et al. 2010; Wieckowski et al. 2009). Furthermore, EVs released by tumor cells have been shown to promote the immunosuppressive tumor niche through transfer of TGFβ, modulation of tumour-associated fibroblasts and promotion of angiogenesis (Park et al. 2010; J. P. Webber et al. 2015; J. Webber et al. 2010). Of relevance to tumour metastasis, EVs from B16-F10 melanoma cells in lymph nodes as well as from a breast cancer cell line containing miR-105 were shown to promote migration through endothelium (Hood, Susana San, and Wickline 2011; W. Zhou et al. 2015).

Evidence has also accumulated that EVs released by mesenchymal stem cells (MSCs) contribute to the effects of MSCs to promote tissue repair and regeneration in models of acute kidney injury (AKI), liver injury, myocardial infarction (MI) and neurological disease (Arslan et al. 2013; Gatti et al. 2011; Herrera et al. 2010). For example, in the mouse glycerol-induced model of AKI, S. Buno et al. demonstrated that MSC-EVs were similarly effective to whole MSCs in enhancing tubular cell regeneration (Bruno et al. 2009). A role for EV-transported RNA was suggested by the fact that the renoprotective effect was eliminated by pre-treating MSC-EVs with RNase. In a mouse model of acute MI, MSC-EVs were shown to reduce infarct size (Lai et al. 2010). Other therapeutic applications of EVs have been suggested in areas such as vaccination, drug delivery and gene therapy (Alvarez-Erviti et al. 2011; Cooper
et al. 2014; Escudier et al. 2005; Kooijmans et al. 2013), While pre-clinical studies indicate potential for clinical use of MSC-EVs for cellular re-programming, a range of translational and regulatory hurdles remain to be addressed before this potential can be realized.

Extracellular vesicles have been shown to have high potential in drug delivery. This was demonstrated by targeted brain delivery and silencing of BACE1 in mouse models for Alzheimer's diseases (Alvarez-Erviti et al. 2011). Loading of EVs with therapeutic nucleotides has also been explored (Lamichhane, Raiker, and Jay 2015). Reported methods for drug loading into EVs include use of lipofectamin, electroporation, extrusion or freeze/thawing, co-incubation methods (Alvarez-Erviti et al. 2011; Cooper et al. 2014; Haney et al. 2015).

1.1.5 Bio-molecular content of EVs
The bio-molecular content of EVs has been the subject of intense study over the past decade with a strong emphasis on profiling technologies including proteomics, transcriptomics and, more recently, lipidomics and glycomics. Figure 1-4 illustrates the diversity of known EV-associated biomolecules.

![Figure 1-4](image)

Figure 1-4: Diversity of biomolecules known to be expressed on the surface or within the inner compartment of EVs. The presence and relative quantity of each biomolecule may vary depending on the EV subtype and on the source and functional status of the parent cells. Adapted from Colombo et al. Annu Rev Cell Dev Biol. 2014;30:255-89.

**Proteins:** Proteomic studies have demonstrated the diversity and specificity of transmembrane and cytosolic proteins present in EVs along with some details of their posttranslation
modifications such as phosphorylation and glycosylation. Among the major types of proteins that were found to be enriched in EVs from multiple sources included tetraspanins (CD81, CD82, CD9 and CD63), heat-shock proteins (HSP70, HSP90), small GTPases (Rab proteins), ESCRT components with accessory proteins (ALIX, TSG101) and endosomal proteins (LAMP-1, flottilin-1). It is worth noting that proteins identified in these studies were initially considered to be exclusively associated with exosomes due to the presence of endosomal markers and ESCRT-machinery (Colombo, Raposo, and Théry 2014; Mathivanan, Ji, and Simpson 2010; Graça Raposo and Stoorvogel 2013). However, in the absence of technologies to definitively separate the different EV subtypes, the available proteomic libraries should be viewed as reflecting the combined protein contents of exosomes, membrane shed vesicles and, potentially, other forms of EV (D.-S. Choi et al. 2014). As shown in figure 1-4, EVs also contain a range of bioactive protein types such as enzymes, transcription factors, chaperones, signaling molecules, ion channels and transporters which may directly reflect the functional nature of their parent cells (Colombo, Raposo, and Théry 2014). Some EV-enriched proteins such as GPI-anchored proteins or flottilin-1 are specifically known to be present in lipid rafts (Allen, Halverson-Tamboli, and Rasenick 2007; Maeda and Kinoshita 2011).

Nucleic Acids: It is now well established that nucleic acids, particularly RNA, are also selectively sorted into EVs. This has been demonstrated in multiple profiling studies using microarray and high-throughput sequencing technologies with in silico alignment of whole cell- and EV-derived RNA preparations. Notably, EVs contain mRNA, miRNA as well as other forms of non-coding RNAs (Miranda et al. 2014; Ratajczak et al. 2006; Valadi et al. 2007). In recent years, most attention has been focused on the miRNA content of EVs (particularly exosomes) due to its selective repertoire, functional capabilities following cell/cell transfer and potential to serve as a disease-specific biomarker (Miranda et al. 2010; Mittelbrunn et al. 2011; Ohshima et al. 2010; Sole et al. 2015). As highlighted by key studies of Valadi et al. and Miranda et al., stringent RNA preparation technique and pre-incubation of EV isolates with RNase are considered to be the gold standard for studies of EV RNA content from biological fluids in order to definitively exclude contaminating, surface-bound nucleic acids (Miranda et al. 2010; Valadi et al. 2007).

Lipids: The lipid content of EVs has been less well investigated than that of proteins and nucleic acids. Nonetheless, it is known the lipid content of EVs differs from that of their cells
of origin. In particular exosomal lipid content has been associated with lipid raft areas in the past (De Gassart et al. 2003; Trajkovic et al. 2008). Specifically, in keeping with the origin of exosomes from the endosomal pathway and MVB, EV content compared to PC-5 cell surfaces indicated a decrease in phosphatidyl choline an increase in phosphatidylserine content. However both cell surfaces and EVs were enriched in sphingomyelin and cholesterol (Llorente et al. 2013). A comparison of EVs derived from MDA, human umbilical vein endothelial cells (HUVECs), human embryonic stem cells (hESCs), human mesenchymal stem cells (hMSCs) revealed cell type variation in composition. The enrichment showed the presence of cholesterol, phosphocholine, sphingomyelin in all cell lines whereas phosphoethanolamine and less phosphatidylserine were present in HUVEC and hESC but not in MDA and hMSC (Fuhrmann et al. 2015). In contrast, membrane shed vesicles contain phosphatidylserine in the outer membrane leaflet in addition to a high content of cholesterol (Heijnen et al. 1999). Also different classes of glycosphingolipids were found in renal cell carcinoma exosomes as well as prostate cancer cell exosomes (Del Boccio et al. 2012; Llorente et al. 2013).

Carbohydrates: Little is known about the carbohydrate content of EVs. Two published studies have applied the lectin microarray (LM) approach to profile surface glycans of EVs derived from cell lines. In 2009 Krishnamoorthy et al. reported that EVs from the H9 T cell line display a similarly glycoprofile to that of budded viral vesicles following human immunodeficiency virus (HIV) infection of the same cell line. The primary glycans detected were N-glycans, N-acetyl-lactosamine, (α2→3) and (α2→6) sialic acid (Neu5Ac or NeuAc) and fucose (Fuc). H9 EVs were also enriched in high mannose structures compared to cell membrane (Krishnamoorthy et al. 2009). Comparison of surface glycosylation of plasma membrane and EVs from human skin cancer cells (SkMel-5) was carried out by Batista et al. 2011. It was found that the EV fraction was enriched for carbohydrates including poly-N-acetyl-lactosamine, high mannose, (α2→6) Neu5Ac and complex N-glycans while blood group motifs and terminal N-acetylated-D-galactosamine (GalNAc) structures were shown to be reduced compared to parent cell membrane (Batista et al. 2011).
1.1.6 Isolation and storage procedures for EVs

A number of techniques have been described for isolation of EVs from biological fluids and cell culture supernatants although no single approach has proven to be universally superior. Multiple considerations come into play in selecting an EV isolation protocol including biological source, sample volume and number, downstream analysis application, degree of purity required, specific EV subtypes of most interest, resources and equipment available, processing time and cost (Tauro et al. 2012). Most isolation procedures will include one or two low-speed centrifugation steps to ensure cells and debris are initially separated from the EV-containing supernatant. This is typically followed by medium-speed centrifugation (10,000-17,000 x g) to ensure removal of apoptotic blebs (Fernández-Llama et al. 2010; Théry et al. 2006a). Subsequent EV isolation techniques can be divided into affinity-based, centrifugation-based, filtration-based and chromatographic.

**Ultracentrifugation:** Centrifugation at speeds above 100,000 x g (ultracentrifugation, UC) is frequently used to generate an EV pellet from biological fluids and culture supernatants (Fernández-Llama et al. 2010; Théry et al. 2006a). The efficiency of this approach is dependent on the viscosity of the fluid sample and optimization of rotor type, speed and centrifugation time is often required to maximize purity and yield (Cvjetkovic, Lötvall, and Lässer 2014; Fatemeh et al. 2012). For cell culture media, 100,000 x g for 70 min may be sufficient for urinary EVs (uEVs) up to 200,000 x g for 1 to 2.5 hours are reported (C. Y. Chen, Hogan, and Ward 2013; Hogan et al. 2014; H. Zhou, Yuen, et al. 2006). Compared to other isolation approaches, EV yields for UC are comparable and isolates are typically suitable for protein and nucleic acid analysis (C. Y. Chen, Hogan, and Ward 2013; Fernández-Llama et al. 2010; Hogan et al. 2014; Miranda et al. 2014; Musante et al. 2013; Théry et al. 2006a). Furthermore, combination of UC with sucrose density gradient centrifugation allows for further refinement of diverse EV subtypes (Hogan et al. 2014). However, UC-based isolation protocols are time- and resource-heavy and not well suited to processing large sample numbers quickly (Van Deun et al. 2014; Hogan et al. 2014; D. Wang and Sun 2014). Additionally several groups have demonstrated that UC does not consistently isolate all subtypes of EVs (Jeppesen et al. 2014; Lobb et al. 2015; Musante et al. 2013). A potential drawback is that non-vesicular proteins and nucleic acid may be co-precipitated, sometimes necessitating further clean up steps such as sucrose density gradient or cushion (Cvjetkovic, Lötvall, and Lässer 2014; Van Deun et al. 2014; Jeppesen et al. 2014; Lobb et al. 2015; Théry
et al. 2006a). As is discussed in greater detail in a subsequent section, isolation of EVs from urine samples by UC (and other techniques) entails the added challenge of fully separating EVs from the abundant urinary glycoprotein Tamm-Horsfall protein (THP, called uromodulin). Among the reported approaches to removing THP from uEV preparations are the use of deuterated water (D$_2$O)-based gradient or addition of 3-((3-cholamidopropyl)dimeethylammonio)-1-propanesulfonate (CHAPS) or dithiothreitol (DTT) (C. Y. Chen, Hogan, and Ward 2013; Fernández-Llama et al. 2010; Musante et al. 2012). Despite these disadvantages, UC-based isolation remains the method of choice for many basic and translational EV research projects.

**Filtration:** Microfiltration has also been widely applied to EV isolation from liquid samples. For example, commercially available centrifugal filter devices with molecular weight cut-off (MWCO) 3,000-100,000 Da have been adapted for this purpose (Cheruvanky et al. 2007; Lobb et al. 2015). Simple vacuum-based ultrafiltration has also been used with trapped EVs released from the filtration membrane by washing with phosphate-buffered saline (PBS) or other elution buffer (Lobb et al. 2015; Merchant et al. 2010). In comparison to UC, advantages of centrifugal filtration (CF) and other filtration protocols include ease of use, shorter time to isolation, lower cost and potential for processing higher numbers of samples (Cheruvanky et al. 2007). However, there are also a number of drawbacks including greater co-enrichment of non-vesicular proteins from biological fluids, aggregation/absorption of EVs in pressure driven cells, lower sample quality/vesicle integrity and, potentially, loss of smaller EVs (Cheruvanky et al. 2007; Gerlach et al. 2013; Lobb et al. 2015; Merchant et al. 2010; Rood et al. 2010).

**Affinity:** Affinity-based isolation of EVs has the potential to selectively purify individual EV subpopulations by targeting and capturing particles with specific surface markers. One such approach, involves incubating an EV-containing sample with magnetic beads coated with antibody or other affinity-based molecules with specificity for one or more EV surface markers then capturing the beads and attached vesicles by placing the sample in magnetic field (Kalra et al. 2013; Théry et al. 2006a). Among the EV markers that have been successfully targeted for affinity-based isolation from specific sample types are CD63 for uEVs and EpCAM as well as A33 with cancer cells (Echevarria et al. 2014; Mathivanan et al. 2010; Tauro et al. 2013). Although specificity and ease of use are distinct advantages of affinity-based EV isolation, the reagents are currently relatively expensive, antibody
dependent and not well suited to large sample size or number. Furthermore, purity and yield can be compromised if non-specific membrane fragments or abundant proteins are not efficiently removed in advance by pre-clarification (Graça Raposo and Stoorvogel 2013).

**Chromatography:** Size-exclusion chromatography (SEC) has been reported as a viable approach for isolation of highly-pure uEVs (Rood et al. 2010). The low molecular weight fractions were shown to contain co-precipitated proteins/peptides while the high molecular weight fractions were enriched for exosomal markers. An advantage of SEC is that it can separate EVs from additional components such as lipoproteins and proteins. This technique has been shown to result in EVs of similar quality to those isolated by sucrose density gradient UC from tissue culture supernatants (Lobb et al. 2015; Nordin et al. 2015). Potential disadvantages are the requirement for an initial concentration step and the collection of EV fractions into relatively large volume which may also necessitate post-isolation concentration steps. Overall, however, the use of chromatography has not, thus far, been widely adopted for translational EV research.

**Other isolation methods**

Commercially available isolation kits such as ExoQuick (System Biosciences), Exo-spin (Cell Guidance System), Total Exosome Isolation (Invitrogen) are now becoming more widely used. Published literature suggests that such products may not yet provide the same isolation efficiency or purity as the isolation techniques above (Van Deun et al. 2014; Lobb et al. 2015; Zubiri et al. 2014). Recently, vesicle isolation using microfluidics was tested but may not be suitable for isolation of larger quantities of EVs (C. Chen et al. 2010; He et al. 2014). Microfluidic devices are based on affinity and may impact diagnostics based on vesicles in near future.

**1.2 Urinary EVs, their bio-molecular content and applicability to clinical medicine**

It has now become clear that healthy urine has an abundant content of EVs and that urinary bio-molecules, which are specifically contained within the EV compartment, may uniquely convey important information regarding renal and urological health (Erdbrugger and Le 2015; Musante, Tataruch, and Holthofer 2014; Salih, Zietse, and Hoorn 2014). Urinary EVs were first clearly described and their importance highlighted in 2004 by the group of Mark Knepper
Comprehensive uEV protein profiling was carried out by this group and, later, by several others (Gonzales et al. 2009; Hogan et al. 2014; Merchant et al. 2010; Moon, Lee, et al. 2011). In addition to providing comprehensive lists of uEV-associated proteins, such proteomic studies have also provided evidence that renal cell type-specific proteins which are of low abundance in urine itself (e.g. the transcription factors ATF3 and WT-1) are enriched in uEVs and, thus, may be of diagnostic and prognostic value in disease settings such as AKI and FSGS (Hua Zhou et al. 2008). Findings such as these have helped to reveal the fact that uEV isolates contain vesicles which derive from all cell types of the nephron and urinary collecting system – highlighting both the clinical translational potential and the high level of complexity of uEV-based analytical research (Hoorn et al. 2005; Pisitkun, Shen, and Knepper 2004).

The growing literature on uEVs has already revealed many other general and specific details regarding their bio-molecular content – some of which may be considered predictable in the context of the broader body of knowledge regarding EVs (summarized in the preceding section of this Chapter). Among the major protein categories now confirmed to be present in uEV isolates are members of the ESCRT complexes, ion channels/transporters, enzymes, injury-induced proteins and components of the epithelial re-absorption complex including megalin and cubulin (Gonzales et al. 2009). Moreover, a number of pilot studies have explored the extent to which uEV-associated proteins may exhibit specific post-translational modifications such as phosphorylation and glycosylation (Echevarria et al. 2014; Gerlach et al. 2013; Gonzales et al. 2009; Saraswat et al. 2015). In addition, a growing number of studies has focused on uEV nucleic acid content – in particular uEV-associated miRNA in health and disease (M. L. Alvarez et al. 2012; Cheng et al. 2014; L.-L. Lv et al. 2013; Miranda et al. 2010; Ramezani et al. 2015). Overall, the main driving factors of uEV research have been its potential to: (a) Shed new light on the biological roles of EVs within the kidney and urinary tract. (b) Reveal new insights into the pathogenesis of kidney disease. (c) Facilitate the discovery of novel biomarkers and diagnostic/prognostics approaches for diverse forms of kidney diseases.

It is important to highlight that the promise of uEV-based research is accompanied by significant technical challenges. One major obstacle has been the establishment of suitable methods for isolation of high quality uEVs (Cheruvanky et al. 2007; Fernández-Llama et al. 2010; Merchant et al. 2010; Musante et al. 2013; Rood et al. 2010). For example, uniform
protocols for uEV isolation by UC have been proposed but not applied consistently, making comparison of the findings among published studies problematic – particularly in regard to potential kidney disease biomarkers (van Balkom et al. 2011; Dear, Street, and Bailey 2013; Dimov, Jankovic Velickovic, and Stefanovic 2009; Erdbrugger and Le 2015; Gámez-Valero et al. 2015; Hoorn et al. 2005; Knepper and Pisitkun 2007; Salih, Zietse, and Hoorn 2014).

Most importantly, the isolation and analysis of low-abundant uEV-enriched proteins is largely dependent on the removal of highly abundant non-vesicular urinary proteins such as Tamm-Horsfall protein, albumin and immunoglobulins (Igs) (Fernández-Llama et al. 2010; Hogan et al. 2009; Hoorn et al. 2005; Merchant et al. 2010; Pisitkun, Johnstone, and Knepper 2006; Zubiri et al. 2014). To achieve a high level of purity, a number of strategies have been reported for separation of uEVs from co-purified urinary proteins. These have included addition of reducing agents (for THP removal), use of albumin/globulin depletion kits and use of extra purification steps via sucrose density gradient UC and size-exclusion columns (C. Y. Chen, Hogan, and Ward 2013; Cheruvanky et al. 2007; Musante et al. 2012; Rood et al. 2010; Zubiri et al. 2014). Although each of these methods has been shown to be effective, it remains the case that uEV yields from typical, single-void urine samples are relatively low and that the isolation of high quality uEVs is a time-consuming and labour-intensive process that has limited the application of high throughput technologies and slowed the pace of discovery in this field (C. Y. Chen, Hogan, and Ward 2013; Fernández-Llama et al. 2010; Rood et al. 2010; Théry et al. 2006a).

Nonetheless, the number of studies in which kidney disease-specific biomarkers have been analyzed in uEV samples from cohorts of healthy and affected human subjects has increased significantly during the past decade, including the time-period during which the project reported in this thesis was performed.

The nature and most significant biomarker-related results of the published studies in this area are summarized in table 1-1.
Table 1-1: Human EV-based kidney disease biomarker studies over the past decade.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Sub-category</th>
<th>Sample No.</th>
<th>EV-Associated Disease Biomarker</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RNA</td>
<td>Protein</td>
</tr>
<tr>
<td>Diabetic Nephropathy</td>
<td>-</td>
<td>Control: n=3; Nephropathy: n=4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type 1 DM</td>
<td>Non-proteinuric: n=30; Proteinuric: n=18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls: n=25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AMBP↑, MLL3↑, VDAC1↓</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Type 2 DM</td>
<td>Control: n=34; Nephropathy: n=127</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Interstitial Fibrosis</td>
<td>-</td>
<td>Control: n=7; IF n=32</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Control: n=7; IF n=30</td>
<td></td>
<td></td>
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<tr>
<td>ADPKD</td>
<td>-</td>
<td>Control: n=7; ADPKD: n=7</td>
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<tr>
<td>Kidney Transplant</td>
<td>Cyclosporine Therapy</td>
<td>Non-CSA Treated: n=8; CSA Treated: n=39</td>
<td>NKCC2 and NCC↑</td>
<td>(Esteva-Font et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>prednisone, mycophenolate, tacrolimus.</td>
<td>mild-to-moderate rejection: n=7; cell-mediated rejection: n=6; antibody-med. reject: n=3; non-spec. findings: n=2</td>
<td>Pathway associated proteins &amp; partial discrimination of pathol. type</td>
<td>(Pasikun et al. 2012)</td>
</tr>
<tr>
<td></td>
<td>Early-Post-transplant Function</td>
<td>Control: n=11; Transplant: n=14</td>
<td>NGAL mRNA↓</td>
<td>(Peake et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>Delayed Graft Function</td>
<td>Transplant: n=15</td>
<td>NGAL↑</td>
<td>(Sonoda et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Donor: n=1; Recipient: n=1</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Light chain Amyloidosis</td>
<td>-</td>
<td>Control: n=8; Multiple myeloma: n=5; LC amyloidosis: n=8</td>
<td>High molecular weight light chain oligomers↑</td>
<td>(Ramirez-Alvarado et al. 2012)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Acute kidney injury</td>
<td>-</td>
<td>Control: n=2; CKD: n=4; AKI: n=4</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Control: n=3; Non-AKI: n=3; AKI: n=3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20
<table>
<thead>
<tr>
<th>Glomerular Disease</th>
<th>Control: n=3</th>
<th>AFT3 mRNA↑</th>
<th>(H.-H. Chen et al. 2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSGS</td>
<td>Control: n=8</td>
<td>PODXL↓</td>
<td>(Cheruvannky et al. 2007)</td>
</tr>
<tr>
<td>FSGS</td>
<td>Control: n=9</td>
<td>miR-1915↓; miR-155↓</td>
<td>(Ramezani et al. 2015)</td>
</tr>
<tr>
<td>FSGS</td>
<td>Control: n=5</td>
<td>WT-1↑ (active FSGS)</td>
<td>(Hua Zhou et al. 2013)</td>
</tr>
<tr>
<td>FSGS</td>
<td>Control: n=7</td>
<td>WT-1↑</td>
<td>(Hua Zhou et al. 2008)</td>
</tr>
<tr>
<td>IgA Nephropathy</td>
<td>Control: n=7</td>
<td>Aminopeptidase N↓; Vasorin precursor↓; Alpha-1 antitrypsin↑; Ceruloplasmin↑</td>
<td>(Moon, Lee, et al. 2011)</td>
</tr>
<tr>
<td>Thin Basement Membrane Disease</td>
<td>Pooled: n=7; TBMD: n=7</td>
<td>Aminopeptidase N↑; Vasorin precursor↑</td>
<td>(Moon, Lee, et al. 2011)</td>
</tr>
<tr>
<td>Membranous Nephropathy</td>
<td>Control: n=3</td>
<td>Nephrin, TRPC6, INF2, IQGAP1, PLA2 R</td>
<td>(Hogan et al. 2014)</td>
</tr>
<tr>
<td>Membrano-proliferative Glomerulo-nephritis</td>
<td>Control: n=3</td>
<td>Nephrin, TRPC6, INF2, IQGAP1, PLA2 R</td>
<td>(Hogan et al. 2014)</td>
</tr>
<tr>
<td>Minimal Change Disease</td>
<td>Control: n=5</td>
<td>miR-1225-5p↑</td>
<td>(Ramezani et al. 2015)</td>
</tr>
<tr>
<td>Lupus Nephritis</td>
<td>Control: n=8</td>
<td>miR-26a↓</td>
<td>(Ichii et al. 2014)</td>
</tr>
<tr>
<td>Nephrotic Syndrome</td>
<td>Control: n=5</td>
<td>WT-1↑ in FSGS WT-1↑ in remission</td>
<td>(Hua Zhou et al. 2013)</td>
</tr>
<tr>
<td>Nephrotic Syndrome</td>
<td>Steroid Sensitive: n=28</td>
<td>WT-1 Not different</td>
<td>(H. Lee et al. 2012)</td>
</tr>
<tr>
<td>Barter’s Syndrome Type I</td>
<td>Control: n=2</td>
<td>NKCC2 absent</td>
<td>(Gonzales et al. 2009)</td>
</tr>
<tr>
<td>Barter’s Syndrome or Gitelman’s Syndrome</td>
<td>Control: n=22</td>
<td>NKCC2 absent in BS-1, NCC absent in GS</td>
<td>(Corpetta et al. 2015)</td>
</tr>
<tr>
<td>Posterior urethral valves</td>
<td>Control: n=20</td>
<td>E-cadherin↓; TGF-β↑; N-cadherin↓; L1CAM↑</td>
<td>(Tmka et al. 2012)</td>
</tr>
<tr>
<td>Congenital unilateral hydro nephrosis in pelviureteral junction obstruction</td>
<td>n=22 postobstructed kidney vs contralateral kidney</td>
<td>AQP-1↓ TGF-β↑</td>
<td>(Li et al. 2012)</td>
</tr>
<tr>
<td>Chronic Kidney Disease</td>
<td>Control: n=7</td>
<td>CD2AP mRNA↓; miR-29c↓</td>
<td>(L.-L. Lv et al. 2013)</td>
</tr>
<tr>
<td>Chronic Kidney Disease</td>
<td>Control: n=11</td>
<td>IL-18 mRNA↑</td>
<td>(Peake et al. 2014)</td>
</tr>
</tbody>
</table>
For the majority of the studies summarized above, uEVs were isolated by basic UC protocols with or without addition of DTT. A limited number of studies applied sucrose density gradient as a further purification step or used alternative isolation techniques such as CF, microfiltration or SEC (Cheruvanky et al. 2007; Hogan et al. 2009; Merchant et al. 2010; Moon, Lee, et al. 2011; Rood et al. 2010; A. Sun et al. 2012). Some of these studies also reported results from relevant animal models alongside analyses of human subject samples, thus enhancing the overall biological significance of the results (Hogan et al. 2009; Sonoda et al. 2009; H. Zhou, Pisitkun, et al. 2006; Hua Zhou et al. 2008; Zubiri et al. 2015). As is obvious from table 1-1, most studies to date have been based on comparison of samples n≤30 of control and affected subjects. Furthermore, a number of studies has focused on a major clinical or pathological classification such as transplantation, fibrosis, AKI and glomerular disease for which multiple specific aetiologies are possible (Benito-Martin et al. 2013; L.-L. Lv et al. 2013, 2014; Sonoda et al. 2009; Hua Zhou et al. 2008). Limitations in sample sets may reflect the low prevalence of particular kidney diseases but also the challenge of coordinating clinical sample collection with laboratory-based isolation procedures. Nonetheless, some of the individual, disease-specific studies have provided initial evidence of clinical translational potential for uEV-associated biomarkers by reporting receiver operating curves for the establishment of sensitivity and specificity in discovery and validation cohorts (Moon, Lee, et al. 2011; Hua Zhou et al. 2013).

As shown in table 1-1, several published studies have focussed on nucleic acid-based biomarker discovery as an alternative to the more commonly pursued protein biomarkers. Interest in uEV-associated nucleic acids has increased significantly since 2010 when a number of published reports convincingly demonstrated the presence of abundant mRNAs and miRNAs in EV isolates, including uEVs (M. L. Alvarez 2014; L. L. Lv et al. 2013; Miranda et al. 2010) and provided deep sequencing of EV-associated RNA (Cheng et al. 2014; Gildea et al. 2013; Miranda et al. 2014). It should be emphasized that, as shown by Miranda et al. in a landmark study, accurate analysis of uEV-associated nucleic acids requires that EV isolates are subjected to RNAase and DNAse digestion steps prior to extraction of RNA from the internal compartment of the vesicles.

While the focus of this thesis is on the kidney in health and disease, uEV isolates may also provide important bio-molecular signatures for other forms of disease including malignant and non-malignant disease of the urinary tract (Duijvesz et al. 2013; Locke et al. 2009;
Mitchell et al. 2009; Principe et al. 2013), cardiovascular disease and even multiple sclerosis (Singh et al. 2015; Zubiri, Vivanco, and Gloria 2013).

Finally, the clinical translation of uEV analysis for biomarker discovery or disease pathogenesis investigation requires close attention to protocols and procedures for urine sample collection, processing and storage. A standardised protocol for collection of urine for uEV studies has been generated by the Human Kidney and Urine Proteome Project (HKUPP) (available online at: http://www.hkupp.org/Urine%20collectiion%20Documents.htm) based on best-available evidence from leading investigators in the field. Among the details of this protocol are the collection of first or second morning, mid-stream urine samples as these have been described as having comparable uEV protein amounts and yields in addition to being comparable to uEVs isolated from 24 hour collection. The addition of protease inhibitors to prevent degradation of uEV-associated proteins is widely practiced (and was used throughout the project reported in this thesis) but is not proven to be necessary and is not recommended by HKUPP for healthy urine at least. Processing of urine samples within 4 hours of collection is widely considered to be optimal although addition of a preservative such as sodium azide may allow for longer storage at room temperature (r.t.) prior to processing or freezing. Pre-clearing of urine prior to storage is recommended as is minimization of the number of freeze-thaw cycles. Pre-cleared urine may be stored for long periods of time at -80°C prior to uEV isolation. In regard to storage of isolated uEV preparations, Zhou et al. demonstrated that they may be stored at 4°C for short periods only and at -20°C for up to 4 days without loss of protein integrity. However, for long-term storage, a temperature of -80°C is necessary (H. Zhou, Yuen, et al. 2006).
1.3 Glycobiology and its importance for kidney health

Glycosylation characteristics ("glycomics") have a large impact on biological events in all living organisms. Carbohydrate structures can be extremely diverse especially in animal, bacteria, plants, yeast and insects (Ajit Varki 2011). The importance of carbohydrates is often underestimated or neglected during scientific investigations. In regard to glycan profiling technologies and disease-specific biomarker discovery, glycomic research is not as advanced as the fields of proteomics and transcriptomics. Factors contributing to this are the structural complexity of carbohydrate moieties (e.g. anomeric center, linkages, ring size and modification of hydroxyl groups), the co-association of glycans with proteins and lipids and the technical challenges of purifying and identifying the range of glycans present within cellular and subcellular biological samples (Laine 1997; A. Varki et al. 2009). In this section of the chapter, the basic principles and details of mammalian glycobiology and its analysis are described along with the specific relevance to the fields to kidney health and disease.

1.3.1 Mammalian glycosylation

The cell surface of mammalian cells is covered with a complex array of carbohydrates which changes during physiological events such as development, differentiation, activation, migration and cell death (Alisson-Silva et al. 2014; Comelli et al. 2006; Fernandez-Valdivia et al. 2011; Gagneux and Varki 1999; Galvan et al. 1998; Ishihara et al. 2014; Kawamura et al. 2014; Y.-J. Liang et al. 2010; Meesmann et al. 2010; Morris et al. 1984; Naito-Matsui et al. 2014; Rampal et al. 2005; Rapoport and Le Pendu 1999; Shi and Stanley 2003; P. Taylor et al. 2014; Van Vliet et al. 2013; Watanabe et al. 2002). This carbohydrate surface coating of cells is often referred to as the glycocalyx (figure 1-5) (Ajit Varki et al. 2009a).

Figure 1-5: Electron microscopic image of the internal surface of a blood vessel showing the carbohydrate coat (glycocalyx) attached to the luminal surface of the endothelial cell. From: Van den Berg, Vink & Spaan, Circulation Research (2003).
Several subtypes of carbohydrate-containing bio-molecules are found in nature (figure 1-6). These are referred to as glycoconjugates and include complexes of carbohydrates with proteins (glycoproteins, proteoglycans) and lipids (glycolipids) as well as secreted free glycans (e.g. glycosaminoglycans) (Ajit Varki et al. 2009e). In the case of glycoproteins, carbohydrate chains may be attached to polypeptides at either asparagine (N-glycosylation) or serine/threonine (O-glycosylation) residues. Carbohydrate structural variety is assumed to have evolved during specification as an adaption against pathogen attack (Gagneux and Varki 1999; Ajit Varki 2011).

Figure 1-6: Illustration of the basic subtypes of surface-bound and secreted carbohydrate structures associated with mammalian cells. Adapted from: Varki, A. Nature (2007).

Carbohydrate attachments to proteins modulate multiple aspects of their function including solubility, stability, susceptibility to enzymatic degradation, binding to other proteins, host-pathogen interactions, and antigen recognition (Bateman et al. 2008; Billings and Pacifici 2015; Fernandez-Valdivia et al. 2011; Garner et al. 2001; Geijtenbeek et al. 2004; Gerken, Butenhof, and Shogren 1989; Kamerling, Boons, Lee, Suzuki, Taniguchi, and J. 2007; Kato et al. 2006; Leung et al. 2012; Lusch et al. 2013; Masayoshi et al. 1990; Rabinovich, Van Kooyk, and Cobb 2012; Rampal et al. 2005; Reinhart, Obedeanu, and Sobel 1990; Rudd et al. 1994; Springer 1990; P. Van Den Steen et al. 1998; Suzuki et al. 2000; Toyoda et al. 2000). Enzymes such as glycosyltransferases and glucosidases are essential for the biosynthesis and processing of glycoconjugates and, thus, their dysfunction can result in disease-causing
abnormalities of carbohydrate structures. Examples of diseases involving inherited or acquired abnormalities of glycosylation include galactosemia, Ehlers-Danlos Syndrome, Tn-syndrome and IgA Nephropathy (IgAN) (Isselbacher et al. 1956; Kresse et al. 1987; Raska et al. 2007; Schadewaldt et al. 2004; Q. Sun et al. 2015; Tongzhong and Cummings 2005). Typically N-glycans are attached to proteins at an N-X-S/T motif in which “X“ may be any amino acid except proline.

In general, N-glycans are built upon three conserved configurations defined as complex-, hybrid- and high-mannose structures (figure 1-7). In mammalian glycosylation, complex N-glycans usually exist in bi- to tetra-branched structures although higher levels of branching may occur (Ajit Varki et al. 2009g). The individual antennae of N-glycans often include LacNAc (N-acetyllactosamine) residues which may be capped with NeuAc/Neu5Ac and/or attached with Fuc residues (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007e; Marth and Grewal 2008; Ajit Varki et al. 2009g). O-glycans are attached to protein backbones at S/T-residues which, in some mucins, may be followed by a proline. Although O-glycan structures are less conserved than N-glycans, eight core structures have been defined (figure 1-7). These may be elongated with polyLacNAc units or blood group antigens as in case of mucins (Gill, Clausen, and Bard 2011; Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007e; P. Van Den Steen et al. 1998; Ajit Varki et al. 2009h).

Figure 1-7: Structures of N-glycans (A), O-glycans (B) with illustrations of branching diversity in both cases. Adapted from: Johnson, J. L. Trends Immunol. (2013).
Importantly, glycosylation varies significantly between species and certain structural characteristics and configurations may be considered typical for human glycoproteins. For example, human N- and O-glycans can be terminally capped with Neu5Ac residues in either an \((\alpha 2\rightarrow 3)\) or an \((\alpha 2\rightarrow 6)\) linkage (Marth and Grewal 2008). Moreover, Fuc residues may appear in the core and/or the antennary structures of N-glycans with \((\alpha 1\rightarrow 6)\) linkages for core and \((\alpha 1\rightarrow 3)\) linkages for antennary branches. Additional linkages of fucosylation (e.g. \((\alpha 1\rightarrow 2)\)) occur in glycoproteins due to blood group associated motifs (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007c). Another specific type of linkage to consider is the bisecting glycan structures often observed in immunoglobulins (Hossler, Khattak, and Li 2009; Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007e; Wormald et al. 1997). Additionally, glycoproteins may frequently contain several carbohydrates attached to their backbones and each glycan attachment may consist of a distinct structure. As a result of this is so-called micro-heterogeneity of glycans, the structural and functional characteristics of glycoproteins can be particularly difficult to investigate (Ohtsubo and Marth 2006).

### 1.3.1.1 Biosynthesis of N-glycosylated proteins

In the classical protein translation and transport process, the attachment of N-linked oligosaccharides to proteins occurs as a regulated process in a co/post-translational manner (summarised in figure 1-8). Ribosomal translation of the protein from mRNA is followed by its translocation into the lumen of the endoplasmatic reticulum (ER) where further modifications are initiated (Helenius and Aebl 2001; Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007d). A precursor N-glycan molecule, \(\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{dolichol pyrophosphate}\), is first attached to N-X-S/T motifs via the action of oligosaccharyltransferase (OST) (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007g; Kornfeld and Kornfeld 1985). Correct folding of the nascent glycoprotein, which is essential for proper function, is initiated by removal of one glucose (Glc) residue via \(\alpha\)-glucosidase I generating the substrate for the carbohydrate binding protein (lectin) malectin (Hettkamp, Legler, and Bause 1984; Schallus et al. 2008). Malectin was suggested to associate with immaturely folded glycoproteins possibly to ensure their retention in the ER (Galli et al. 2011). Subsequently, \(\alpha\)-glucosidase II removes a second Glc residue from the immature glycoprotein. This facilitates interaction with specific chaperones such as calnexin/calreticulin in addition to the oxidoreductase ERp57 (Frickel et al. 2004; Hammond, Braakman, and Helenius 1994; Jessop et al. 2009; Oliver et al. 1999; Ware et al. 1995). Together, these
proteins control glycoprotein folding and prevent aggregation within the secretory pathway which, in the case of some proteins can lead to cell stress (Halperin, Jung, and Michalak 2014). Once a protein is properly folded the final terminal Glc residue is removed by α-glucosidase II which allows further transport towards to the cis-Golgi network (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007d, 2007h; E. S. Trombetta and Helenius 1998). Removal of mannose (Man) by ERMan-I may also promote interaction with cargo receptors such as ERGIC-53, a lectin-domain containing protein which further promotes transport through the Golgi network via the ERGIC pathway (Appenzeller et al. 1999; Appenzeller-Herzog et al. 2005; Gonzalez et al. 1999; Itin et al. 1996; Kamiya et al. 2008; Tremblay and Herscovics 1999).

By contrast, improper protein folding is detected by UDP-glucose:glycoprotein glucosyltransferase which adds a new terminal Glc residue to the glycoprotein leading to its re-entry into the calnexin/calreticulin cycle until folding is corrected (Hammond, Braakman, and Helenius 1994; Hebert, Foellmer, and Helenius 1995; Ritter et al. 2005; S. E. Trombetta and Parodi 1992). Alternatively, degradation of improperly folded proteins may occur (Słomińska-Wojewódzka and Sandvig 2015). Although complete details of how degradation is mediated are lacking, it is known that EDEM proteins 1 to 3 interact with oligosaccharides on misfolded glycoproteins, removing them from the calnexin/calreticulin cycle and promoting their degradation by the ER-associated degradation (ERAD) pathway (Hirao et al. 2006; Hosokawa et al. 2010; Mast et al. 2005; Molinari et al. 2003; Sousa, Ferrero-Garcia, and Parodi 1992). Glycans are further truncated by the mannosidase activity of EDEM which enhances interactions with MRH lectin domains of the proteins OS-9 and XTP3-B (Fujimori et al. 2013; Groisman et al. 2011; Hosokawa et al. 2009; Satoh et al. 2010; Yamaguchi et al. 2009). While OS-9 contributes to the removal of misfolded proteins, XTP3-B was recently suggested to be a negative regulator of the ERAD system (Fujimori et al. 2013; Mikami et al. 2010). Misfolded proteins that are targeted for degradation are retro-translocated to the cytosol by proteins such as Derlin-1 where final degradation by cytosolic PNGase and the proteasome occurs (Benyair, Ogen-Shtern, and Lederkremer 2015; Cho, Lee, and Jun 2013; Greenblatt, Olzmann, and Kopito 2011; Hirsch, Blom, and Ploegh 2003; J.-H. Lee et al. 2005; Oda et al. 2006; F. Wang, Olson, and Shyng 2012).

Overall, the Golgi compartments comprise about 200 glycosyltransferases and glucosidases that contribute to the trimming and elongation of the ends of the glycan precursor (Marth and
Grewal 2008). This set of glycoenzymes is also responsible for the assembly and processing of O-glycans, N-glycans and glycolipids (figure 1-9) (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007h; Y.-C. Lee et al. 1993). Fully synthesised glycoproteins are directed to the cell membrane or to the secretory machinery (Helenius and Aebi 2001; Moremen, Tiemeyer, and Nairn 2012; Ajit Varki et al. 2009c).

Figure 1-8: Glycoprotein biosynthesis and degradation pathways in the endoplasmic reticulum for N-glycans. Adapted from: Moremen, Tiemeyer and Nairn, Nat Rev Mol Cell Biol. (2012).
Figure 1-9:Trimming and processing of N-glycan precursors in the Golgi apparatus yielding in mature N-glycan structures. Adapted from: Varki et al., Essentials of Glycobiology (2009).

1.3.1.2 Biosynthesis of GPI-anchored proteins

Proteins referred to as GPI-anchored are inserted into the plasma membranes via a phosphatidylinositol (PI) linker which may dictate the localisation and functional characteristics of the protein (T Kinoshita, Murakami, and Morita 2007; Taroh Kinoshita, Fujita, and Maeda 2008). Such proteins are known to display specific glycosylation features in the anchor region and may also be subject to N- and O-linked glycosylation of their polypeptide backbone (Brewis et al. 1995; Nakano et al. 1994). In general, GPI-anchors consists of a PI molecule which is further modified by three mannose (Man), one glucosamine (GlcN) and one ethanolamine phosphate (EtN-PO₄) unit. The biosynthetic steps involved in the generation of GPI-anchored proteins are summarised in figure 1-10. In mammalian cells, a typical GPI-anchored protein contains an unsaturated lipid core structure as well as two diacyl or 1-alkyl and 2-alkyl residues per PI-unit, respectively (Brewis et al. 1995). In addition two to three EtN-PO₄ residues are attached mainly to the first, second and/or terminal
Man residues of the GPI-anchor, respectively. The main events in the GPI-anchored protein biosynthetic pathway are: (a) Addition of N-acetylglucosamine (GlcNAc) nucleotide to the PI molecule, (b) deacetylation of GlcNAc to generate glucosamine (GlcN), (c) flipping of the precursor anchor from the cytosol towards the lumen of the ER, (d) generation of a linear chain of Man residues including EtN-PO$_4$ modifications (e) transfer of the protein with its C-terminal end to the terminal EtN-PO$_4$ within the GPI anchor after cleavage of a signal peptide in the protein by a translocase, and (f) deacetylation of the GPI anchor (Ashida et al. 2005; Taroh Kinoshita 2014; Kostova et al. 2000; M. Takahashi et al. 1996; Taron et al. 2004; Young Kang et al. 2005). Finally the GPI-anchored protein is transported to the Golgi network where the lipid and glycan structures may be further modified (Brewis et al. 1995; Fujita et al. 2011). Carbohydrate substrates required during biosynthesis of GPI-anchored proteins are delivered either as nucleotide sugar or connected to dolicholphosphate (Ashida, Maeda, and Kinoshita 2006; Kostova et al. 2000; Tomita et al. 1998). Examples of GPI-anchored proteins reported to be present within uEV preparations include THP and CD24 – both of which are also known to have heavily glycosylated polypeptide backbones (Bleckmann et al. 2009; Fernández-Llama et al. 2010; Keller et al. 2007; Kristiansen, Sammar, and Altevogt 2004; Olczak and Olczak 1999; Rindler et al. 1990; Van Rooijen, Kamerling, and Vliegenthart 1998).

![Figure 1-10: Summary of the sequential steps involved in the biosynthesis of GPI-anchors in the endoplasmic reticulum.](image)

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**Legend:**
- PI: Phosphatidylinositol
- GlcN: glucosamine
- GlcNAc-N-acetylglucosamine
- Dol-P-Dolicholphosphate
- Man-Manose
- EtNP-ethanolamine phosphate
- PE-phosphatidyethanolamine
- GIP-glycosyolphosphatidylinositol

---

**Biochemical Reactions:**
- Addition of GlcNAc to PI
- Deacetylation of GlcNAc to GlcN
- Flipping of the precursor anchor from the cytosol to the ER lumen
- Generation of a linear chain of Man residues
- Transfer of the protein with its C-terminal end to the terminal EtN-PO$_4$
- Deacetylation of the GPI anchor

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**Examples of GPI-anchored proteins reported to be present within uEV preparations:**
- THP and CD24
- Both heavily glycosylated polypeptide backbones
1.3.1.3 Biosynthesis of O-glycosylated proteins

In contrast to N-glycan biosynthesis, O-glycans are attached in a post-translational manner to serine or threonine residues of the protein backbone by a process that occurs exclusively in the Golgi network. Biosynthesis of O-linked core 1 and core 2 structures (see figure 1-7B) can be carried out by most cell types while core structures 3 and 4 are primarily confined to the colon and are not discussed further here (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007b). More core structures 5-8 do exist in human but are reported to be extremely rare present (Ajit Varki et al. 2009h). Synthesis of both core structures 1 and 2 involves the initial attachment of GalNAc to the serine or threonine residue. Nucleotide sugars (e.g. UDP-GlcNAc, UDP-Gal) are required as substrates for the glycosyltransferases (Brockhausen et al. 1985; Gantt et al. 2011; Meynial, Paquet, and Combes 1995). In mucin-type glycans, GalNAc is preferentially transferred to threonine residues by core 1 (β1→3) galactosyltransferase(C1GalT) with a requirement for the core 1 β3GalT specific molecular chaperone (Cosmc) (Ju and Cummings 2002; Ju et al. 2002; Qin et al. 2005). This O-glycan is often instantly sialylated in the following step by specific sialyltransferases or further branched into core 2 structures by core 2 (β1→6) N-acetylglucosaminyltransferase (Bierhuizen and Fukuda 1992). Core 1 and core 2 structures may be further modified with galactose (Gal), Fuc, GlcNAc and Neu5Ac/NeuAc in a sequence-specific manner, although direct addition of Gal to core 1 O-glycan structure does not occur (Bierhuizens, Maemura, and Fukuda 1994; Ohtsubo and Marth 2006). The final structure of O-glycans depends on a variety of factors including the S/T binding motif, expression and activity of key enzymes, availability of nucleotide-linked monomer substrates and precise location within the Golgi network (Bennett et al. 1999; Gerken, Ten Hagen, and Jamison 2008; Gerken et al. 2006; Granovsky et al. 1994; Hassan et al. 2000; Ju and Cummings 2002; Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007b; Perrine et al. 2009; Qin et al. 2005; Skrincosky et al. 1997; Wandall et al. 2007). Taken together, this is a highly complex process with the potential to generate a wide range of permutations of the final O-glycan structure (Gill, Clausen, and Bard 2011; Laine 1997; P. Van Den Steen et al. 1998).

1.3.1.4 Mammalian glycolipids

Glycolipids comprise one or several saccharide units covalently attached to a lipid moiety. The first glycolipid identified, galactosylceramide (GalCer), consists of a single Gal molecule linked to the C-1 hydroxyl group of a lipid moiety ceramide (Ishizuka 1997; Ajit Varki et al.
Two classes of mammalian glycolipids exist, namely glycoglycerolipids and glycosphingolipids (Hermansson, Hokynar, and Somerharju 2011; Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007f; Maccioni, Giraudo, and Daniotti 2002). The lipid component in glycoglycerolipids and glycosphingolipids comprise ceramide and acylalkylglycerol, respectively. Of the two, glycosphingolipids are the most abundant and biologically important in mammalian tissues whereas glycoglycerolipids were reported in rat brain as well as mammalian testis (van Gestel et al. 2015; De Haas and Lopes-Cardozo 1995; Ajit Varki et al. 2009d; Y. Zhang et al. 2005). Glycolipids are primarily found within “lipid raft” domains of cell membranes and may display blood group motifs (Haslam and Baenziger 1996; Ajit Varki et al. 2009d). The core structure for glycosphingolipids is glucosylceramide (Glc-Cer) with the major sub-types being distinguished by subsequent addition of additional saccharides to this core as summarised in table 1-2 (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007f).
Table 1-2: Summary of the structures of various glycosphingolipids Based on: Kamerling, Boons, Lee, Suzuki, Taniguchi and Voragen, Comprehensive Glycoscience Vol 1-4 (2007e).

<table>
<thead>
<tr>
<th>Glc-Cer</th>
<th>Common core structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganglio-</td>
<td>Gal(β1→3)GalNAc(β1→4)Gal(β1→4)Glc(β1→1’)Cer</td>
</tr>
<tr>
<td>Globo-</td>
<td>Gal(α1→4)Gal(β1→4)Glc(β1→1’)Cer</td>
</tr>
<tr>
<td>Iso-globo-</td>
<td>Gal(α1→3)Gal(β1→4)Glc(β1→1’)Cer</td>
</tr>
<tr>
<td>Lacto-</td>
<td>Gal(β1→3)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1’)Cer</td>
</tr>
<tr>
<td>Neolacto-</td>
<td>Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1’)Cer</td>
</tr>
</tbody>
</table>

The biosynthesis of glycosphingolipids is illustrated in figure 1-11. As shown, generation of various series of glycosphingolipids occurs according a series of alternative enzymatically-controlled pathways (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007a).

Figure 1-11: Summary of the biosynthesis of glycosphingolipids. Adapted from: Kamerling, Boons, Lee, Suzuki, Taniguchi and Voragen, Comprehensive Glycoscience (2007a).
Extensions to individual glycosphingolipid core structures can be large and lead to terminal elongations including negatively charged (by addition of Neu5Ac/NeuAc or sulphate in case of sulfatides) and neutral monosaccharides (Ishizuka 1997; Ajit Varki et al. 2009d). Typical elongations include carbohydrate units of Fuc, Neu5Ac/NeuAc, Gal, GlcNAc. Glycosphingolipids are synthesized initially on the cytosolic surface of the ER following which they are flipped onto the internal side of the ER membrane then transported to the Golgi network for further modification by glycosyltransferases (Ajit Varki et al. 2009d). Currently, it remains unclear to what extent the glycosyltransferases responsible for glycosphingolipid synthesis overlap with those responsible for truncating and processing N-glycans (Ajit Varki et al. 2009d).

The biological roles of glycosphingolipids include signalling, cell recognition, immune response and modulation of receptors (Hakomori and Igarashi 1995; Iwabuchi et al. 2012; Pontier and Schweisguth 2012; Sonnino et al. 2009; Yoshizaki et al. 2008). As might be expected from this range of functions, the glycosphingolipid composition of individual cell types is regulated during development and differentiation (Y.-J. Liang et al. 2010; Maccioni, Giraudo, and Daniotti 2002). In contrast, glycoglycerolipids are less investigated than glycosphingolipids thus their function in mammals remains less clear. However, glycoglycerolipids of germ cells appear to be required for spermatogenesis (Honke 2013).

1.3.2 Lectins and their application to glycoanalysis

Certain proteins with structural and functional characteristics that are distinct from those of antibodies and enzymes have specific, reversible binding affinities for carbohydrates (Gabius et al. 2011; Pusztai 2008a). These proteins, which have been recognised for over 100 years, are referred to as lectins (Els J. M. Van Damme et al. 1998; Komath, Mannem, and Swamy 2006). Lectin-carbohydrate interactions have been described with dissociation constants (Kd) mainly between the milli- and micro-molar range (Hirabayashi et al. 2013). In addition, lectins often demonstrate high specificity for carbohydrate ligands and this property has been adapted for use in a wide range of analytical and diagnostic approaches, including tissue staining by immunohistochemistry, blood group typing and the microarray platform used in this project (Faraggiana et al. 1982; Gerlach et al. 2013; F. Khan et al. 2002).

Lectins have been identified broadly across the spectrum of living organisms including fungi, viruses, bacteria, plants and animals (Komath, Mannem, and Swamy 2006; Sharon and Lis
The diversity of known lectins and their sources has led to difficulty in developing a unified classification system. The advantages and disadvantages of the different lectin classification systems that have been proposed are well discussed by Komath et al. (Komath, Mannem, and Swamy 2006). Briefly, an early classification by Sharon was based on specific carbohydrate ligands such as D-Man, D-Gal/D-N-GalNAc, L-Fuc, Neu5Ac, D-GlcNAc. Other classification approaches are based on primary protein sequence and/or topology leading to particular families (S-type (galectins), C-type, I-type, L-type, M-type, P-type, R-type lectin grouping). Additionally, lectin structure can be used for classification as suggested by Lis and Sharon. This latter classification includes three lectin groups simple, mosaic and macromolecular structures. The majority of lectins also contain hydrophobic regions that may bind to specific protein ligands (Pusztai 2008b). To date, no overall classification has established that can be readily applied to all lectins but each of the existing systems may be of value for individual areas of research (E.J.M. Van Damme et al. 1997a).

Some lectin-carbohydrate interactions were reported to be very specific allowing for discrimination of carbohydrate epimers as well as 2’OH modifications as seen with Gal and GalNAc residues. The ligand binding affinity and specificity of lectins are based on structural and chemical features of their carbohydrate binding domains (Hamelryck et al. 1999; Ravishankar et al. 1999; Sharma and Surolia 1997). The tertiary and quaternary structures often contain β-sheets with barrel roll (prism type I or type II, β-trefoil folds), hevein or jelly roll architectures (Sinha et al. 2007). Chemically, the main contributing factors to the binding of lectins to carbohydrates are van der Waals forces as well as hydrophobic interactions and non-covalent interactions (π-stacking) between C-H bonds of carbohydrate rings with aromatic residues of the proteins. In addition, molecules such as water and cations such as Ca\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\) may stabilize lectin structure and/or improve ligand binding (Kamerling, Boons, Lee, C., et al. 2007; Komath, Mannem, and Swamy 2006; Pusztai 2008c). The stabilizing role of cations is of particular importance for ligand binding to C-type lectins (Drickamer 1999). The biological activities of lectins on cell surfaces are often complex as a result of the co-recognition of oligosaccharides and the proteins to which they are attached. For example, lectins may bind carbohydrates in a monovalent or multivalent manner depending on the architecture of their binding domains (E.J.M. Van Damme et al. 1997b). Multivalent interactions may lead to clustering effects of proteins on the cell surface with high potential for triggering intracellular signalling or other functional responses (Sacchettini,

The project described in this thesis was almost exclusively based on the use of plant lectins to interrogate the surface glycome of uEVs. The natural functions of many plant lectins remain poorly understood. One beneficial effect that has been proposed is the capture of nitrogen-fixing bacteria (Bohlool and Schmidt 1974; Diaz et al. 1989; Rodríguez-Navarro, Dardanelli, and Ruiz-Sainz 2007). Experimental work with plant lectins such as WGA has also demonstrated antifungal function (Ciopraga et al. 1999). Additionally, lectins may participate in growth-dependent regulations and may exhibit similar function as storage proteins in plants (Kamerling, Boons, Lee, C., et al. 2007; Peumans and Van Damme 1995b). Regardless of their physiological roles, a wide range of plant lectins have been identified and are typically given abbreviated names that reflect the plants from which they are derived (Kamerling, Boons, Lee, C., et al. 2007). Over several decades, plant lectins have found a wide range of applications across different scientific disciplines and industries and are highly desired due to their stability and abundance (Ambrosi, Cameron, and Davis 2005; Peumans and Van Damme 1995a).

Individual plant lectins have been used for some time to purify bio-molecules, identify tissue or categorise cell stages based on their carbohydrate affinities (Batisse et al. 2004; Faraggiana et al. 1982; S. Feng et al. 2009). Only quite recently, however, have larger panels of lectins been adapted to high-throughput profiling platforms such as LMs based on the microarrays that revolutionized the field of genomics in the mid 1990s (Schena et al. 1995). Advanced LM technology was developed in 2005 and was initially used to screen glycoproteins for their lectin affinities (Angeloni et al. 2004; Kuno et al. 2005; Pilobello et al. 2005; Zheng, Peelen, and Smith 2005). This approach allowed for small quantities of purified protein (nM range) to be broadly and rapidly analysed for glycosylation characteristics in a high throughput format (Hirabayashi et al. 2013). Today, this state-of-the-art technology is frequently used for a wider range of applications including profiling of complex mixtures of glycoproteins and of the surface glycomes of intact, viruses, bacteria, eukaryotic cells and subcellular particles (Ebe et al. 2006; Gerlach et al. 2013; K.-L. Hsu, Pilobello, and Mahal 2006; Krishnamoorthy et al. 2009). In addition to plant lectins, glyco-analytical arrays have also been developed for various purposes using libraries of mammalian lectins, antibody-lectin sandwich, glycans, glycolipids, glycopeptides and other affinity reagents (S. Chen et al. 2007; Love and
Recently LM technique has been expertly reviewed by Jun Hirabayashi et al. (Hirabayashi et al. 2013). Briefly, the main principle of LM is the covalent attachment of multiple individual purified lectins to chemically-modified glass or hydrogel surfaces (see figure 1-12). This allows for simple and reproducible anchoring of lectins onto specific locations (“spots”) within a small surface area using printing techniques that typically produce spots (or features) of less than 500 µm diameter (Gerlach et al. 2013; Pilobello et al. 2005). A key challenge during this printing process is the maintenance of stable lectin architecture to avoid loss of binding affinity. Once the printing conditions are optimised, however, batches of microarray slides may be efficiently printed and stored or used immediately for glycan profiling. For performance of a LM analysis, the free spaces on the slide are quenched, a fluorescently-labelled analyte is applied to the slide surface and the array is imaged and quantified using a standard microarray scanners and analytical software package. From the image, the relative amount of fluorescence at each lectin feature is quantified for subsequent comparative analysis. Analysis approaches such as hierarchical clustering that are frequently utilized in fields of genomics and proteomics can be applied to LM datasets.
Figure 1-12: Overview of the lectin microarray technique.

Left: Distinct surface anchors that may be used for the attachment of lectins or other affinity reagents.
Right: Following immobilization of affinity molecules (1), the surface is blocked, exposed to fluorescently labelled analyte, scanned and converted to image files (2) and the resulting dataset is statistically “mined” using software-based analysis strategies such as principal component analysis and hierarchical clustering (3). Adapted from: Hirabayashi et al., Chemical Society Reviews (2013).

Further advancements in LM in the past ten years include the development of highly-sensitive evanescent fluorescence technique (allowing for analysis of proteins in the picomolar range) and dual-colour analysis for comparison of experimental and reference samples in competitive assays (Krishnamoorthy et al. 2009; Kuno et al. 2005; Uchiyama et al. 2006). Both approaches were used to discriminate the cellular surfaces of wild-type Chinese Hamster Ovary (CHO) cells from those of the glycosylation-defective CHO mutant cell lines lec8 which lacks the expression of GlcNAc (Ebe et al. 2006; Pilobello, Slawek, and Mahal 2007). The glycomic array field is also developing towards engineered lectins to avoid the seasonal variability in the availability purified plant lectins, to enlarge the repertoire of specific lectins and to increase affinity available for detection of glycan moieties (Arnaud, Audfray, and Imberty 2013; Ribeiro and Mahal 2013).
1.3.3 Kidney function, structure and specialized cell types

The kidneys function primarily to excrete metabolic waste products by generating approximately 180 L/day of cell-free filtrate from the blood and processing it into urine. In addition, however, they also play a key role in controlling blood pressure, water, electrolyte and acid-base balance, reabsorption of peptides/proteins, activation of the vitamin D hormonal system and regulation of red blood cells through the production of erythropoietin (Boron and Boulpeap 2005; Curthoys and Moe 2014; Haraldsson, Nyström, and Deen 2008; J. L. Zhou and Xiao 2013).

Each kidney comprises many individual functional units called nephrons, the absolute number of which is estimated to vary in healthy humans from approximately 0.4 to 1.5 million/kidney depending on genetic and environmental factors as well as on the method used to estimate nephron number (J. L. Zhou and Xiao 2013). The overall functional efficiency of the kidneys, termed glomerular filtration rate (GFR), represents the combined filtration capacities of the individual nephrons (Levey, Inker, and Coresh 2014). Each nephron consists of a filtration structure called the glomerulus connected to a complex tubular structure comprising several functionally and phenotypically distinct segments (figure 1-14A, table 1-2) (R. Lifton et al. 2009).

The glomerulus consists of afferent and efferent arterioles connected by a capillary “ball” containing fenestrated endothelium, basement membrane (GBM) and unique external epithelial layer (podocytes) which, together, form the glomerular filtration barrier (figure 1-14B) (Boron and Boulpeap 2005; Jalanko et al. 2001). The initial cell-free filtrate which passes through this barrier is collected into an extra-vascular compartment (Bowman’s space) from which it passes into the lumen of the proximal tubule for subsequent processing (R. Lifton et al. 2009). Water, ions and small proteins with a positive charge readily pass into Bowman’s space but large proteins and negatively charged molecules may be retained in the blood due to the charge selectively of the glomerular basement membrane which contains several proteoglycans, glycosaminoglycans as well as other glycoconjugates (Haraldsson, Nyström, and Deen 2008). Thus, the highly complex and as-yet incompletely understood structure of the glomerular filtration barrier is essential for normal kidney function.

The proximal tubular segments are responsible for the greatest amount of reabsorption of water, ions, peptides/proteins and amino acids (Christensen and Verroust 2002; Curthoys and
Moe 2014). Subsequently, the loop of Henle, distal tubule, connecting tubule and collecting duct “fine tune” the resulting urine via complex transport mechanisms and specialized cell types to appropriately control the final excretion rates of water, sodium, acid, potassium, calcium, phosphorous, magnesium and other tightly-regulated molecules (R. Lifton et al. 2009). The tubule is surrounded throughout its length by a dense network of peri-tubular capillaries which are contiguous with the glomerular vessels and facilitate the return of reabsorbed water, ions and bio-molecules into the blood stream (Boron and Boulpeap 2005; R. Lifton et al. 2009). Individual tubular functions leading to final concentrated urine are summarized in table 1-2.

Two subtypes of nephron have been described, superficial cortical and juxtamedullary, based on their location within the kidney as well as the length of the loop of Henle. In general, juxtamedullary nephrons play a greater role in modulating blood pressure via a specialized interface between the distal convoluted tubule and the afferent arteriole called the macula densa region or through the influence of the renin/angiotensin system (Boron and Boulpeap 2005; Mount 2014).

Once it has undergone the final stages of processing in the collecting duct, filtrate from all nephrons is channeled through several papillae into the pelvis of the kidney and from there, via the ureter into the bladder (figure 1-14C). Urine released periodically from the bladder through the urethra contains water, electrolytes, metabolites, protein/peptides as well as EVs, intact cells and cell debris which may originate from any point between the glomerulus and the urethra (Boron and Boulpeap 2005; Decramer et al. 2008; Moon, You, et al. 2011).
Figure 1-13: Basic features of the nephron and renal collecting system.

A. Simplified diagram of the basic components of the nephron including the glomerulus and Bowman’s capsule and the major tubular segments. Adapted from an image available at: http://www.biologycorner.com/anatomy/urinary/urinary_labeling_key.html


C. The macro-anatomy of the kidneys collecting system and lower urinary tract. Adapted from: image available at: http://www.uofmchildrenshospital.org/healthlibrary/Article/40091
<table>
<thead>
<tr>
<th>Tubular region</th>
<th>Primary function</th>
<th>Overall body function</th>
<th>Cell type</th>
<th>Receptor/pores/functional proteins</th>
<th>Lit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulus</td>
<td>Blood filtration, exclusion of highly charged molecules via slit diaphragm and glomerular basement membrane</td>
<td>Center of blood filtrations, part of macula densa region for blood filtration and GFR regulation</td>
<td>Mesangial cells, podocytes, endothelial cells</td>
<td>Nephrin, podocin, podocalyxin, ZO-1, CD2AP, P-cadherin; collagen type V, fibronectin, entactin, proteoglycans, laminin</td>
<td>(Haraldsson, Nystrom, and Deen 2008)</td>
</tr>
<tr>
<td>Proximal tubules</td>
<td>Reabsorption: NaCl, HCO₃⁻, Ca²⁺, PO₄³⁻, SO₄²⁻, K⁺ proteins/peptides Glc, amino acids; uptake: NH₄⁺ vs. HCO₃⁻ organic cationic and anionic molecules, water absorption</td>
<td>Regulation of blood pressure; gluconeogenesis; homeostasis for fluid &amp; electrolytes; acid-base balance; water permeability; passive process</td>
<td>3 distinct cell types (S1,S2,S3) with apical brush border</td>
<td>Na⁺/K⁺-ATPase, NHE3, SLC5A1, SLC3A2, NCX1, NCX2, Na⁺/HCO₃⁻-transporter, AQP-1, organic cationic and anionic transporters, Megalin,Cubulin, Kir6.1, Kir7.1, claudins</td>
<td>(Giebisch 2001; Hou, Rajagopal, and Yu 2013; J. L. Zhou and Xiao 2013)</td>
</tr>
<tr>
<td>Thick ascending limb and loop of Henle</td>
<td>Reabsorption: Na⁺; Ca²⁺/Mg²⁺ homeostasis, Cl⁻, HCO₃⁻; ammonium, K⁺ transport, THP expression (30-50 mg/day); &lt;20 mg/day albumin secretion, generation of hypertonic environment via special. juxtamedullary nephron; water uptake (thin descending limb)</td>
<td>Acid-base balance, modulation of urine concentration; water uptake; part of macula densa region for blood filtration and GFR regulation</td>
<td>S cells (smooth surface cell type) R cells (rough surfaced cell type)</td>
<td>NKCC2, ROMK, Carbonic anhydrase, 30 pS, 70 pS, BK channel. NHE3, AQP-1, Na⁺/K⁺-ATPase (not desc limb). KCC4, claudins, Cl⁻/HCO₃⁻-exchanger and K⁺/HCO₃⁻-exchanger, NBC1n1, NHE4, THP</td>
<td>(Mount 2014)</td>
</tr>
<tr>
<td>Distal tubule</td>
<td>Production of kallikrein (hormone); secretion: H⁺/HCO₃⁻; absorption of K⁺ (via IC) reabsorption: Na⁺,Cl⁻; secretion of K⁺ (via PC) homeostasis: Ca²⁺/Mg²⁺ regulated water uptake by ADH; secretion of H⁺/NH₄⁺ ions</td>
<td>pH-regulation of urine, tightly regulated uptake of electrolytes, concentration of urine (ADH dependent), part of macula densa region</td>
<td>Intercalating cells (IC), connecting tubule (CT), principal cells (PC)</td>
<td>ROMK, NCC, SLCl2A13,SLCl2A7, KCC4, NDBCE, SLC4A8, ENaC TSC TRPM6, Na⁺/K⁺-ATPase, Kir4.1, Kir4.2, Kᵢr5.1, claudins, Cl⁻/K⁺-K⁺, BK, TRPV5, Calcium ATPase, NCX1, Klotho, SLC4A11, Kv1.1</td>
<td>(Subramanyan and Ellison 2014; Weiner and Verlander 2011)</td>
</tr>
<tr>
<td>Collecting duct</td>
<td>Water uptake, urea excretion regulation via hormones e.g. aldosterone, arginine, vasopressin; absorption: Mg²⁺, K⁺, Na⁺ transport of HCO₃⁻,Cl⁻ modulation of intravascular volume, secretion of H⁺ production of prostaglandins, secretion of NH₄+/H⁺</td>
<td>Water reabsorption, acid-base balance, immune defense by IC against E.coli; production of NGal during UTI and antimicrobial peptides, modulation of Na⁺, K⁺ levels in plasma, extracellular fluid control, blood pressure</td>
<td>IC, cortical principle cells (PC)</td>
<td>ENaC, Na⁺/K⁺-ATPase, BK, H⁺-ATPase, H⁺/K⁺-ATPase, SLC4a1, pendrin, carbonic anhydrase II, AQP-2, AE1,BK, SLC26a11, GPR4, NDBCE, AE4/SLC4a9, ROMK, CFTR, AQP3 and AQP4, claudins</td>
<td>(Pearce et al. 2015; Roy, Albateineh, and Pastor-Soler 2015; Thébault, Hoendeno, and Bindels 2006; Weiner and Verlander 2011)</td>
</tr>
</tbody>
</table>
1.3.4 Chronic kidney disease: epidemiology, outcomes and diagnosis

The frequency of life-style associated diseases such as hypertension, obesity, cardiovascular disease and diabetes mellitus has increased dramatically in industrialised societies in recent decades. As reported by the World Health Organisation (WHO), the rise in such chronic non-communicable diseases reflects population aging combined with unhealthy diet, reduced exercise, smoking and consumption of alcohol (Jha et al. 2013). This has resulted in a very large increase in the prevalence of chronic kidney diseases (CKD) for which the commonest causative factors include diabetes mellitus, hypertension and aging. The 2013 United States Renal Data System (USRDS) report highlighted the fact that the prevalence CKD, defined as urine albumin creatinine ratio (ACR) $\geq 30$ mg/g and/or estimated glomerular filtration rate (eGFR) $\leq 60$ml/min/1.73m$^2$, among the 2005-2010 National Health and Nutrition Examination Survey (NHANES) cohort was 13.1%. When sub-divided into age groups, CKD prevalence within this cohort was 5.7% for 20-39 year-olds, 9.1% for 40-59 year-olds and 35.0% for those > 60 years old (“2013 USRDS Annual Data Report -CKD in the General Population” 2014).

Furthermore, in 2011, Medicare records from the US indicated that 9.2% of those insured had CKD and that these individuals accounted for 18.2% of the Medicare budget. The high costs for medical care are generated by the requirements for on-going complex medical management as well as for renal replacement therapies (RRT) such as dialysis and kidney transplantation (KTx) (Fishbane et al. 2015). The annual mortality risk for adults over the age of 66 with advanced (Stage 4-5) CKD is as high as 74 per 1000. This risk rises further to 139 per 1000 when CKD is associated with cardiovascular diseases or diabetes. In addition to carrying high risk for mortality, individuals with CKD patients are at risk for end-stage renal disease (ESRD) which requires the initiation of RRT to maintain life (“Précis: An Introduction to Chronic Kidney Disease in the U.S.” 2014). In Ireland, CKD has also increased in recent decades with the prevalence among the Irish population estimated to be 7%. According to the Irish National Renal Office annual report in 2013, the incidence of ESRD in the Republic of Ireland was 88 patients per million population (pmp) with 81% of these initiating haemodialysis (HD), 14% initiating peritoneal dialysis (PD) and 5% receiving a KTx. The prevalence of ESRD in this report was 862 patients pmp, representing a total of 3960 people (Epidemiology of ESKD in Ireland - Data from 2013 Census 2013).
In recent years, CKD has come to be recognised as a significant global health problem rather than as a relatively uncommon medical illness. The need for greater public awareness is driven by the fact that the most common causative factors for CKD are potentially preventable as well as the fact that progression of CKD may be slowed with early recognition and treatment (Levey and Coresh 2012; Wouters et al. 2015). The term CKD encompasses all pathologies that result in reduced kidney function or alter morphology in the nephron. The full range of aetiologies for non-diabetic CKD is very broad and includes individual forms of glomerular and tubulointerstitial disease as well as vascular and inherited cystic diseases (“Part 9. Approach to Chronic Kidney Disease Using These Guidelines” 2002). Furthermore, the course of CKD can be highly variable ranging from rapid failure within weeks to months to clinically silent disease that progresses over years to decades (Levey and Coresh 2012; Wouters et al. 2015). One category of kidney disease that is dealt with in the final section of this thesis is glomerulonephritis (GN). This represents a family of renal diseases that share the common feature of acute or chronic glomerular injury mediated by inappropriate intraglomerular activity of elements of the immune system. Pathological examination of kidney tissue from clinical or experimental GN typically demonstrates infiltration of macrophages, neutrophils or T cells; deposition of antibody and/or complement components; fibrin deposition and activation/proliferation of native glomerular cell populations (Chadban and Atkins 2005). Clinically, the extent of renal damage caused by GN and other causes of CKD is estimated and monitored by measurement of biomarkers such as creatinine and albumin in blood and urine respectively. According to the 2002 National Kidney Foundation guidelines, CKD is defined by a reduction in creatinine-based eGFR to 60ml/min/1.73 m² or less or by functional or structural abnormalities without decreased GFR for longer than 3 months (National Foundation DOQI 2002). Other guidelines have incorporated the presence of abnormal excretion of albumin in the urine, most commonly measured as ACR in a first morning “spot” urine sample (Regeniter et al. 2009). The most commonly used cut-off between normal and abnormal ACR is 30 mg/g (Eckardt et al. 2013; National Foundation DOQI 2002; Wouters et al. 2015).

As described above, diagnosis of kidney disease typically occurs via clinical tests such as serum creatinine and urine protein but other clinical and laboratory such as measurement of blood pressure and microscopic examination of the urine sediment provide additional essential information (“Part 9. Approach to Chronic Kidney Disease Using These Guidelines”
A basic schematic for CKD diagnosis and management is presented in figure 1-6. Importantly, in order to fully diagnose the cause for abnormal kidney function, provide appropriate treatment and monitor therapeutic response, more comprehensive diagnostics are typically required. This may include specific serological tests (especially in the case of suspected GN), imaging studies (e.g. ultrasound, computed tomography (CT)-scan and magnetic resonance imaging (MRI)-scanning) and renal biopsy (Chadban and Atkins 2005; Levey and Coresh 2012; Wouters et al. 2015). Family history and genetic testing for inherited diseases may also key to accurately defining CKD aetiology ("Part 8. Recommendations for Clinical Performance Measures" 2002). Regardless of aetiology, classification of CKD has become an important clinical tool for monitoring progress and standardising management. The most frequently used classification system, most recently defined by the kidney disease: improving global outcomes (KDIGO) guidelines published in 2013, is based on the initial assessment of eGFR typically using the creatinine-based MDRD or CKD-EPI formula along with urine ACR. According to this system, CKD can be classified into 5 stages linked to risk for adverse outcomes as shown in table 1-3 (Levey, Stevens, et al. 2009; National Foundation DOQI 2002; Rate 2006; “Summary of Recommendation Statements” 2013). Early stages of CKD may present as asymptomatic disease discovered during routine health screening (Qaseem et al. 2013). Currently, there is a need to identify new biomarkers that can better discriminate early stage CKD and define future risk for mortality or ESRD with the aim of improving initial treatment and reducing overall healthcare costs for CKD and its complications (Wouters et al. 2015).
Unfortunately CKD is often associated with high mortality, primarily due to cardiovascular diseases, in addition to increased risk for hospitalisation due to AKI and infection (Bellomo, Kellum, and Ronco 2012; Malhotra, Beniwal, and Pursnani 2012). Even in the absence of such complications, reduced GFR over an extended period of time may progress to ESRD requiring dialysis and/or KTx (National Foundation DOQI 2002; “Part 9. Approach to Chronic Kidney Disease Using These Guidelines” 2002; Peralta et al. 2011; Wouters et al. 2015). Treatment for CKD can be considered to be both generalized and disease-specific depending on the aetiology. Generalized CKD therapy focuses on evidence-approaches for preventing cardiovascular events and slowing the progression of eGFR decline. Among the treatments proven to be of generalized value in CKD are blood pressure lowering agents (especially renin angiotensin system inhibitors in the case of diabetic and other proteinuric causes of CKD), dietary modifications (reduced sodium, low protein), cholesterol lowering drugs, correction of chronic acidosis and improved glycaemic control in diabetic mellitus (DM). For specific aetiologies, immunosuppressive and anti-proliferative agents,
corticosteroid and anti-coagulants and other interventions may stabilise or improve kidney function (Qaseem et al. 2013; Wouters et al. 2015). In all cases, the potential benefits of each therapeutic intervention must be weighed against the possible adverse effects (Chadban and Atkins 2005).

Overall, CKD is a challenging, complex disease that is difficult to access by clinical trials due to slow progression and that may remain asymptomatic until late stage. Additional, complexity comes from the heterogeneity of underlying cause (Eckardt et al. 2013; Levey, Cattran, et al. 2009). The identification of new biomarkers of kidney function and risk for progression such as cystatin C, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1) has generated very strong interest as a means to more quickly and accurately detect beneficial effects of novel therapeutic agents in clinical trials for CKD (Z. Khan and Pandey 2014; Shlipak and Day 2013; Wouters et al. 2015). Indeed, a combination of several biomarkers may prove to be most suitable for more powerful prediction of outcome (Z. Khan and Pandey 2014; Peralta et al. 2011). The project described in this thesis focused on the possibility of harnessing new knowledge of uEVs, specifically their surface glycosylation, as a new source of biomarker discovery in the setting of kidney disease.

### 1.3.5 Glycosylation in the kidney and in urine extracellular vesicles

As the focus of the project described in this thesis was on glycosylation of uEVs, it is important to highlight the relevance of diverse glycosylation events and glycoproteins to specific aspects of renal anatomy, physiology and disease pathogenesis. One of the most significant roles for carbohydrates within the kidney occurs in the glomerulus and is linked to the filtration process. In the first place, the glomerular endothelial surface has a proteoglycan layer which can be partially removed by hyaluronidase, chondroitinase and heparinase as shown by Hjalmarsson et al. and Jeansson et al. (Hjalmarsson, Johansson, and Haraldsson 2004; Jeansson and Haraldsson 2006). In vivo attenuation of this endothelial glycocalyx by heparanase has been linked to proteinuria and microvascular dysfunction in diabetic nephropathy (Garsen et al. 2014). Glycans also play an important part in the normal function of the podocyte. For example, removal of Neu5Ac residues from podocyte surfaces using neuraminidase, as performed by Andrews et al., led to loss of the slit diaphragm (Andrews 1979). In addition, the podocyte expresses multiple individual glycoproteins which are essential for maintenance of normal podocyte morphology and of the integrity of the
glomerular filtration barrier. These include podocalyxin, nephrin and alpha-dystroglycan
(Drozdova, Papillon, and Cybulsky 2013; Harrison et al. 2012; Holzman et al. 1999;

In the renal tubule, ion channels, receptors and transporters such as the aquaporins, megalin,
urea transporter A1 (UT-A1) and transient receptor potential vanilloid type 5 (TRPV-5) have
been shown to undergo glycosylation and the regulation of this process may play key roles in
the localization and functional state of these proteins (G. Chen et al. 2011; Leunissen et al.
2013; Morelle et al. 2000; Sonoda et al. 2009; Wolf et al. 2014). As an example, TRPV-5, an
ion channel involved in calcium uptake in the distal tubule, was shown to be endocytosed
after removal of Neu5Ac by glycosidases on the cell surface (Cha et al. 2008). This
endocytosis of TRPV-5 from the epithelial cell surface may be prevented by the carbohydrate
binding protein galectin 3 allowing for continued calcium uptake (Leunissen et al. 2013). A
role in this process for the extracellular domain of α-Klotho through its glucoronidase activity
has been suggested although this remains controversial (Andrukhova et al. 2014). A similar
Klotho-dependent regulatory process has been described for the potassium channel ROMK1
(Cha et al. 2009). Thus, glycosylation and its regulation is likely to be an essential factor in
normal ion and water balance as well as protein reabsorption in the renal tubule. Importantly,
glycosylation patterns of renal proteins have been shown to be altered in disease settings with
associated changes in protein functions. For example, Ravidà et al. used lectin
immunoblotting, lectin affinity chromatography and mass spectrometry to demonstrated
differential expression of glycoproteins in the kidneys of diabetic compared to healthy rats.
For the enzyme dipeptidyl peptidase-IV, glycosylation changes due to diabetes were
associated with altered function in rats (Ravidà et al. 2015).

Glycoproteins are also known to contribute to renal protection from infection. The heavily
glycosylated protein THP (also called uromodulin), which is expressed and shed largely in the
thick ascending limb of the loop of Henle, exhibits the ability to interact with E. coli and
therefore may prevent attachment of the bacteria (Devuyyst, Dahan, and Pirson 2005; Pak et al.
2001). Finally, glycolipids of various types as well as sulfatide have been shown to be present
in abundance in renal tissue and may play a role in acid-base homeostasis (Kamerling, Boons,
Lee, Suzuki, Taniguchi, and Voragen 2007f; Stettner et al. 2013; T. Takahashi and Suzuki
2012).
In summary, some of the glycoproteins such as AQP-1, AQP-2, megalin, CD24, nephrin, podocalyxin are expressed in specific sections of the nephron and have also been detected in uEVs by immunoblotting or mass spectrometry. In most of these studies the carbohydrate component in these proteins was often neglected (Hogan et al. 2014; Keller et al. 2007; Pisitkun, Shen, and Knepper 2004; Sonoda et al. 2009; H. Zhou, Yuen, et al. 2006). Considering the wide variety of glycosylated proteins present within the kidney and the specialized functions carried out along the length of the nephron, it is not surprising that lectins have been used for some time as staining reagents in the identification of specific nephron sections and cell types within kidney tissue samples.

The presence of these and other glycoproteins within uEVs and the known presence in uEVs derived from all cell types along the nephron would suggest that uEV isolates contain a complex and biologically important glycome. Furthermore, the functional relevance of many glomerular and tubular glycoproteins to various disease states provides incentive to analyse uEV-associated glycans in both health and disease. As discussed in Section 1.1.5 of this Chapter, the carbohydrate content of EVs has been studied to a limited extent during the past 5 years. However, at the time this project was initiated, and even as it progressed few published studies broadly addressed uEV glycosylation and its potential alterations during kidney disease (Echevarria et al. 2014; Saraswat et al. 2015; Staubach et al. 2012).

Thus, based on the scientific and clinical background described in this Introductory Chapter, the topic of uEVs and uEV glycosylation can be considered to: (a) Reflect a complex biological process with significance for normal and abnormal cell-cell communication. (b) Relate to an area of human health that is of growing global significance. (c) Carry potential for identification of novel biomolecular signatures of kidney health within a readily accessible biological fluid. (d) Address existing technical and knowledge gaps.
Chapter Two

Aims and Hypotheses
2 Aims and hypotheses

2.1 Rationale and novelty

During the past decade, a strong interest in uEVs has emerged. This was driven both by the desire to better understand the role of EVs in the kidney and by the more pragmatic goal of identifying new and better kidney disease biomarkers within urine. To date, however, the field has placed a large emphasis on analysis of uEV proteins and nucleic acids with relatively little attention given to another relevant biocomponent - carbohydrates. Although lectins have been used for decades to study the nephron – primarily through immunohistochemistry – the application of lectin-based glycoprofiling technologies to better understand the carbohydrate content of uEVs had not been performed at the time this project was initiated. Thus, the central rationale for the project was that the combination of techniques for EV isolation and lectin-based glycoprofiling would yield new knowledge of the surface glycome of intact uEVs. By initially studying samples from healthy individuals we sought define the normal uEV surface glycome. Comparison with uEV samples from patients with known kidney disorders (polycystic kidney disease and gomerulonephritis) was planned as a means to evaluate the potential for uEV glycomic analysis to serve as a platform for discovering new, non-invasive biomarkers of renal disease.

2.2 Overarching goal

Define the normal surface glycosylation profile of human urinary extracellular vesicles and determine whether this profile is detectably altered in the setting of specific kidney diseases.

2.3 Hypotheses

1. Urinary EVs express a complex array of carbohydrate moieties in their surfaces that is consistent among healthy individuals.
2. Kidney disease is associated with specific alterations to uEV surface glycosylation.

2.4 Specific aims

1. To apply lectin-based profiling to characterize and validate a surface glycosylation signature of uEVs isolated by different protocols from urine of healthy adults.
2. To develop an approach for applying LM technology to compare the surface glycoprofiles
of density gradient-separated fractions of healthy adult uEVs.

3. To determine whether uEV density, size and surface glycosylation characteristics are altered in the urine of adults with CKD due to biopsy-proven GN.
Chapter Three

Material and Methods
3 Material/Methods

3.1 Materials

Details of reagents and materials used, buffer solution compositions and equipment items employed for the project are listed in the Appendix to the thesis (Page 205 pp.).

3.2 Methods

3.2.1 Collection and pre-clarification of urine samples

Roche protease inhibitor tablets (1 tablet per 50 ml) were placed in a 120 ml collection jar (Sarstedt, Germany) and provided to healthy adult volunteers and patients enrolled by informed consent at outpatient clinics. Subjects were provided with instructions for collecting a “clean catch” urine sample. For healthy adult volunteers, first morning void urine samples were collected. For enrolled patients, “spot” urine samples were collected during morning outpatient clinics at Merlin Park University Hospital, Galway. Patient samples were collected and relevant clinical details recorded from the medical records by informed consent under a protocol approved by the Galway University Hospitals Ethics Committee (copies of the patient information leaflet and consent form are included in the Appendix).

3.2.2 High-speed (ultra) centrifugation

Frozen urine samples were thawed at r.t. then subjected to centrifugation at 17,000 x g for 10 minutes at to remove large subcellular particles such as apoptotic blebs. The supernatants were transferred into high-speed centrifugation tubes which had been pre-rinsed twice with PBS to remove any residual material on the tube walls. The high-speed centrifugation carried out for 2.5 hours at r_{max} 150,000 x g and at 17 °C. Subsequently, each pellet containing uEVs was carefully re-suspended in sterile PBS and frozen at -80 °C to be further processed within a maximum of one year.

For experiments in which Tamm-Horsfall protein was removed from the uEV high-speed pellet using the reducing agent TCEP, frozen uEV samples were thawed slowly on ice followed by concentration in PBS using Amicon® ultra-centrifugal filters at 10,000 x g for 10 minutes at 4 °C. Next, TCEP·HCl was pre-dissolved in 100-200 µl PBS and the concentrated uEV suspensions were added to a final concentration of 0.6 M. The solution was vortexed for 30-60 seconds followed by brief (1 minute) centrifugation at RT on a bench-top microcentrifuge. This cycle of vortexing and centrifugation was repeated up to 10 times until
the foaming of the uEV solution was absent or minimal. Subsequently, the uEV/TCEP·HCl suspensions were added immediately to pre-rinsed high-speed centrifugation tubes filled with PBS to a total volume of 30 ml. The uEV samples were then re-pelleted by high-speed centrifugation (150,000 x g for 2.5 hours at 17 °C). The TCEP-treated uEV pellets were re-suspended in PBS and protein content was estimated with a Pierce® Bicinchoninic acid (BCA) protein assay kit.

### 3.2.3 Centrifugal concentration

*This method was developed and performed for the project by Dr. Jared Q. Gerlach.* Pre-clarified urine samples (see 3.2.1) were treated with 350 mg DTT per 15 ml urine at 37 °C for 30 min. Urine was aliquoted into 2 ml volumes and chill on ice for 15 min. Aliquots were spun at 17,000 x g to remove apoptotic blebs for 15 min at 4 °C. Supernatant was transferred into centrifugal concentrators (4 ml, 100 kDa MWCO, Millipore) for centrifugation at 2000 x g at 4 °C until 50 µl of urine volume remained. The concentrate was removed and Centrifugal filter walls were washed with 100 µl PBS prior storage at -80 °C in PBS buffer.

### 3.2.4 Precipitation of Tamm-Horsfall Protein

*This method was developed and performed for the project by Dr. Jared Q. Gerlach.* Isolation of Tamm-Horsfall protein was carried out according to Tamm and Horsfall (Tamm and Horsfall 1952). Pre-clarified urine was supplemented with 34 mg of NaCl per 1 mL urine and vortexed. The urine was placed on ice for 2 h to allow precipitation of THP which was then pelleted by centrifugation at 1800 x g, 15 min at 4 °C. The pellet was re-dissolved in water and NaCl precipitation was repeated three times. The final pellet was resuspended in 10 ml water and dialized twice against water with a 15 kDa MWCO membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA). The dialized protein was lyophilized and stored at 4 °C until use.

### 3.2.5 AlexaFluor 647 labelling of Tamm-Horsfall Protein

*This method was performed for the project by Dr. Jared Q. Gerlach.* Purified THP was labelled with AlexaFluor 647 as NHS-ester according to manufacturer’s protocol (Life Technologies). The excess dye was removed by dialysis against water (Gerlach et al. 2013).
3.2.6  Protein estimation by BCA assay

Aliquots of frozen uEV samples thawed on ice or freshly prepared uEV suspensions were subjected to protein estimation using the Pierce™ BCA protein assay kit according to manufacturer’s protocol. Briefly, 20 µl of deionized water and 5 µl of uEV suspension or protein standard were added into a 96-well microtest-plate. The BCA reagent was prepared and added according to product instructions and the microtest-plate was shaken at 37 °C for 30 minutes in a LEEC compact incubator, model: K2N. The absorbance of standard and unknown samples was then detected at the wavelength of 562 nm using a SpectraMax® M5e plate-reader (Molecular Devices, UK). Protein concentrations of unknown samples were calculated from the standard curve using proprietary Excel software.

3.2.7  Bis-Tris Polyacrylamide gel electrophoresis (PAGE)

Samples were diluted with PBS or deionized water to a minimum volume of 10 µl and 5X reducing buffer was added as required. Next, samples were briefly vortexed and centrifuged using table-top instruments then heated to 95 °C for 10 minutes using a heating block. Next, samples or Seeblue2 Pre-stained standard were briefly centrifuged and transferred into pre-cast Bis-Tris NuPAGE® 4-12% gradient gels (Invitrogen, Life Technologies) and subjected to electrophoresis in MOPS buffer at 200 V for 55 minutes. The gels were washed twice for 5 minutes with deionized water, fixed for 15 minutes by shaking at RT in 30% (v/v) ethanol / 10% (v/v) acetic acid and then washed twice in 10% (v/v) ethanol for 10 minutes. Finally the fixed gels were stored overnight in deionized water with silver staining carried out the following day as described below.

3.2.8  Silver stain

For silver staining of NuPAGE® gels, the manufacturer’s protocol was followed. Briefly, 50 µl of sensitizer solution were added to 25 ml deionized water. The gel with sensitizer solution was shaken intermittently for 1 minute. Gels were washed twice for 1 minute each in deionized water then incubated in 25 ml silver stain containing 500 µl enhancer solution at RT for 30 minutes with intermittent shaking. Next, the staining solution was removed and the gels were washed twice in deionized water for 20 seconds. Finally, the silver stain was developed with 25 ml development solution supplemented with 500 µl enhancer solution for 2 to 3 minutes following which the staining reaction was stopped by transferring the gels to 5% (v/v) acetic acid. Images of stained gels were acquired on a CanonScan LiDE90 scanner.
3.2.9 Immunoblotting

For immunoblotting, NuPAGE® gels were transferred to PVDF membranes (Immobilon®-P SQ, 0.22 µm pore size) immediately following electrophoresis according to Bunkelmann et al. (Brunkelmann, Corpas, and Trelease 1995). Four pieces of extra thin blotting paper (15 cm x 15 cm) were prepared and two each were soaked in cathodic or anodic transfer buffer. The hydrophobic surface of the PVDF membrane was broken by immersing in methanol following which membranes were soaked in anodic solution for 5 minutes. The transfer construct was then assembled in the following order: Two anodic solution-soaked blotting papers, 1 PVDF membrane, 1 NuPAGE® gel, 2 cathodic solution-soaked blotting membranes. The transfer was performed using a Trans-Blot®SD Semi-Dry Electrophoretic Transfer Cell at 10 V for 1 hour at RT.

Following transfer, the membranes were immersed overnight in 50 ml 1.5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) with 0.05% (w/v) Tween20® (TBST) at 4 °C. The next day, the membranes were washed twice with TBST for 15 minutes with intermittent shaking. Primary antibody incubations were carried out with solutions of anti-human AQP-2 (13 µl in 17 ml TBST/3% (w/v) bovine serum albumin (BSA)) or anti-human CD24 (1.9 ml in 20 ml TBST/5% (w/v) BSA) for 3-4 hours at 4 °C with gentle shaking followed by two 15 minutes washes in TBST. Secondary antibody incubations were then performed in solutions of horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG (4 µl in 20 ml 1X TBST) for 1 hour at RT with gentle shaking followed by two 15 minutes washes in TBST. Membrane signals were then developed by applying enhanced chemical luminescent (ECL) substrate solution containing HRP to the membranes. The chemiluminescent signal was detected and imaged using a Kodak Image-station 4000 MM Pro®.
3.2.10 Lectin blotting

Protein transfer was performed as described above. Membrane blocking was carried out in TBST, 1.5% (w/v) BSA on an orbital shaker at RT for 30 minutes followed by two 10 minute washes in TBS and one wash in TBST containing 1 mM each of MgCl₂, CaCl₂ and MnCl₂. Next, biotinylated lectin solutions (1 µg/ml PNA, SNA-I, GSL-I, PHA-E, or AAA in TBST) were added and the membranes were incubated at RT for 1 hour on an orbital shaker followed by three 10 minutes washes in TBS. The membranes were then incubated with avidin-alkaline phosphatase solution (1:10,000 in TBS) for 1 hour at RT followed by three 10 minute washes in TBS. Finally, the colour reaction was developed using 1 SIGMA-FAST® BICP/NBT tablet in 10 ml of water. Images of stained gels were acquired on a CanonScan LiDE90 scanner.

3.2.11 Flow cytometry analysis

*Binding of uEVs to beads:* Attachment of uEVs to aldehyde latex beads was performed in 1.5 ml Eppendorf® tubes. Aliquots of freshly prepared or thawed uEV suspensions containing 5 µg of protein (as determined by BCA assay) were diluted in PBS to final volumes of 40 µl following which 10 µl of a 4% (w/v) stock suspension of sulfated aldehyde latex beads (ø= 4 µm) were added and incubated for 15 minutes at RT. Next, 950 µl of PBS were added and the suspensions were incubated either overnight in a cold room with rotation or for 2 hours at RT. Blocking of the bead surfaces was carried out by adding 110 µl of 1 M glycine in PBS followed by incubation at RT for 30 minutes, following which the beads were pelleted by centrifugation in a bench-top microcentrifuge at 1700 x g for 3 minutes at RT. The supernatant was removed carefully using a hand-held pipette and 1 ml of PBS, 0.5% (w/v) BSA was added to the bead pellet which was then gently re-suspended and re-pelleted. The washing step was repeated 3 times following which the uEV-coated beads were stored at 4 °C until further use. Aliquots of beads incubated with PBS alone were processed through the entire procedure to serve as “non-uEV coated” control samples.

In some experiments, uEVs were fluorescently labelled with PKH26 prior to coating of beads or, in the case of experiments carried out using samples provided by collaborators at Mayo Clinic, USA, uEV samples were pre-labelled with the fluorescent dye CM-DiI. Additionally, in some experiments, beads were coated with THP or with other proteins (BSA, fetuin, asialo-fetuin) by incubating them with solutions of purified proteins according to the same protocol.
**Staining of bead-bound uEVs:** The staining strategy for flow cytometric analysis of uEV-coated beads is summarized in figure 3-1.

**Figure 3-1:** Labelling strategy for FCM.

Optimal concentrations of biotinylated lectins in PBS, 0.5%(w/v) BSA were first determined in preliminary staining experiments [summarized in table 4-2 Result Chapter 4 (page 96)].

Staining assays were carried out in 96-well microtiter plates. First 50 µl of PBS, 0.5% (w/v) BSA or 50 µl of the appropriate primary lectin or antibody solution were added to individual wells. Next, 10 µl of uEV-coated, purified protein-coated or control (uncoated) beads were added to wells as appropriate. The plates were incubated at 4 °C for 30 minutes following which 150 µl of PBS, 0.5% (w/v) BSA were added and the beads were pelleted by centrifugation of the plates for 5 minutes at 450 xg in a table-top centrifuge. The solutions were then dumped from the plates by rapid inversion over a sink, the plates were briefly vortexed to loosen the pellets and the wells were refilled with 200 µl of PBS, 0.5% (w/v) BSA. A total of 3 washes was performed in this manner after which the beads were re-supended in 50 µl PBS, 0.5% (w/v) BSA and 10 µl of either (a) 1:40 dilution of PE-Cy7- or APC-conjugated Streptavidin (SA) to detect biotinylated lectins or (b) 1:100 dilution of FITC-conjugated F(ab')2 anti-mouse IgG (Fc) to detect mouse anti-human CD24 monoclonal antibody. Following incubation in the dark at 4 °C for 30 minutes, 140 µl PBS, 0.5% (w/v) BSA were added and the plates were washed as described above. Finally, beads were re-suspended in 150 µl of PBS, 0.5% (w/v) BSA, the individual samples were transferred to 5 ml FACS tubes and were analysed using a FACSCanto® flow cytometer (BD Biosciences) and Flowjo® version 7.6.4 (TreeStar, Inc., Ashland, OR, USA) software. For each sample, 5000 events were acquired. Data was analysed as median fluorescence intensities (MFI) of the appropriate fluorescence. Non-specific background & binding was substracted and data was presented as fold-increase of MFI over control sample.
3.2.12 PKH26 labelling of uEVs

Labelling reactions were carried out using a protocol recommended by the manufacturer. Briefly, uEV samples were subdivided into 50 µl aliquots in 2 ml Eppendorf® tubes and 200 µl Diluent C were added. Separately, 246 µl of Diluent C were added to 3 µl ethanol along with 1 µl of PKH26 stock solution. The 250 µl PKH26 stock solution was added to the uEV solution for a final volume of 500 µl which was then mixed by pipetting for 1 minute prior and rested at RT for a further 1 minute. Next, 500 µl PBS were added, mixed and rested for 1 minute at RT. Finally, an additional 1 ml of PBS was added and the labelled samples were kept on ice before being concentrated with Amicon® centrifugal filtration units (0.5 ml, 100 kDa MWCO; 10,000 x g, 4 °C, 10 minutes). The centrifugal filters were rinsed with PBS and the final, concentrated PKH26-labelled uEV suspensions were kept on ice before use in flow cytometry experiments or loading onto a sucrose density gradient.

3.2.13 Sucrose density gradient

The linear sucrose density gradient (8.5 - 50%) was hand-poured in 1 ml fractions. The 2 M sucrose/HEPES stock solution was diluted with 20 mM HEPES buffer until the appropriately percentage sucrose was achieved. Each 1 ml sucrose fraction was poured slowly along the wall of a high-speed centrifugation tube (Beckman #344059). To achieve diffusion between the fractions, gradients were poured 4 hours before usage and stored at 4°C. Suspensions of PKH26-labelled uEVs, prepared as described above, were carefully layered on top of the gradients and the final volume was noted exactly to ensure reproducibility of fractions to be collected following centrifugation.

The gradient was subjected to centrifugation in a Sorvall® 100SE Ultra Centrifuge using a Thermo-Fisher® Surespin 630; TH-641 rotor at 200,000 x g (rav, k-factor 120) for 16 hours at 4 °C. Following centrifugation, the gradient fractions were immediately collected from the top beginning with a first loading fraction of ~ 2 ml (exact volume depending on the sample volume loaded) which was discarded. All other 1 ml fractions were removed very slowly from the top maintaining the pipette tip just below the meniscus at all times. The individual fractions were diluted in 20 mM HEPES buffer supplemented with Roche® protease inhibitors and were re-centrifuged at 150,000 xg (rav, k-factor 161) for 1 hour 15 minutes at 4 °C. The recovered pellets were re-suspended in PBS and stored at -80 °C. Subsequently, the fractions were thawed on ice and protein estimation was performed using BCA estimation as previously described prior to their use in specific experimental procedures.
3.2.14 Lectin microarrays

**Printing of microarrays:** All LM slides used for this project were printed by Dr. Jared Q. Gerlach, postdoctoral researcher, Glycosciences Laboratory, NUI Galway. Briefly, a collection of individual purified lectins were printed in 0.5 mg/ml PBS solutions supplemented with 1 mM sugars (Glc, Man or Gal) on functionalised Nexertion® H glass slides (Schott, Germany). The pre-activated slides contained terminal N-hydroxysuccinimide ester (NHS)-groups on the 3D gel coated glass surface for instant reaction with the lectin once printed with a Scienion S3 non-contact spotter (Scienion, Berlin, Germany) under constant (62 ± 22%) relative humidity at 20 °C. The reactions were allowed to complete over night in a humidity chamber filled with saturated 50% (w/v) NaCl solution. Next day, the slides were blocked for 1 hour with 100 mM ethanolamine in a 50 mM sodium borate solution, pH 8.0 using a Coplin jar. Subsequently, slides were three times washed in PBS/Tween20 and once in TBS. Finally the slides were dried at 450 xg for 5 min. Lectin slides were stored until use at 4 °C in the presence of hygroscopic CaCl$_2$. All printed slide batches did undergo quality prior use with standard AlexaFluoro-647(AF647)- or AF555-labelled glycoproteins to ensure appropriate CV% values. Internal array quality controls were applied at all times according to quality control.

**Incubation with PKH26-labelled uEV samples and scanning:** The compositions of incubation and washing buffer for LM assays are detailed in the Appendix. Triplicate experiments with PKH26-labelled uEVs (PKH26-uEVs) applied to LMs were carried out following sample titration (0.25-1.5 µg/ml) to ensure an optimised fluorescence to noise ratio. Optimized PKH26-uEVs or control protein (asialo-Fetuin-AF555) were diluted in 70 µl incubation buffer TBS with 0.025% (w/v) Tween20® and added by hand pipetting into one well of an eight-well gasket positioned in a metal gasket holder. Up to eight different samples could be analysed in parallel per gasket slide. Once the eight-well gasket was filled, it was closed from the top with the lectin-containing surface facing towards the gasket glass cover. The gasket holder was closed and positioned into a hybridisation oven at 23 °C for 40 minutes with overhead shaking at 4 rpm. Next, the gaskets were submersed and opened in TBS with (0.025% (w/v) Tween20® and transferred carefully using tweezers to a Coplin jar filled with TBS with 0.025% (w/v) Tween20® in which they were allowed to stand for 2 minutes at RT. The buffer was replaced with fresh TBS with 0.025% (w/v) Tween20® and a second 2 minute standing wash was performed following which the slides were quickly rinsed with TBS with
0.025% (w/v) Tween20® and transferred into 50 ml tubes in which they were dried by centrifugation at 450 x g for 5 minutes at 10 °C. Finally, the uEV-incubated slides were placed into an Agilent® plastic holder and scanned at 543 nm (green channel) or 633 nm (red channel) with 90% laser power, 70% PMT (Photomultiplier tube) and 5 µm resolution using the Agilent® G2505 microarray scanner. The resulting images were saved as TIFF files and were later analysed using GenePix Pro® v.6.1 software. For inhibition studies, LMs assays were carried out as described above with relevant haptenic sugars added to the incubation solution to final concentrations of 100 mM.

Data Extraction and Analysis: The TIFF image files were opened in GenePix Pro® v.6.1, the images were flipped 180 ° and the appropriate .gal files were opened to align an array mask of the printed slides. The printing mask was orientated as required using an adaptive feature size ranging in diameter from 70% to 100% per circular feature. The subarrays were automatically aligned and individually adjusted according to fluorescence. A .txt file was generated incorporating the appropriate fluorescence values obtained for each lectin feature. The .txt file was opened in Microsoft Excel® and a developer tab was applied. Next, the lectins were sorted according to print list and normalized. The data was analysed as histograms and further processed using Hierarchical Clustering Explorer v3.5 (http://www.cs.umd.edu/hcil/hce/hce3.html). Hierarchical clustering of normalized datasets was performed with relative fluorescence units (RFU) between 0 and 65,500. Normalization procedures varied depending on the data set used. For uEVs D100 and D115 data (figure 5-5 and figure 5-6) was averaged and HCE scaled and normalized. Data for donor 211 and 221 (figure 5-9) was tested as single sample with only one biological and one technical replicate using HCE for scaling and normalization. For analysis of t H-uEVvs GN-uEVs in Chapter 6 (figure 6-11) normalisation based on total mean values generated by the entire dataset.

3.2.15 Particle sizing

IZON® qNano Analysis: Dilute samples of uEVs from a single healthy donor enriched by CF and UC methods were subjected to particle size determination by the IZON qNano (Izon Science, Ltd., Christchurch, NZ) particle analysis system. The device was calibrated using calibration beads in the range of 100-1000 nm. Stretch was set to 47.02. Nanopore ID A15479 was used.
Nanoparticle Tracking Analysis (NTA): NTA analysis was carried out in collaboration with Drs. Ciaran M. McGuire and Adriele Prina-Mello of the Nanomedicine Group, Institute of Molecular Medicine, School of Medicine, Trinity Centre for Health Sciences, St. James’s Hospital including the Advanced Materials and BioEngineering Research (AMBER) and Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN), Trinity College Dublin, Dublin, Ireland. Selected aliquots of fractionated uEV samples were thawed and analyzed on a NanoSight® NS 500 System using a 405 nm laser, 430 nm long pass filter and NTA v.2.3 build version 2.3.5.0033.7.-Beta 7 software (Malvern, UK). This device allows for compensation of artificial broadening of distribution of particles using the Finite Track Length Adjustment (FTLA) algorithm. First, background recordings were taken for PBS alone to ensure lack of particles in the buffer system. Next, uEV samples were diluted 1:10,000 to a final volume of 1 ml PBS prior to loading and recording of 60 second video files. A dilution resulting in 20 to 70 particles per field was considered to be acceptable for video recording. Only videos with >200 particles were accepted for further analysis. For each samples, 6 videos were merged to generate final statistics and graphs via NTA v.2.3 build version 2.3.5.0033.7.-Beta 7 software (Malvern, UK).

3.2.16 Transmission electron microscopy
The samples were handled with the droplet method on Parafilm®. Copper grids (200 mesh, formvar coated, Agar Scientific) were used as supporting material. For each sample, 25 µl of uEV suspension in PBS containing 1 µg protein (as estimated by BCA assay) were diluted 1:1 with 4% (w/v) paraformaldehyde in PBS and incubated for 20 minutes. Next, the grids were washed twice for 2 minutes each in 100 µl PBS followed by cross-linking with 50 µl of 1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 5 minutes and three 1 minutes washes with 100 µl PBS. Finally, 50 µl of uranyl oxalate, pH 7.0 solution in 900 µl methylcellulose was added and incubated for 5 minutes on ice. The samples were imaged at 80 kV with an Hitachi H100,000 transmission electron microscope.

3.2.17 Immunogold labelling
For immunolabelling, all samples were again handled with the droplet method on Parafilm®. Gold or copper grids (200 mesh formvar coated) were placed onto 10-15 µl drops of PBS containing 0.75 - 1 µg of uEV samples mixed with 4% (w/v) paraformaldehyde in a 1:1 ratio and were incubated for 20 minutes. The grids were washed twice for 3 minutes each in 100 µl
PBS and free aldehyde groups were blocked with three sequential incubations with 100 µl of 50 mM glycine in PBS for 3 minutes each followed by a 10 minute incubation with 100 µl PBS, 5% (w/v) BSA and two 3 minutes washes in PBS, 0.1% (w/v) BSA. For primary antibody incubation, antibody stock solutions were diluted 1:10 in PBS, 3% (w/v) BSA and the grids were incubated in 20-50 µl for 30 minutes at 4 °C followed by six 3 minutes washes in PBS, 0.1% (w/v) BSA. For secondary antibody incubation, 10-20 µl of gold particle (5-9 nm or 11 nm) conjugated-anti-mouse IgG or anti-rabbit IgG antibody stock solutions were diluted 1:5 in PBS, 0.5% (w/v) BSA and added to the grids for 30 minutes at RT followed by six 3 minutes washes in PBS, 0.1% (w/v) BSA. Cross-linking was achieved by incubation in 50 µl of 1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 5 minutes. The grid was transferred for washing onto a 100 µl drop of water for 2 minutes incubation and this step was repeated a total of eight times. Finally, the samples were contrasted in 2% (v/v) phosphotungstic acid in PBS for 10-15 seconds or 0.5% (v/v) uranylacetate for 4 minutes and were imaged on an Hitachi H7000 transmission electron microscope at 75 kV or an Hitachi H100,000 transmission electron microscope at 80 kV.

Immunogold labelling data were counted from uEVs with clearly visual shapes. Immunogold labels were counted for each fraction from ~50-100 uEVs per sample. Values were presented as mean of total sum of gold counts per 17 particles per fraction per donors for CD24 and 50-100 particles per fraction per donor for CD63 with biological replicates of n=3. Differences in immunogold labels per uEV particle was analysed using paired Student’s t-test.

### 3.2.18 Statistical analysis

Statistical analyses were performed using Excel (Microsoft) and GraphPad Prism ® version 5.01 software.

For experiments with low sample number (n ≤ 5), data were assumed to follow normal distribution with equal variances and were analysed as means rather than medians. Flow cytometry (FCM) and LM data were expressed as median fluorescence intensities which were averaged from appropriate technical replicates. Nanoparticle tracking analysis variables were analyzed as means due to comparision of uEVs in of multiple fractions.

For clinical pilot studies, unpaired Student’s t-test was used to compare healthy versus diseased. For experiments involving comparison of data for individual uEV samples, paired Student’s t-test was used. For comparisons of data across multiple sucrose gradient fractions,
one-way ANOVA followed by Tukey’s post-hoc test was performed.

For all statistical analyses, significance was assigned to p-values < 0.05.
Chapter Four

Results

*Lectin-based Characterisation of the Surface Glycome of Unfractionated Human Urine Extracellular Vesicles*
4 Results

4.1 Introduction

Following the first comprehensive description of “exosomes in human urine”, (Pisitkun, Shen, and Knepper 2004) it has become evident that uEVs isolated by various methods from urine encompass various subtypes of nanovesicles and have features in common with EVs present in other bodily fluids such as plasma, breast milk and amniotic fluid (Admyre et al. 2007; Caby et al. 2005; Hogan et al. 2009; Keller et al. 2007; Lasser et al. 2011; Ogawa et al. 2011). Of the different EV subtypes that have been characterized to date, “true” exosomes (Ø 40-100 nm) and membrane-derived vesicles (Ø 20-1000 nm) have been most extensively studied (Pilzer et al. 2005; Théry, Ostrowski, and Segura 2009). Over the last decade, scientific and clinical interest in EVs has increased dramatically with the realization that EVs contain a repertoire of selected biomolecules that is representative of the phenotype of their parent cells. Thus, parent cell-specific material such as proteins, lipids, surface-associated carbohydrates, messenger (m)RNA and micro (mi)RNA can be isolated from EV preparations and, potentially, correlated with the function and health of specific organs and tissues (Cheng et al. 2014). However, discrimination of distinct EV subtypes remains challenging and is mainly based on vesicle size along with the presence of proteins of endosomal/multivesicular body origin (CD63, ALIX, TSG101).

Several groups have reported detailed protocols for isolation of EVs from bodily fluids and cell culture supernatants based on techniques including ultracentrifugation, filtration, centrifugal concentration, affinity purification, precipitation, microfluidics and size exclusion chromatography (SEC) (Cheruvanky et al. 2007; Fernández-Llama et al. 2010; Kanwar et al. 2014; Lobb et al. 2015; Mathivanan et al. 2010; Merchant et al. 2010; Miranda et al. 2010; Ravi, Khosroheidari, and DiStefano 2015; Rood et al. 2010). Each of these methods has the potential to successfully enrich nanovesicles but the degree of purity, the overall EV yield and the diversity of vesicle subtypes contained within the isolates may vary widely. For uEVs in particular, co-enrichment of abundant, non-vesicular proteins remains a significant barrier to their isolation and analysis for research purposes (Fernández-Llama et al. 2010; Jeppesen et al. 2014; Musante et al. 2012; Rood et al. 2010; Zubiri et al. 2014). At the time this project was initiated (2010), UC was the most commonly used technique for uEV isolation from
human samples and, despite the emergence of less time-intensive approaches, this continues to be the case (Cvjetkovic, Lötvall, and Lässer 2014; Jeppesen et al. 2014; Théry et al. 2006a, 2006b). Among the alternatives, CF and other filter-based methods have been most extensively reported and, as the materials and time required for CF were considered to be more compatible with subsequent high through-put analyses and clinical applications, we elected to perform side-by-side comparisons of UC- and CF-based protocols as part of the initial approach to investigating uEV surface glycosylation (Cheruvanky et al. 2007; Merchant et al. 2010).

A variety of carbohydrate moieties may are displayed on the surfaces of mammalian cells. Such carbohydrates are typically attached to specific biomolecules to form so-called glycoconjugates (Kamerling, Boons, Lee, Suzuki, Taniguchi, and J. 2007; Ajit Varki et al. 2009e). These may include integral membrane proteins and lipids as well as surface-associated proteoglycans, GPI-anchored proteins (Curry and Adamson 2012; Ajit Varki 1993; N. M. Varki and Varki 2007). Together these glycoconjugates build up a negatively charged, dynamic and complex physical barrier known as glycocalyx which may extend between 0.5 and 3 µm from the cell surface (Reitsma et al. 2007). Importantly, cell-surface carbohydrates are known to vary based on the stage of development, differentiation, cell activation and apoptosis (Gagneux and Varki 1999; Sahin and Balcan 2009; Ajit Varki 2008). Thus, it is reasonable to explore the variability in glycosylation of tissues, cells and subcellular particles as a potential indicator of health, stress response and disease. In the case of the kidney, which is the primary organ of interest for this thesis, glycosylation may be of broad relevance to renal development and homeostasis as well as to specific biological components of kidney function including tubular ion transport and glomerular filtration (Andrews 1979; G. Chen et al. 2011; Doyonnas et al. 2001; Kakani et al. 2012; Morelle et al. 2000). Additionally, kidney associated diseases such as IgAN, lupus nephritis (LN), SLE, diabetic nephropathy and certain inherited conditions are known to involve abnormalities of glycosylation (Hashii et al. 2009; Oortwijn et al. 2006; Ravidà et al. 2015; Xie et al. 2013). At the time this project was initiated, only a few published studies had linked EVs directly or indirectly with surface carbohydrates and even basic details about the spectrum and variability of uEV-associated glycoproteins and glycolipids in the setting of health and disease was lacking (Keller et al. 2007; Krishnamoorthy et al. 2009; Pisitkun, Shen, and Knepper 2004).
A long-established approach to the analysis of glycosylation in tissue samples and cells is the use of lectins as detection agents (Batisse et al. 2004; Faraggiana et al. 1982). Lectins can be used to discriminate among their various carbohydrate ligands (Ambrosi, Cameron, and Davis 2005; Goldstein 2002). Screening formats which have been adapted for lectin-based screening of carbohydrates include immunohistochemistry, enzyme-linked immunosorbent assays (ELISA), immunoblotting, surface plasmon resonance, flow cytometry and microarrays (Batisse et al. 2004; Katrlík et al. 2011; Krishnamoorthy et al. 2009; Moldoveanu et al. 2007; Ravidà et al. 2015). Based on the specific expertise and technical resources available to us and taking into consideration the unique characteristics of EVs as well as the limited amount of existing literature, we selected FCM and LM as the approaches of choice for the project described in this and subsequent chapters of the thesis. This project was the first work to be performed on EVs in our laboratories and we anticipated specific challenges such as validating the presence of intact EVs; developing a cost- and time-effective isolation protocol; consistently achieving acceptable yield and purity of EVs; excluding the contribution of co-purified non-EV proteins to the glycosylation profiles detected and interpreting the results of lectin-based carbohydrate detection. An important starting-point for the protocols developed for this Chapter was the highly detailed 2006 publication “Isolation and characterization of exosomes from cell culture supernatants and biological fluids” (Théry et al. 2006a). Much of the data described in the Chapter has been published (Gerlach et al. 2013).
4.2 Hypothesis, aim and objectives

Recent literature highlights the potential for human urine extracellular vesicles (uEV) to be used as a non-invasive source of biomarkers for kidney diseases. While proteomic techniques had been extensively applied to uEVs at the time of initiation of this project, no published techniques or definitive studies existed in regard to the uEV glycome in health or disease.

4.2.1 Hypotheses

1. Extracellular vesicles isolated from urine of healthy adults display carbohydrate-associated structures on their surface.

2. The method of isolation employed influences the surface glycosylation attributed to uEV isolates from healthy adults.

3. A distinctive surface glycosylation signature can be assigned to uEVs from healthy adults and adults with known kidney disease through the use of lectin-based profiling techniques.

4.2.2 Aim

The overall aim of this section of the thesis was to apply lectin-based profiling to characterize and validate a surface glycosylation signature of uEVs isolated by different protocols from urine of healthy adults.

4.2.3 Objectives

1. Directly compare UC- and centrifugal filtration (CF)-based approaches to generate uEV preparations for analysis of glycosylation.

2. Develop a lectin-based carbohydrate binding assay for the detection and relative quantification of specific carbohydrate moieties on the surface of human uEVs.

3. Generate a specific glycoprofile for uEVs from healthy adults and perform a pilot study of uEV glycosylation changes in adults with known kidney disease.
4.3 Results

4.3.1 Isolation, characterization and quality control of uEVs

For the isolation of uEVs from urine of healthy individuals literature was first reviewed for appropriate isolation methods. To begin with, a rapid, simple isolation protocol based on CF recently presented by A. Cheruvanky et al. (2007) was chosen for isolation of uEVs (Cheruvanky et al. 2007). This procedure has high potential in higher sample throughput and was reportedly also suitable for clinical application as isolation method. In addition, a second common and more time consuming protocol based on UC was followed as described by P. Fernández-Llama et al. (Fernández-Llama et al. 2010). A flow diagram summarizing both selected procedures is presented in figure 4-1. For the enrichment of uEVs from urine of 3 healthy individuals, urine samples were collected and processed according to methods 3.2.1 to 3.2.3. Each sample was pre-clarified to remove cell debris before freezing the samples at -80 °C until further processing. After thawing, the collected samples were subdivided and processed by using the two selected isolation approaches.

![Figure 4-1: Workflow of centrifugal filtration (CF) and ultracentrifugation (UC) protocols for the isolation of uEVs.](image-url)
For CF isolation, a starting volume of 15 ml of first morning urine were used for each of the 3 healthy adults to yield a uEV concentrate (CF-uEVs). For UC isolation, 50-100 ml of first morning urine were used to yield a uEV pellet (UC-uEVs). The estimated protein contents of these isolates (by Bradford assay) were 1.05 ± 0.5 µg/ml (CF-uEVs) and 1.71 ± 0.12 µg/ml (UC-uEVs) of urine.

The protein profiles of these uEV preparations were analysed by Bis-Tris PAGE and silver staining as shown in figure 4-2 A. Protein bands appeared over a broad range of molecular weights (14-191 kDa) with one specific high-abundance band around 85 kDa consistent with THP in both isolation methods. Interestingly, the proteins were differently distributed within the total uEV profiles when comparing both isolation methods for individual donors. In general, UC-uEVs showed a more defined protein pattern while CF-uEVs contained several abundant proteins most likely overlaying other less abundant protein bands. Nonetheless, immunoblot experiments confirmed the presence of two renal epithelial cell markers CD24 (30-40 kDa) and AQP-2 (~20 kDa and 30-40 kDa) in uEVs generated with both isolation methods as shown in figures 4-2 B-1 and B-2. CD24 is a GPI-anchored membrane glycoprotein with mucin-like properties that is highly expressed in humans by lymphocytes. In the kidney it is expressed by glomerular parietal epithelial cells in addition to proximal tubular epithelial cells and cells in the collecting duct as well as bladder epithelium (Angelotti et al. 2012; Y.-L. Choi et al. 2007; Droz et al. 1990; Kristiansen, Sammar, and Altevogt 2004; Lindgren et al. 2011; Smeets et al. 2013). Expression of recombinant human CD24 in CHO cells demonstrated the presence of O-glycans and N-glycans in this protein (Motari et al. 2009). Human AQP-2 is primarily expressed on the apical membrane of the principal cells of the renal collecting duct. It has one site for N-glycan attachment when expressed in madin darby canine kidney type I cells (Hendriks et al. 2004). Thus, it is not surprising that CD24 and AQP-2 stains appeared as broad and/or multiple bands consistent with the behaviour of glycoproteins. The detected bands for CD24 and AQP-2 were consistently more intense for UC-uEVs compared to CF-uEVs.

The highly-abundant protein band at approximately 85 kDa corresponded with the expected position of THP, a protective glycoprotein located in the thick ascending limb loop of Henle which is known to be co-purified with uEVs and to confound some analyses of uEV protein content (Hogan et al. 2009; Horsch et al. 2014; Keller et al. 2007; Van Rooijen, Kamerling, and Vliegenthart 1998). As shown in figure 4-2 C, the amounts of THP detected by
immunoblotting were variable but were comparable for the two uEV isolation methods. Only weak THP bands were observed in the pre-clarified urines. In contrast, while albumin was also detected by immunoblotting in both CF-uEV and UC-uEV preparations as well as in pre-clarified urine, the amount of albumin present was strikingly higher within CF-uEV preparations (figure 4-2 C).

Figure 4-2: Comparison of uEVs derived from 3 healthy adults isolated by ultracentrifugation (UC) and centrifugal filtration (CF).

A. 4-12% NuPAGE run in 3-(N-morpholino)propansulfonic acid (MOPS) running buffer followed by silver stain (5 µg CF-uEVs, 5 µg UC-uEVs, 1 µg THP, 1 µg human serum album (HSA) per well).

B. Immunoblots with rabbit anti-human APQ-2 (B-1) and mouse anti-human CD24 (B-2) (AQP-2: 4 µg CF-uEVs, 4 µg UC-uEVs, 4 µg 1800 x g urine supernatant (SN 1800 x g) per well; CD24: 5 µg CF-uEVs, 5 µg UC-uEVs, 5 µg SN 1800 x g per well).

C. Immunoblots of the same samples with mouse anti-human THP and rabbit anti-HSA (4 µg CF-uEVs, 4 µg UC-uEVs and 4 µg SN 1800 x g per well).

Abbreviations: D1, D2, D3 = donor 1, donor 2, donor 3; HMW = high molecular weight, LMW = low molecular weight.
From these initial isolation and characterization experiments it was concluded that: (a) Urine EVs may be successfully isolated from healthy adult samples by both CF and UC approaches and contain specific glycoproteins likely to originate from renal epithelial cells. (b) A greater amount and purity of uEV-specific proteins can be captured by the UC- compared to the CF-based method. (c) While a variable amount of THP contamination occurs with both isolation methods, CF-uEVs are further contaminated by heavy co-purification of albumin.
Following comparative Bis-Tris PAGE and immunoblotting analyses of the CF-uEV and UC-uEV samples, an initial visualisation of the structure and size of particles contained in these isolates was performed by transmission electron microscopy (TEM). As shown in figure 4-3, the TEM images obtained for both CF-uEVs (4-3 A) UC-uEVs (4-3 B, C) showed vesicular structures in the approximate range of 50-100 nm with some having an expected cup-shaped appearance after fixation and counter staining with uranyl acetate.

![Image of TEM images of uEVs](image)

Figure 4-3: Transmission electron microscopy (TEM) images of uEVs derived from healthy individuals after isolation by A. CF or B.+ C. UC. The samples were fixed with paraformaldehyde, cross-linked with glutaraldehyde and counter-stained with uranyl acetate on a 200 mesh copper grid. Recordings were taken at 80 kV. n=1 per isolation method.

For further evaluation, particle sizing and counting was carried out during a demonstration session provided by Izon Science, Ltd. using the IZON qNano® device (http://www.izon.com/products/qnano-2/). As shown in figure 4-4 and table 4-1, particles were detected separately in both CF- and UC-uEV isolates from the same individual. The particles detected in the CF- and UC-uEV isolates were of similar size ranges from 75 to 350 nm in accordance with published studies (Hogan et al. 2014; Keller et al. 2007). The diameters of particles in both isolates were >100 nm and, as such, were somewhat larger than expected for uEVs based on the landmark study in the field (Pisitkun, Shen, and Knepper 2004). This may reflect technical differences between the IZON instrument and other methodologies (such as nanoparticle tracking) used for EV sizing. Importantly, as shown in table 4-1, the calculated vesicle concentration for the UC-uEV sample was approximately 7-fold higher than that of the CF-uEV sample.
In summary, uEVs with expected shape and size characteristics were obtained using both CF- and UC-based isolation methods. Consistent with the results for Bis-Tris PAGE and immunoblotting, the lower particle concentration for CF-uEVs compared to UC-uEVs suggested a substantially higher contamination of co-purified urinary proteins in samples isolated by the CF-based protocol. Thus, UC appeared to offer significant advantages over CF as regards sensitivity and specificity in experiments directed toward analysis of uEV-specific biomolecules.
4.3.2 Surface carbohydrates moieties of uEVs originate partially from glycoproteins

To confirm the presence of glycoproteins on uEV surfaces, four lectins (SNA-I, PHA-E, AAA and PNA) with diverse carbohydrate specificities - were used for lectin blotting of uEV proteins and selected purified control glycoproteins as described in Methods 3.2.10 (page 59) and shown in figure 4-5.

Figure 4-5: Lectin blots results for UC-uEVs, CF-uEVs and purified control glycoproteins.

Lectin blots prepared using 6 µg aliquots of CF-uEV protein, 4 µg aliquots of UC-uEV protein and 2 µg aliquots of control glycoproteins per well are shown. Biotinylated lectins SNA-I (1 µg/ml), PHA-E (0.5 µg/ml), AAA (1 µg/ml) and PNA (1 µg/ml) were followed by avidin-conjugated alkaline phosphatase and finally visualized using FAST™ BCIP/NBT tablets.

Abbreviations: THP = Tamm-Horsfall protein (positive control for SNA-I, PHA-E, AAA), Fet = sialo-fetuin from bovine calf serum (positive control for SNA-I and PHA-E), bIgG = bovine immunoglobulin G (positive control for AAA)

Lectin blots with SNA-I (specificity for \(\alpha2\rightarrow6\)Neu5Ac) revealed a single strong band at 64 kDa for fetuin and a faint band between 64 kDa and 97 kDa for THP as expected. For proteins derived from CF- and UC-uEVs, SNA-I blotting revealed multiple bands over the entire molecular range with minor differences in distribution between the two isolation methods.

Lectin blots with PHA-E (specificity for biantennary glycans) demonstrated strong bands for both fetuin and THP and also strongly stained a broad range of CF- and UC-uEVs proteins with some differential band patterns for the two isolation methods.

Lectin blots with AAA (specificity for \(\alpha\)-Fuc) were characterised by strong staining of bovine
immunoglobulin G (IgG) but not THP. However, AAA staining of UC-uEV proteins revealed several well-defined glycoproteins across the entire molecular range while bands detected among CF-uEV proteins were lower in number and generally less intense.

Finally, lectin blots with PNA (specificity for Gal(β1→3)GalNAc) demonstrated no staining of THP (the high molecular weight band observed in these blots was considered to represent unidentified protein contaminant of the purified THP preparation). Interestingly, PNA bound to multiple proteins in the very high marker range (>191 kDa) in both CF- and UC-uEVs with the intensity of these bands being greater in UC-uEV associated proteins. Of note, several very high molecular weight proteins including megalin, cubilin, polycystin 1, maltase-glucoamylase, fibronectin-I, CD35, IgG Fc-binding protein and dynel (anoxemal heavy polypeptide 8) corresponding to the high molecular range were identified in the uEV proteome by Pisitkun et al. (Pisitkun, Shen, and Knepper 2004). Strong binding of PNA to proteins of this size range, suggest the presence of high levels of O-glycosylation.

From these lectin blots it was concluded that: (a) Lectins demonstrated largely expected specificity of binding to purified glycoproteins. (b) Binding of individual lectins to uEV proteins occurred in distinctive patterns consistent with a broad repertoire of vesicle-derived glycoproteins. (c) Consistent with the other basic analysis techniques applied, the signal intensity and diversity of bands detected among UC-uEV protein preparations were greater than those of CF-uEV-derived lysates.

Thus, for subsequent experiments in which lectin binding was adapted to detect surface glycosylation signatures of intact uEVs, samples prepared by UC were exclusively employed.
4.4 Development of a bead-based flow cytometry method for analysis of uEV lectin binding

A FCM assay for lectin binding to uEVs was developed based on the encouraging results of the lectin blots described above. Due to their physical size, individual uEVs cannot be reliably detected by a conventional flow cytometer (Nolte-t’ Hoen et al. 2012; Graça Raposo and Stoorvogel 2013; H. B. Steen 2004). Thus, a bead-based assay protocol was adapted from that reported by Thery et al. (2006) (Théry et al. 2006a). Specifically, the protocol was modified for use with a 96-well format for multiple parallel incubations.

For the development of the FCM assay, preparations of UC-uEVs from healthy adults were used. Aliquots of uEV suspensions calculated to contain 5 µg of total protein were attached to 4% (w/v) sulfated aldehyde latex beads (ø = 4 µm). The binding of uEVs to latex beads was initially confirmed by incorporation of the lipophilic membrane dye PKH26, which can be detected in the FL2 (PE) channel of a flow cytometer, into the surface of the vesicles (PKH26-uEVs).

**Figure 4-6** illustrates the gating strategy and provides an example of the results obtained by FCM analysis. First, single beads were gated in the forward scatter area (FSC-A) and side scatter area (SSC-A) plot. Next, doublets were excluded by gating around the single bead population in a FSC-A versus forward scatter height (FSC-H) plot. Finally, non-labelled UC-uEV coated beads were used to set a threshold gate for the detection of PKH26-positive beads. As shown, beads coated with PKH26-labelled uEVs demonstrated clearly higher fluorescence in the FL-2 (PE) channel compared to beads coated with unlabelled UC-uEVs.

This observation was consistent for UC-uEV preparations from multiple healthy donors (**figure 4-7**).
Figure 4-6: Gating strategy for the detection of PKH26-labelled UC-uEV coated latex beads by flow cytometry. UC-uEVs were PKH26 labelled, washed and coated to latex beads. Free aldehyde groups on the latex beads were blocked with glycine for 30 minutes. The PKH26 labelled uEV coated beads were analyzed in the cytometer along with glycine-coated and unlabelled uEV-coated beads as controls. After gating for size (left dot plot – FSC-A vs. SSC-A) and doublets (middle dot plot – FSC-A vs. FSC-H), the single bead population was analyzed for fluorescence in the FL-2 channel (right dot plots – PE-A vs. FSC-A – upper, unlabelled UC-uEV-coated beads; lower, PKH26-labelled UC-uEV-coated beads). The fluorescence shifts for glycine-coated, unlabelled uEV-coated and PKH26-labelled uEV-coated beads are presented together in the histogram plot (far right).

Figure 4-7: Repeat of FCM detection of PKH26-labelled UC-uEVs coated on latex beads with relevant controls using UC-uEV samples from three healthy adults.

A. Fluorescence shifts in the PE-channel of three individual samples of PKH26-labelled UC-uEV-coated beads (shaded histograms) are shown along with those of relevant controls.

B. Mean ± SD of the median fluorescence intensities (MFI) of unlabelled uEV-coated beads and PKH26-labelled uEV-coated beads for the three healthy adult samples with two technical replicates each. Two-tailed, paired Student’s t-test showed no significance.

Thus, it was concluded that the strategy of coating UC-uEVs directly to latex beads represented a viable option for subsequent analysis of the surface proteins and carbohydrates expressed by uEVs. After this demonstration of uEV attachment to latex beads using PKH26 labelling, all further experiments were carried out with non-labelled uEVs.

Next, UC-uEVs were analysed for surface expression of a glycoprotein known to be expressed by renal tubular epithelial cells as well as Bowman’s capsule using mouse anti-
human CD24 antibody combined with a FITC-conjugated goat F(ab’)2 anti-mouse IgG-Fc (Angelotti et al. 2012). Nonspecific binding was excluded using glycine-coated beads incubated with primary followed by secondary antibody. Figures 4-8 and 4-9 illustrate the gating strategy used for this analysis (figure 4-8) and the results obtained for three individual UC-uEV samples (figure 4-9). As shown, strong, specific binding of anti-CD24 to uEV-coated beads was observed for each sample indicating that uEVs display the GPI-anchored glycoprotein CD24 consistent with renal epithelial cell origin.
Figure 4-8: Gating strategy for the detection of CD24 expression on UC-uEV-coated beads.

Samples were stained with mouse anti-human CD24 followed by goat anti-mouse IgG-Fc-FITC. After gating for size (left dot plot – FSC-A vs. SSC-A) and doublets (middle dot plot – FSC-A vs. FSC-H), the single bead population was analyzed for fluorescence in the FL-2 channel (right dot plots – PE-A vs. FSC-A – upper, glycine-coated beads; lower, uEV-coated beads). The fluorescence shifts for fully-stained UC-uEV-coated beads along with relevant staining controls are presented together in the histogram plot (far right).
Figure 4-9: Repeat of FCM detection of CD24 on UC-uEV-coated latex beads with relevant controls using UC-uEV samples from three healthy adults.

A. Fluorescence shifts in the FITC-channel of three individual samples of UC-uEV-coated beads (shaded histograms) are shown along with those of relevant controls.

B. Mean ± SD of the median fluorescence intensities (MFI) of uEV-coated beads without (uEVs) and with anti-CD24 staining (CD24 uEVs) for the three healthy adult samples and 3 technical replicates each. Two-tailed, paired Student’s t-test showed no significance.

Following these proof-of-principle experiments, it was concluded that the use of aldehyde-functionalized latex beads as a carrier for FCM analysis provided the opportunity for simple, fast screening for post-/co-translational modifications, such as glycosylation, of bead-bound proteins.

The assay was, therefore, next tested by coating beads with purified proteins with known glycosylation characteristics and staining with two lectins, SNA-I and PNA, having known specificities for (α2→6)Neu5Ac and Gal(β1→3)GalNAc respectively. Positive binding, indicating the presence of specific carbohydrate moieties was achieved by incubation of uEV-coated beads with biotinylated lectins followed by fluorochrome-coupled SA. A detailed gating strategy is shown in figure 4-10.

As expected PNA bound with moderate intensity to beads coated with asialo-fetuin but not to beads coated with fetuin or with the non-glycosylated protein BSA (see figure 4-11). Similarly, SNA-I bound strongly to beads coated with fetuin but not to those coated with
asialo-fetuin or BSA. These analyses confirmed that lectins were compatible with the FCM-based assay platform and provided readily detectable staining of specific glycan structures displayed on the surface of uEV-coated latex beads.
Figure 4-10: Example of flow cytometric gating strategy for lectin binding to glycoprotein-coated beads.

First protein-coated beads (in this case asialo-fetuin) were gated for size (left upper dot plot – FSC-A vs. SSC-A) and doublets (left lower dot plot – FSC-A vs. FSC-H). Next a control sample stained with streptavidin-APC alone was used to set a fluorescence threshold gate in the APC channel (middle dot plot – APC-A vs. FSC-A). Finally, protein-coated beads stained with biotinylated lectins PNA (upper right dot plot) and SNA-I (lower right dot plot) were analysed with positive lectin staining appearing in the APC positive gate. In this case, PNA, but not SNA-I, was shown to bind strongly to asialo-fetuin-coated beads.
Figure 4-11: Analysis of PNA (top, left) and SNA-I (top, right) binding ranges to three purified proteins coated on latex beads.

Left: Bar graph (upper panel) and individual histograms (lower three panels) of PNA fluorescence intensities in the APC channel demonstrates high-level binding to asialo-fetuin-coated beads with absence of binding to beads coated with BSA and fetuin.

Right: Bar graph (upper panel) and individual histograms (lower three panels) of SNA-I fluorescence intensities in the APC channel demonstrates high-level binding to fetuin-coated beads with absence of binding to beads coated with BSA and asialo-fetuin.

Error bars for the graphs represent ± SD of 3 technical repeats. Fluorescence intensities are shown for relevant controls in the histograms.
To demonstrate that lectin binding to glycoproteins was indeed carbohydrate-mediated, the experiment was repeated including a haptenic sugar as a competitive inhibitor. As shown in figure 4-12, PNA binding to bead coated with both 5 and 10 µg of asialo-fetuin was strongly inhibited in the presence of 100 mM lactose (Lac). Thus, PNA binding to asialo-fetuin was most likely to represent a bona fide carbohydrate-based interaction rather a non-specific protein-protein interaction.

Figure 4-12: Inhibition of PNA binding to asialo-fetuin coated beads in the presence of haptenic sugar (100 mM lactose).

Beads coated with 5 µg (left) or 10 µg (right) asialo-fetuin were stained with biotinylated PNA in the presence or absence of 100 mM lactose followed by streptavidin-APC. The histograms demonstrate high fluorescence intensities for fully-stained beads in the absence of lactose (asialo-fetuin coated beads + lectin + SA-conjugated APC) that is reduced to the level of relevant negative controls in the presence of lactose (asialo-fetuin coated beads + biotinylated lectin + SA-conjugated APC + 100 mM lactose)
Finally, a series of experiments was carried in which uEVs coated on latex beads were screened with lectins for the presence of surface exposed carbohydrates.

Initially, three biotinylated lectins with distinct binding specificities, WFA, Jacalin, and SNA-I, were used at 5, 10, 15 and 20 µg/ml followed by SA-APC to determine the optimized working concentration for each. Next, aliquots of UC-uEV-coated beads were stained, along with relevant controls with each individual lectin in the optimized working concentration. In addition, beads coated with purified BSA and THP were stained.

As shown in **figure 4-13**, each of the three lectins bound to uEV-coated beads compared to the appropriate unstained controls with the order of binding intensity being WFA > Jacalin > SNA-I. In contrast, SNA-I and Jacalin demonstrated no binding above background intensity to THP- and BSA-coated beads. One lectin, WFA, showed a low level of background fluorescence following staining of control (glycine-coated) beads but this was substantially lower that the fluorescence associated with its binding to uEV-coated beads. In subsequent analyses, lectin fluorescence results for uEV-coated beads were expressed as fold-change over the fluorescence intensity of lectin-stained non-uEV-coated beads (“MFI/control”).

Overall, this experiment was successful and demonstrated clearly that UC-uEVs could be screened for the presence of surface carbohydrates once attached to latex beads. It was concluded at this stage, that the carbohydrate moieties displayed on the surface of uEVs derived from healthy adults included (α2→6)Neu5Ac (SNA-I), GalNAc (WFA), and sialylation independent Gal (Jacalin). Interestingly, these structures can be found in N- and O-glycans as modifications of proteins as well as glycolipids and other glycoconjugates (Kamerling, Boons, Lee, Suzuki, Taniguchi, and Voragen 2007a, 2007e; Marth and Grewal 2008; Ajit Varki et al. 2009h).
Figure 4-13: Flow cytometric analysis of lectin binding to uEV-, BSA- and THP-coated beads.

Upper: Bar graphs of SNA-I, WFA and Jacalin (Jac) fluorescence intensities, expressed as mean MFI/control in the APC channel for uEV- (left), BSA- (middle) and THP-coated (right) beads. The results demonstrate variable intensity binding of all three lectins to 3 distinct uEV samples and minimal to absent binding to BSA and THP. Error bars of BSA and THP represent SDs of 3 technical replicates whereas uEVs were three biological sample replicates. * = p < 0.05 compared to non-uEV-coated beads; *** = p < 0.001 compared to non-uEV-coated beads (paired Student's t-test). Lower: Individual fluorescence histograms of SNA-I, WFA and Jacalin binding to uEV-, BSA and THP-coated beads along with fluorescence histograms for relevant controls. In all panels, the shaded histograms represent 3 technical replicates of the fully-stained uEV-coated beads.
Unexpectedly, this experiment showed no significant binding of WFA, SNA-I and Jacalin to purified THP coated beads despite the fact that THP is known to be heavily glycosylated and represents a significant contaminant of uEVs isolated by most methodologies (Cheruvanky et al. 2007; Fernández-Llama et al. 2010; Gerlach et al. 2013; Musante et al. 2012; Van Rooijen, Kamerling, and Vliegenthart 1998).

In order to further evaluate the capacity of the bead-based FCM assay to detect glycosylation associated with bound THP, experiments in which beads were coated with 5, 10, 15 and 20 µg of purified THP then stained with PHA-E (specificity for biantennary glycans predicted to be present on THP) were carried out. The results, as shown in figure 4-14, confirmed a dose-dependent increase in PHA-E-specific fluorescence compared to relevant controls. Thus, it was concluded that THP is capable of binding to the latex beads used for this assay but demonstrates a more restricted lectin binding pattern (positive for PHA-E but negative for WFA, SNA-I and Jacalin) compared to uEV-coated beads.

![Figure 4-14: FCM detection of dose-dependent binding of biotinylated PHA-E/SA-APC to THP-coated latex beads.](image)

Left panel: Bar graph of PHA-E-specific fluorescence intensities, expressed as mean ± SD MFI/control in the APC channel. PHA-E staining was carried out at an optimized working concentration (20 µg/ml) and beads were coated with 5, 10, 15 or 20 µg of purified human THP. Results shown represent three biological replicates and two technical replicates for all conditions except the result for 20 µg THP beads which represents two biological replicates.

Right Panel: Fluorescence histograms of PHA-E binding to 5 (shaded), 10, 15 or 20 µg THP-coated beads along with histograms for relevant negative controls.
Next, the binding of additional lectins (PNA, SNA-I and RCA-I) to UC-uEV-coated beads was analysed along with competitive inhibition with haptenic sugar in order to confirm carbohydrate-mediated binding (figure 4-15). As shown, partial or complete competitive carbohydrate inhibition with 100 mM Lac led to reduced binding intensities for all three lectins. Inhibition of uEV-lectin binding using Lac ranged from 78 to 95% (SNA-I) and reached 97% (PNA) as well as 99% (RCA-I). It was concluded that lectins bind to UC-uEVs on the surface of latex beads predominantly in a carbohydrate-dependent manner and that the carbohydrates displayed on UC-uEV surfaces contain typical binding motifs often found in glycoproteins such as Gal(β1→3)GalNAc and LacNAc associated with O- and/or N-glycans, respectively.
Figure 4-15: Fluorescence histograms of SNA-I, PNA and RCA-I binding to three individual preparations of uEV-coated beads in the presence and absence of 100 mM lactose. Positive binding of all three lectins to each preparation of uEV coated beads (uEV1, uEV2, uEV3) is evident (shaded histograms with dotted outlines) that is partially or completely lower to the fluorescence intensities of relevant negative controls in the presence of lactose (shaded histograms with dashed outlines). For each sample, 3 technical replicates were performed for non-lactose- and lactose-inhibited conditions. Two-tailed, paired Student’s t-test showed significance with uEVs: p-values 0.007 for PNA, p-values 0.053 for SNA-I, p-values 0.015 for RCA-I.

To summarise this sequence of experiments, UC-uEVs and purified glycoproteins were detectable by FCM when coated to latex beads. Higher sample throughput for uEV screening was achieved by using a 96 well format during sample preparation. Multiple biotinylated lectins proved to be compatible with the flow cytometric technique and surface carbohydrates of UC-uEVs were readily detected with evidence of high specificity. It was possible to show that UC-uEVs from healthy adults displayed the renal epithelial cell-expressed glycoprotein CD24 and that UC-uEV surfaces contained carbohydrate motifs associated with N- and O-glycans. In the following section, more comprehensive screening for carbohydrates on uEV surfaces using lectins in this assay format is presented.
4.5 Profiling of surface glycosylation of uEVs and THP derived from 3 healthy adults.

A panel of nine lectins was selected to more broadly profile terminal binding motifs of glycoconjugates exposed on uEV surfaces and to compare with purified THP. For this profiling, it was also decided to compare UC- and CF-uEV preparations.

First, all lectins were titrated in a working range from 5-20 µg/ml using the staining protocol and relevant controls as described previously. The lectins that were chosen along with their optimized staining concentrations and primary carbohydrate affinities are summarized in table 4-2.
Next, isolated CF-uEVs, UC-uEVs and purified THP from urine samples from the same three healthy adults were coated to beads and screened for lectin binding using the optimized assay protocol and gating strategy (see figure 4-10). The results of these analyses are presented in figure 4-16 A (CF-uEVs and UC-uEVs) and figure 4-16 B (THP). For UC-uEVs multiple lectins, WFA, PHA-E, GSL-Ib4, RCA-I, jacalin and PNA, all showed medium to high binding while others, MAA, SNA-I, AAA, bound at readily-detectable but lower levels. The lectin-binding profile of CF-uEVs, while broadly similar to that of UC-uEVs, was characterized by medium to high binding of only 3 lectins, RCA-I, GSL-Ib4, and PHA-E with all others binding at low levels. In contrast to uEV samples, the lectin binding profile of purified THP was far more restrictive with only PHA-E binding at moderate levels and RCA-I at low levels.
Lectin binding to UC-uEV and CF-uEV coated beads

Lectin binding to Tamm-Horsfall Protein coated beads

Figure 4-16: Comparative bead-based FCM profiles of lectin binding to: A. UC-uEVs and CF-uEVs and B. purified THP.

Lectins were used in optimized working concentrations ranging from 5-20 µg/ml. UC-uEV, CF-uEV and THP-coated beads were incubated with biotinylated lectins followed by incubation with streptavidin-APC. Data are presented as mean ± SD of the APC median fluorescence intensities (MFI) for samples from 3 healthy adults expressed as fold-change over the MFI of the negative control. Three technical replicates were performed for each individual sample.

* = p < 0.05 for UC-uEVs vs. CF-uEVs by two-tailed, unpaired Student’s t-test.
It was concluded that: (a) Urine EVs isolated by CF and UC methods display a complex lectin-binding profile indicative of a broad range of N- and O-linked protein glycosylation as well as glycolipids. (b) The differences in lectin binding intensities observed between CF-uEVs and UC-uEVs likely reflect higher purity of UC-uEVs as well as a greater degree of contamination of CF-uEV samples by PHA-E-binding, non-EV glycoproteins. (c) The results obtained for beads coated with purified THP suggest that, while data interpretation may be confounded for a limited number of lectins in assays such as these, the overall complexity of the uEV surface glycome is not broadly obscured or altered by co-purified THP—particularly in the case of UC-uEVs.

4.5.1 Lectin binding studies on uEVs derived from ADPKD patients in FCM

The previously developed FCM assay was applied to UC-uEVs isolated from 5 autosomal dominant polycystic kidney disease (ADPKD) patient samples and their age-matched controls generated by our collaborators at Mayo Clinic, Rochester, USA, as shown in table 4-3. All individuals had an eGFR above 60 ml/min/1.73 m² indicating well-preserved kidney function. Two technical replicates were performed for all ADPKD samples and three for all control samples. The UC-uEVs used for these analyses were labelled with the fluorescent dye SP-DilC18(3).

Table 4-3: List of UC-uEVs from 5 subjects with ADPKD (PKD-A to E) with preserved renal function and their age-matched healthy controls (N-A to E) received from collaborators at Mayo Clinic, Rochester, US.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Exosomal protein received in μg</th>
<th>Exosomal protein in μg available after lectin microarrays</th>
<th>Gender</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKD-A</td>
<td>5.5</td>
<td>4.4</td>
<td>Male</td>
<td>37</td>
</tr>
<tr>
<td>PKD-B</td>
<td>6.3</td>
<td>5.04</td>
<td>Female</td>
<td>33</td>
</tr>
<tr>
<td>PKD-C</td>
<td>5.9</td>
<td>4.72</td>
<td>Male</td>
<td>35</td>
</tr>
<tr>
<td>PKD-D</td>
<td>5.6</td>
<td>4.48</td>
<td>Female</td>
<td>22</td>
</tr>
<tr>
<td>PKD-E</td>
<td>3.9</td>
<td>3.12</td>
<td>Male</td>
<td>33</td>
</tr>
<tr>
<td>N-A</td>
<td>2.9</td>
<td>2.32</td>
<td>Male</td>
<td>34</td>
</tr>
<tr>
<td>N-B</td>
<td>3.6</td>
<td>2.88</td>
<td>Male</td>
<td>33</td>
</tr>
<tr>
<td>N-C</td>
<td>4.7</td>
<td>3.76</td>
<td>Male</td>
<td>35</td>
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<td>N-D</td>
<td>6.3</td>
<td>5.04</td>
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<td>24</td>
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<tr>
<td>N-E</td>
<td>4.9</td>
<td>3.92</td>
<td>Male</td>
<td>25</td>
</tr>
</tbody>
</table>
Results for the FCM assays of this sample collection using anti-CD24 antibody and four lectins (PNA, Jacalin, RCA-I and SNA-I) predicted to reflect uEV surface expression of Gal, GalNAc and \((\alpha 2 \rightarrow 6)\)Neu5Ac was verified using SNA-I are shown in figure 4-17.
CD24

FITC-A

PNA

Jacalin

RCA-I

SNA-I

APC-A

glycine coated beads + biotinylated lectin + SA-conjugated APC
uEV coated beads
uEV coated beads + biotinylated lectin + SA-conjugated APC
Figure 4-17: Flow cytometry-based comparison of surface CD24 and glycosylation profiles of UC-uEVs from age-matched subjects without kidney disease (healthy, n = 5) and with ADPKD (PKD or “diseased”, n = 5).

Left Panels: Histograms (shaded) of binding of anti-CD24, PNA, Jacalin, RCA-I and SNA-I to UC-uEV-coated beads from all subjects in the two groups compared to histograms of relevant negative control. Right Panels: Graphs of the results for individual samples expressed as fold-change of mean of median fluorescence intensity (MFI) over the unstained control. Two technical replicates were recorded per sample.

Two-tailed, unpaired Student’s t-test p-value CD24: 0.2148; p-value SNA-I: 0.1529; p-value RCA-I: 0.1686; p-value PNA: 0.4115; p-value Jacalin: 0.5146.

The lectins selected for analysis in this pilot study indicated the presence of terminal Gal as often presented in O- and N-glycans. Galactose residues can be further linked to terminal Neu5Ac in a ($\alpha$$2$$\rightarrow$$6$) or ($\alpha$$2$$\rightarrow$$3$) manner. The presence of ($\alpha$$2$$\rightarrow$$6$)Neu5Ac is recognizable by SNA-I and this lectin did bind to Control and ADPKD uEVs to a variable degree. Although there were some interesting trends (higher staining for CD24, RCA-I and SNA-I in some PKD samples compared to all Controls and a trends toward lower staining for PNA and jacalin PKD samples), the small number of available samples precluded making any definitive conclusion regarding alterations to uEV surface glycosylation in early ADPKD. Nevertheless, it was possible to conclude that uEV surfaces in both groups expose Gal residues on their surfaces with evidence of O- and N-glycan origin and some degree of ($\alpha$$2$$\rightarrow$$6$)Neu5Ac capping.

4.5.2 Correlation of lectin binding to uEVs in two distinct platforms FCM and lectin microarray

In parallel LM experiments carried out on the age-matched control and ADPKD samples by Dr. Jared Q. Gerlach, some significant differences were observed between control and ADPKD groups for individual lectins and by principal component analysis of the full LM dataset (Gerlach, Krüger et al. 2013). This observation brought up the question of how qualitatively and quantitatively consistent the data generated by FCM assays (in which the lectins are present in solution) are with data generated by LM (in which the lectins are attached to the surface of a glass slide). In the final analysis for this Chapter, a comparison was made of quantitative lectin binding data (fluorescence intensities) obtained for seven different lectins by FCM and LM using aliquots of UC-uEV preparations from three healthy adults (table 4-4).
Table 4-4: Comparison of fluorescence intensities and binding rank order of 7 lectins in LM and FCM platforms for aliquots of three healthy adult UC-uEV samples.

<table>
<thead>
<tr>
<th>Platform</th>
<th>LMA (normalized data)</th>
<th>FCM (mean MFI fold over control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>healthy UC (n=3)</td>
<td>healthy UC (n=3)</td>
</tr>
<tr>
<td>method</td>
<td>(mean ± stdev) -10^4³</td>
<td>rank no.</td>
</tr>
<tr>
<td>lectin</td>
<td></td>
<td>(mean ± stdev) -10^4³</td>
</tr>
<tr>
<td>PNA</td>
<td>2.33 ± 0.55</td>
<td>6</td>
</tr>
<tr>
<td>Jacalin/AIA</td>
<td>9.77 ± 2.33</td>
<td>2</td>
</tr>
<tr>
<td>RCA-I</td>
<td>12.23 ± 2.08</td>
<td>1</td>
</tr>
<tr>
<td>SNA-I</td>
<td>6.93 ± 1.64</td>
<td>3</td>
</tr>
<tr>
<td>MAA</td>
<td>3.79 ± 0.56</td>
<td>5</td>
</tr>
<tr>
<td>PHA-E</td>
<td>6.58 ± 2.17</td>
<td>4</td>
</tr>
<tr>
<td>GSL-IB4</td>
<td>1.87 ± 0.18</td>
<td>7</td>
</tr>
</tbody>
</table>

All of the seven lectins for which the two platforms could be compared demonstrated clear and specific binding to uEVs. Thus, in the broadest sense, the methodologies provided consistent information regarding the complexity of uEV surface glycosylation. For three of the seven lectins (MAA, PHA-E and Jacalin/AIA) the rank orders of fluorescence intensity were similar indicating close compatibility between the two platforms. For the remaining 4 lectins (PNA, SNA-I, GSL-IB4, RCA-I) the rank orders for FCM and LM differed. Most notably, PNA and GSL-IB4 were 1\textsuperscript{st} and 3\textsuperscript{rd} highest binders in the FCM assay compared to 6\textsuperscript{th} and 7\textsuperscript{th} in the LM assay.

It was concluded that: (a) Both FCM and LM platforms represent sensitive techniques for screening the surface glycan characteristics of EVs and have potential to be applied to uEV samples from healthy individuals and those with kidney disease. (b) Interpretation of the quantitative results obtained for individual lectins from a single platform should be made with caution due to platform-specific variability in their binding intensities.
4.6 Discussion

4.6.1 Development of protocols for isolation and quality control of uEVs

The initial objective of this chapter was to enrich uEVs of high quality with reliable and efficient isolation procedures for the purpose of applying lectin-based glycosylation profiling techniques. Thus, UC and CF protocols as described in literature by two distinct groups were tested. In our hands, UC consistently provided samples containing vesicles of the previously-reported size and cup-shaped morphology by TEM (Hogan et al. 2014; Pisitkun, Shen, and Knepper 2004; Théry et al. 2006a). Furthermore, protein yields from the UC protocol were similar to or greater than those reported for nanofiltration (Merchant et al. 2010). In the case of the CF protocol, protein yields were comparable to those of UC-uEV preparations and vesicles of expected size were detectable by TEM but, in keeping with the findings of others, lacked a cup-shaped morphology (L.-L. Lv et al. 2014). Moreover, as we subsequently observed in down-stream applications such as lectin blots and antibody-based immunoblots, particle counting and FCM analysis, the major drawback of CF-uEV samples was co-enrichment of abundant, urine-derived proteins associated with significantly lower vesicle purity and density. These key findings led us to exclude CF-based isolation of uEVs for subsequent experiments throughout the project.

The experimental work for this Chapter also provided important insights into the challenges of performing down-stream analyses of uEVs generated from urine samples of limited volume – as might be anticipated for large-scale patient-orientated research and clinical applications. Typical “spot” urine samples are in the range of 50-200 ml volume with expected uEV yields ranging from 20 to 100 µg of protein using UC or other isolation methods. Thus, the potential to apply conventional assays such as immunoblotting for individual uEV-associated glycoproteins/proteins was restricted. In our hands, only a limited number of such markers could be reliably analysed in each sample by immunoblotting. As our experiments progressed, we observed that two glycoproteins – CD24 and AQP-2 – were most consistently detectable across multiple analysis techniques (immunoblot, TEM, FCM) and antibodies against these specific markers became important tools for validating and comparing samples that were subsequently subjected to profiling of glycosylation. Of note, FCM-based detection of CD24 and AQP-2 on the surface of EVs has also been reported by others (Echevarria et al. 2014; Keller et al. 2007).
4.6.2 Surface glycosylation of uEVs

The experiments performed for this Chapter using uEVs from healthy adults provided one of the first detailed analyses of the surface glycosylation characteristic of human uEVs. Initially glycoproteins of uEVs were detected with lectin blots using AAA (α-Fuc), PHA-E (biantennary N-glycan structures), SNA-I ((α2→6) linked-Neu5Ac) and PNA (Gal(β1→3)GalNAc). Both UC-uEVs and CF-uEVs showed similar binding profiles with these lectins. The findings suggested the presence of glycoproteins containing biantennary N-glycan structures and high molecular weight protein-associated O-glycan structures. In general, N- and O-glycan structures may be fucosylated or sialylated in humans. The presence of Fuc and Neu5Ac in uEV-associated glycoproteins was confirmed by detection of multiple bands in AAA and SNA-I lectin blots and was in keeping with the published studies in this area (Echevarria et al. 2014; Staubach et al. 2012). Very recently, an additional detailed study revealed even proportions of fucosylated N-glycans (total fucosylation 65%) core only and core plus antennae with less antennae-only fucosylation (Saraswat et al. 2015). Overall, our initial observations regarding human uEV protein glycosylation were in accordance with the small number of studies that were published during the time that the project was on-going and highlight the need for additional research in this area.

Further efforts to develop a lectin-based screening assay for glycoprofiling of uEVs using bead-based FCM were successful. Flow cytometry is a fast, convenient method for cell or uEV screening and we were able to demonstrate its potential for higher sample throughput by adapting a published protocol to a 96 well format. We were also the first to report the use of soluble lectins to detect and profile uEV surface glycans in a FCM format and to validate the carbohydrate-specificity of the lectin binding using haptenic sugar competition. Recent publications reveal that uEV glycosylation is rich in complex N-glycan as well as high-mannose N-glycan structures (Saraswat et al. 2015; Staubach et al. 2012). The selected lectin panel for this study primarily covered terminal glycans motifs. The binding profiles we observed for UC-uEVs included lectins with affinity for terminal Neu5Ac, α-Fuc and α- and β-Gal and/or GalNAc which suggest the presence of complex or hybrid N- and O-glycan structures as well as glycolipids on UC-uEV surfaces - observations which were later confirmed by comprehensive LM studies in our own laboratory and by others (Echevarria et al. 2014). Although not evaluated by FCM, additional studies in our laboratory in which uEVs...
were treated with α-sialidase (Neu5Ac-specific exoglycosidase also called α-neuraminidase) and endo Tv (high-mannose structures specific endo-β-N-acetylgalactosaminidase) prior to LM profiling demonstrated increased binding intensity for multiple lectins, in keeping with an “unmasking” phenomenon by which removal of terminal residues provided lectin access to a greater array of underlying glycan structures (Gerlach et al. 2013).

Our profiling study of UC-uEVs from healthy adults, along with other studies published while our work was on-going, showed that the uEV surface displays glycans associated with proteins, lipids and, potentially, other glycoconjugates. Multiple lectins bound differently to UC-uEV and CF-uEV isolates – most likely due the heavy protein contaminations observed with the CF-uEVs. As demonstrated in our pilot study using uEVs from ADPKD patients, the bead-coupled FCM method developed for this Chapter could also be applied to the analysis of uEV glycosylation in individuals with a specific kidney condition.

4.6.3 Comparison of FCM and LM glycosylation assay platforms

Two main platforms for glycoprofiling of uEVs were chosen in our laboratories: (a) the already-established LM technique and (b) the bead-based FCM technique, the development of which is described in this Chapter. Comparison of lectin binding to the same set of UC-uEVs for both techniques elucidated the fact that particular lectins (e.g. PNA, GSL-IB4) appeared to have reduced affinity when surface-bound (LM) than when they were in soluble form (FCM). Similarly, biotinylation of certain lectins (e.g. SNA-I) used in the FCM assays, may have compromised carbohydrate binding in this platform based on the observed higher binding intensity in the LM assays. As biotin (and fluorochrome) coupling to detection agents tends to occur randomly on the protein surface and may differ for individual batches, this must be considered as a limitation in interpreting the FCM results for lectins with apparent low-level or negative binding. In contrast, the LMs used for our uEV studies were printed with the appropriate haptenic sugar to exclude deformation of the binding domain (Gerlach et al. 2013). The fact that some of the results obtained for individual lectins by FCM were at variance with those obtained for LM as well as the differences observed between UC-uEVs and CF-uEVs highlights the importance of validating novel findings regarding EV biomolecular content through the use of multiple analytical approaches.
4.7 Conclusion

A UC-based protocol for uEV isolation was established that fulfilled most of our experimental requirements. A complex range of carbohydrate moieties was shown to be displayed on the uEV surface using a novel FCM-based technique which was broadly consistent with results obtained separately using LM. Throughout the experiments presented in this Chapter, non-specific lectin binding was ruled out by inclusion of relevant controls and via binding inhibition by haptenic sugars. Our experience indicated that FCM is a relatively cost effective, flexible platform for profiling surface glycosylation of uEVs. Nonetheless, for the subsequent stages of this project (described in Chapters 5 and 6) it was decided to employ the LM platform in preference to FCM. The primary reasons for this were the broader range of lectins and lower amounts of uEV samples that could be utilised in LM.
Chapter Five
Results

*Optimised Subfractionation of Human Urine Extracellular Vesicles for Surface Glycoprofiling*
5 Results

5.1 Introduction

In the five years prior to the initiation of this project, research on EVs had increased dramatically but published studies on uEVs focused primarily on technical issues such as isolation procedures and on the characterisation of EV-associated proteins/peptides and RNA in healthy urine (Merchant et al. 2010; Miranda et al. 2010, 2014; Pisitkun, Shen, and Knepper 2004; Rood et al. 2010). Large clinical studies on uEVs derived from subjects with specific kidney diseases were lacking. However, another important limitation to the application of EV analysis to clinical research and diagnostics has been the heterogeneity of vesicles present in isolates from biological fluids following conventional isolation approaches such as the UC and CF protocols applied in Chapter 4 (Hara et al. 2010; Hogan et al. 2009, 2014; Mitchell et al. 2009; Prunotto et al. 2013).

Cells along the nephron may release EVs of widely varying sizes and biomolecular content via exocytosis, direct shedding from the cell surface or, potentially, by other mechanisms (Hara et al. 2010; Hogan et al. 2014; Pisitkun, Shen, and Knepper 2004). In addition, epithelial cells of the entire urinary tract including the nephron, pelvis/ureter, bladder and organs of the male or female genital tract may contribute to the final composition of uEVs. For example, a study on prostate cancer using sucrose-cushion purified uEVs encountered this complexity problem leading possibly to false-negative results (Mitchell et al. 2009). Thus, the UC-uEV glycoprofiles identified by our group as described in Chapter 4 and by others (Echevarria et al. 2014; Gerlach et al. 2013; Saraswat et al. 2015; Staubach et al. 2012) are likely to represent the combined surface glycosylation characteristics of a broad range of vesicle types. Indeed, each cell is surrounded by a protective sugar coat which consists of many distinct carbohydrate-associated molecules inserted into the cell membrane and the repertoire of glycoproteins and glycolipids varies widely among cell types (Ajit Varki et al. 2009b, 2009e). Furthermore, carbohydrates may be differentially modified during biogenesis by different cell types (Gagneux and Varki 1999).

Batista et al. showed that glycosylation profiles of a parent cell and its shed microvesicles are not identical raising important questions regarding the biological role of carbohydrates in uEVs (Batista et al. 2011). The concept of uEVs playing a specific role in intercellular
communication along the nephron in health and disease is supported by recent reports of urinary exosomes containing a wide range of mRNA and miRNA species and by the demonstration that uEVs expressing the ADPKD-associated proteins PKD1 and PKD2 preferentially interact with the primary cilia of epithelial cells (Hogan et al. 2009; Lasser et al. 2011; Miranda et al. 2010; Valadi et al. 2007). Ultimately, distinct uEV subpopulations may display specific glycosylation profiles for preferential receptor binding to recipient neighboring cells as recently discussed for exosomes derived from ovarian cancer cells (Escrevente et al. 2011). In this section of the project we began to address uEV heterogeneity as it relates to surface glycosylation by developing approaches for subfractionating UC-uEV pellets generated from health adult urine to the LM platform.

A further challenge to this specific project was the potential confounding of glycoanalytical studies of UC-uEVs or uEV subfractions due to THP and other co-enriched non-vesicular glycoproteins (Fernández-Llama et al. 2010; Merchant et al. 2010). This challenge in particular is likely to be greater when analyzing urine samples from patients with acute or chronic kidney disease in whom pathological damage to the glomerular barrier or defective tubular reabsorption of proteins is common and may greatly enhance urine content of solubilised protein (Rood et al. 2010). Tamm Horsfall protein is the most abundant glycoprotein in urine with a normal excretion rate of 20-160 mg/day (Kobayashi and Fukuoka 2001). It is expressed on the apical surfaces of cells lining the thick ascending limb of the loop of Henle and distal convoluted tubule and occurs as GPI-anchored protein that can be released by proteolysis (Kreft et al. 2002; Rindler et al. 1990). Its documented functions within the distal nephron include prevention of crystallisation, regulation of water and ion flux and direct and indirect anti-microbial effects with some of these properties being glycosylation-dependent (Horton et al. 1990; Kreft et al. 2002; Pak et al. 2001; Reinhart, Obedeanu, and Sobel 1990; Rhodes 2000; Thomas et al. 1993). The glycosylation of THP is more complex and exceeds the usual glycosylation profile of simple glycoproteins as shown by the presence of high-mannose structures, sulfated residues, the Sdα–antigen in addition to repetitively elongated lactosamine antennas (Easton et al. 2000; Hard et al. 1992; Van Rooijen, Kamerling, and Vliegenthart 1998; Staubach et al. 2012).

Multiple approaches to the removal of THP from uEV isolates have been reported. Most commonly, the reducing agent DTT has been used for THP removal through the reduction of disulfide bonds in the zona pelludica region of the target glycoprotein as first described by
Fernandez et al. (Fernández-Llama et al. 2010). A practical disadvantage of this approach is the requirement to use relatively large amounts of a toxic, irritant compound. An alternative protocol was recently reported by Musante et al. based on the use 1% (w/v) CHAPS solution which also yielded intact uEV isolates free of significant THP contamination (Musante et al. 2012). Ultimately, we posited that successful removal of THP would be an important requirement for developing a protocol for glyco-profiling of subfractionated UC-uEVs from healthy urine and, in particular, from urine of individuals with kidney disease. Therefore the removal of THP during UC-uEV subfractionation and its importance for successful application of glycoanalytical studies to UC-uEV subfractions was specifically addressed in this Chapter.

Finally, as noted in the conclusion to Chapter 4, it was decided to focus on the LM platform for analyzing and comparing the surface glycomes of UC-uEV subfractions. This relatively simple, high-throughput technique has been well described over the past decade as a method to study the carbohydrate content of fluorescently-labelled glycoprotein solutions, biological samples and cell lysates as well as the diversity of glycans displayed on the surfaces of bacteria, viruses and eukaryotic (Ebe et al. 2006; Gerlach et al. 2013; K.-L. Hsu, Pilobello, and Mahal 2006; Krishnamoorthy et al. 2009). Key advantages of the LM platform which were of relevance to this project included the small amount of sample (nano molar range) required to generate a “fingerprint” profile based on lectin-glycans interactions (Hirabayashi et al. 2013); the relatively large number of lectin affinities that can be measured in a single array; the ease of analysis of data based on directly measured fluorescence intensity; the reproducibility of the method and the fact that it had previously been employed in the discovery of disease-associated glycan signatures (Fry et al. 2011; Kuno et al. 2011; Wu et al. 2012). Furthermore, as described in Chapter 4, our group had successfully applied LM to profile the carbohydrates exposed on the surface of unfractionated UC-uEVs (Gerlach et al. 2013).

In this Chapter, I describe the characterisation of fluorescently labelled UC-uEV subfractions prepared using a sucrose density gradient protocol modified to allow for consistent THP removal along with proof-of-principle experiments in which high quality LM profiles were generated from a small number of test samples.
5.2 Hypothesis, aim and objectives

Original research described in Chapter 4 established that uEVs isolated from healthy adult urine samples exhibit a complex surface glycosylation profile using lectin-based techniques. This Chapter focuses on further exploring the basis for uEV surface glycome complexity and supporting future kidney disease-associated uEV glycosylation signatures through development of techniques for comparative analysis of density-based subfractions.

5.2.1 Hypothesis
Urine EV subfractions generated by density gradient ultracentrifugation from healthy adult urine samples have distinct surface glycosylation profiles which may be of value for resolving uEV heterogeneity.

5.2.2 Aim
The overall aim of this section of the thesis was to develop an approach for applying LM technology to compare the surface glycoprofiles of density gradient-separated fractions of healthy adult uEVs.

5.2.3 Objectives
1. Fractionate fluorescently-labelled UC-uEV pellets based on physical density using sucrose density gradient ultracentrifugation and perform initial protein analyses of all fractions.
2. Validate the application of LM technology for comparative analysis of sucrose density gradient separated uEV fractions.
5.3 Results

5.3.1 Sucrose density gradient fractionation of uEVs from pooled healthy adult samples

In order to subfractionate uEVs from human urine samples for the purpose of investigating variability in surface glycoprofile, the sucrose density gradient centrifugation approach, as described by Thery et al. in 2006, was established. Initially, test gradients (8.5-50% sucrose) were poured by hand, loaded with PBS and subjected to UC for 16 hours followed by collection of 1 ml fractions from the top down as described in Methods 3.2.13 (Théry et al. 2006a). Optical densities were measured for fractions 3 to 12 (with fraction 12 representing the lowest fraction from the gradient). As shown in figure 5-1, there was a linear relationship between density and fraction number for fractions 3 to 11.

![Figure 5-1: Relation of density to fraction number for sucrose gradient prepared according to Thery et al. with PBS loading (Théry et al. 2006a).](image)

Data expressed according to the Brix scale in respect to 20 °C. Each point represents mean ± SD for n=4 gradients prepared in 2 independent experiments.

It was concluded that the hand-poured, 8.5-50% sucrose density gradient could be used to generate up to 10 individual fractions corresponding to a density range from 1.03 to 1.24 g/cm³.

Thus, the technique was next applied to UC-uEVs prepared from urine samples of healthy adults as described in Chapter 4.

UC-uEVs were isolated from 200 ml urine samples of three healthy adults and were then pooled and fluorescently labelled with PKH26. The pooled PKH26-UC-uEVs were loaded onto a sucrose density gradient which was subjected to UC and separated into 10 fractions as described above.
The protein content of each fraction was quantified by BCA assay. As shown in figure 5-2 A, fractions 3 to 5 (F3 to F5) contained very low amounts of protein and, in subsequent analyses of this gradient, these fractions were not analysed. The highest protein yield was found in F7 (~1.14 g/cm³) and F8 (~1.17 g/cm³) while F12 reflected the protein content of material pelleted at the bottom of the gradient.

Protein aliquots from fractions 5 to 12 were then separated on a 4-12% Bis-Tris PAGE followed by silver staining (figure 5-2 B). This demonstrated a large diversity of protein bands for all fractions across a wide range of molecular weights. The band patterns were broadly similar across the fractions although specific bands could be seen to vary between lower and higher fractions. A moderately strong band of the approximate molecular weight of THP was observed in F6-8 and strong band of the approximate molecular weight of albumin was observed in F12.

Next, protein aliquots from the same fractions were analysed by immunoblotting for the uEV markers CD24 (figure 5-2 C) and AQP-2 (figure 5-2 D). Both proteins were readily detected in all fractions but the band intensities were strikingly stronger in F6-F7 for CD24 and in F6-F8 for AQP-2 consistent with enrichment in these fractions of EVs expressing these specific glycoproteins.

Overall, it was concluded from these initial experiments that: (a) PKH-labelled UC-uEVs from healthy adults could be readily subfractionated by density gradient UC with excellent preservation of protein quality and diversity. (b) Of the 10 fractions obtained, sufficient uEVs for subsequent analyses could be most reliably obtained from F6, F7, F8, F9 and F10 as well as the “pellet” fraction F12 (although in subsequent gradients derived from larger samples it was also possible perform analyses of PKH-UC-uEVs from F3, F4 and F5). (c) The higher intensities of CD24 and AQP-2 bands in immunoblots of lower density compared to higher density fractions were in keeping with uEV and uEV-glycoprotein diversity across the gradient fractions.
Figure 5-2: Protein analyses of sucrose density gradient fractions of PKH26-UC-uEVs pooled from three healthy adult urine samples. (n=1)

A. Protein content of fractions 3 to 12 estimated by BCA assay. The density of each fraction is indicated.

B. Silver stained 4-12% Bis-Tris PAGE of 0.4 µg protein aliquots of fractions 6 to 12.

C. Immunoblot of 0.45 µg protein aliquots of fractions 6 to 12 for CD24.

D. Immunoblot of 0.4 µg protein aliquots of fractions 6 to 12 for AQP-2.
5.3.2 Surface glycoprofiling of density gradient subfractions from healthy adult urine

For the next set of experiments, UC-uEVs were prepared from 400 ml urine samples from four healthy adults, labelled with PKH26 and subjected to sucrose density gradient separation as described above. Protein content of individual fractions was compared by silver staining of Bis-Tris-PAGE gels and by immunoblotting for CD24 and AQP-2 (figures 5-3 and 5-4). As shown in figure 5-3, THP was strongly co-purified in fractions 8-12 of two of these preparations. Processing of repeat samples from the same donors resulted in a similar outcome suggesting that THP contamination of sucrose density gradient-separated uEVs may be subject-specific (data not shown).

![Silver stained 4-12% Bis-Tris PAGES of sucrose density gradient subfractions (F3 to F12, 0.25 µg protein each fraction/well) of PKH26-UC-uEVs from four healthy adult urine donors (D100, D115, D101 and D208). Ori = original UC-uEV preparation. A. Samples without THP contamination. B. Samples with THP contamination.](image-url)
For the two subfractionated samples not affected by THP contamination, immunoblots for CD24 and AQP2 demonstrated similar patterns of strong staining of fractions 4 to 8, weak staining of fractions 9 to 11 and moderate staining of fraction 12 (figure 5-4).

![Immunoblots for CD24 (A) and AQP-2 (B) of sucrose density gradient subfractions (F3 to F12) of PKH26-UC-uEVs from two healthy adult urine donors (D100, D115) lacking THP contamination. Ori = original UC-uEV preparation.](image)

It was concluded that: (a) Tamm-Horsfall protein cannot be consistently eliminated from UC-uEV preparations by a standard sucrose density gradient centrifugation protocol. (b) Lower density uEV fractions (1.03 to 1.17 g/cm³) are enriched for vesicles containing high amounts of CD24 and AQP-2 suggesting that sucrose density gradient subfractionation has the potential to enrich for vesicles of different subtypes and/or vesicles derived from different cell types along the nephron and urinary tract.

Despite low sample quantities, LM profiles were obtained from the THP-free PKH26-UC-uEV gradient fractions of D100 and D115. The results of these LMs are shown in figure 5-5.
as heat maps of normalized fluorescence intensities based on 3 technical replicates for each individual fraction.

Figure 5-5: Lectin microarray results for individual fractions (F3 to F11) of PKH26-UC-uEVs isolated from 400 ml urine samples from two healthy adult “donors” (A = D100, B = D115).

Data were generated in HCE 3.5 software after mean normalization based on 3 technical replicates per fraction and 6 spots per lectin for each technical replicate.

Although this was viewed as a preliminary experiment, it was possible to make several conclusions: (a) The profiles observed were broadly similar for fractions from the two healthy adult samples. (b) All fractions generated profiles that were characterized by binding to a broad range of lectins but also included multiple non-binding lectins. (c) One subset of lectins showed relatively consistent, medium-to-high intensity binding across all the gradient fractions. This included SNA-II, DBA, ACA, ABL DSA, LEL, Calepsa, WGA and RCA-I. (d) Certain lectins demonstrated a pattern of higher binding intensity to fractions of higher density. These included PA-I, VVA-B4, BPA, WFA, NPA, GNA, HHA, PHA-L, PHA-E and MOA. (e) Two lectins, SNA-I and STA, demonstrated a pattern of higher binding to fractions of lower density. In summary, lectin binding to PKH26-uEV subfractions showed complexities consistent with those observed for unfractionated uEVs in Chapter 4 with initial evidence for both density-independent and density-dependent lectin binding intensities.
To clarify these glycoprofile trends, the PKH26-uEVs subfractions from the two healthy donors were simplified to three samples each - Low-density (pooled F3-F6), Mid-density (F7) and High-density (pooled F8-F10) – which were then re-analysed by LM. Heat maps for this analysis are shown in figure 5-6.

![Heat map](image)

Figure 5-6: Lectin microarrays results for low (F3-6), medium (F7) and high (F8-10) density PKH26-UC-uEV samples from two healthy adult “donors” (D100 and D115).

Panel A: Heat map generated in HCE 3.5 software after mean normalization based on 3 technical replicates per fraction and 6 spots per lectin for each technical replicate.

Panel B: Unsupervised hierarchical cluster map. Zero corresponds to no similarity between samples or lectin binding and one corresponds to total similarity.

As shown, this simplified analysis confirmed the density-dependent trends in binding intensity to certain lectins, particularly those of NPA, GNA and HHA (higher binding with higher vesicle density) and those of SNA-I and STA (higher binding with lower vesicle density). In addition, MAA was also seen to bind with higher intensity to the lower density pool in this experiment.
5.3.3 THP removal from uEVs for accurate glyco-profiling with lectin microarrays

As previously shown in figure 5-3B, 2 of the initial four UC-uEV preparations from healthy adult urine samples contained significant THP contamination despite density gradient subfractionation. Indeed, repeated experiments showed the appearance of THP in medium to high density fractions of about 8.5-50% sucrose gradients (not shown). Thus, THP cannot be consistently eliminated from UC-uEVs by applying a sucrose density gradient based on 20 mM HEPES buffer. When pre-clarified, first morning void urine from eight additional healthy adults was analysed by Bis-Tris-PAGE and silver staining, the THP content was highly variable with at least 50% having high THP content likely to contaminate subsequent UC-uEV isolates (figure 5-7A). In contrast, albumin content was similar across all samples.

To develop a method for THP removal without altering the surface glycosylation/lectin binding pattern of the resultant uEVs, the reducing agent tris(2-carboxyethyl)phosphine (TCEP), first described by Levison in 1969 in relation to reduction of Gamma-Globulin, was tested (Levison, Josephson, and Kirschenbaum 1969). TCEP reduces disulfide bridges in a similar manner to DTT but is more efficient in pH ranges < 8.0 and is more compatible in sulfhydryl-based labelling reactions and nickel protein purification columns. Furthermore, it can be used at r.t. and is non-irritant (Getz et al. 1999; Han and Han 1994). TCEP was also reported in regards to glycopeptides and glycoproteins (Gong Chen et al. 2007; Rebecchi et al. 2011). Thus, TCEP was added prior to density gradient UC of a pooled PKH26-UC-uEV sample from two healthy adults with high initial THP contamination (figure 5-7A, 211 and 221). As shown in figure 5-7B, no THP was evident in the resulting fractions which otherwise contained the expected complex band patterns.
Variable contamination of first morning urine samples with THP and its removal by addition of TCEP to gradients.

A. Silver stained 4-12% Bis-Tris PAGE of 8 µl aliquots of pre-clarified urine of 8 healthy adults (lanes 2-9), purified THP (lane 11) and gradient-separated UC-uEVs (lane 12) from one urine sample. Samples with THP contamination that were pooled to test the use of TCEP are indicated by blue arrows.

B. Silver stained 4-12% Bis-Tris PAGE of individual fractions (F3-F12) from TCEP-treated density gradient of pooled UC-uEVs.

Immunoblotting of all fractions from this gradient for CD24 showed the expected high level of this EV-associated protein in lower density fractions (F4-F7) with lower-level expression in F8-F11 (figure 5-8).

CD24, Donor 221, 211

Figure 5-8: Anti-CD24 immunoblot of fractions 3-12 from TCEP-treated density gradient of pooled PKH26-UC-uEVs from two healthy adult urine samples with initial high THP contamination.
Furthermore, LM of three fractions (F5, F7 and F8) from same gradient demonstrated broadly similar patterns of lectin binding to those observed with non-TCEP-treated gradients (figure 5-9).

Figure 5-9; Heat map showing LM glycoprofiles of fractions 5, 7 and 10 from TCEP-treated density gradient of pooled PKH26-UC-uEVs from two healthy adult urine samples (211/221) with initial high THP contamination.

In summary, (a) A high proportion of tested individuals showed high THP excretion in the first morning void that resulted in contamination of multiple fractions from standard sucrose density gradients. (b) Successful removal of THP was achieved by addition of the reducing reagent TCEP to sucrose density gradients allowing for purification of PKH26-uEV fractions suitable for surface glycoprofiling by LM.

5.4 Discussion

5.4.1 Gradient subfractionation of uEVs

In this Chapter, a protocol allowing the separation of uEVs into subfractions based on density (8.5-50%) was successfully developed. The value of subfractionating uEVs in order to enrich and better characterise specific vesicle populations in particular regions of the gradient is exemplified by the work of Hogan et al. using a 0-30% sucrose density gradient prepared with deuterated (“heavy”) water (D₂O) (Hogan et al. 2014). In this study, subfractionated uEVs demonstrated high diversity including distinct vesicle types and evidence for specificity of cell source from different regions of the nephron and urinary tract.

There are, of course, disadvantages to gradient (and other) subfractionation approaches including the requirement for larger volumes of urine and the relatively labour-intensive and time-consuming protocols involved. Additionally, as we have shown here, the final uEV content is not distributed evenly across the gradient fractions and the amount of material
acquired for analysis of lower-density fractions in particular tends to be very low and limits the range of analysis techniques that can be applied. Nonetheless, in samples not heavily contaminated by THP, it was possible to perform immunoblots of all fractions for two proteins, AQP-2 and CD24 with known distribution patterns along the nephron and we observed that these were more abundant in low-to-medium density fractions. Expression of AQP-2 is restricted to the principal cells of the cortical collecting duct while CD24 is expressed by parietal epithelial cells of the glomerulus as well as immature proximal tubular epithelial cells and, at low level, by epithelial cells of the ureter and bladder. Cells expressing CD24 are known to be increased in the developing kidney and during regeneration and repair following acute kidney injury. Increased expression of CD24 has been also reported in multiple cancers including carcinomas of the kidney and urothelium (Angelotti et al. 2012; Y.-L. Choi et al. 2007; H. J. Lee et al. 2008; Lindgren et al. 2011; Nielsen et al. 2002; Smeets et al. 2013). Our observation that these proteins were primarily present in the lower density fractions from gradients of healthy adult urine suggests that these fractions may be enriched for specific sub-types of uEV and/or uEVs derived from specific cell types. It also provides supportive evidence for subfractionation as an intermediary step toward quantifying potential disease-associated biomolecules in uEVs.

Thus, our initial observations in this chapter were in keeping with the concept that uEVs can be separated using sucrose density gradients into subpopulations with distinct and biologically relevant characteristics.

5.4.2 Removal of THP during gradient subfractionation

Consistent with some previous reports, we observed that sucrose density gradient did not consistently result in THP-free uEV subfractions. While there is substantial inter-individual variability, THP is frequently excreted in high amounts into urine (Fernández-Llama et al. 2010; Hunt et al. 1985). In this Chapter, our screening of urine derived from several individuals confirmed this assumption. Specifically, we demonstrated that, in urine samples with high initial THP content, relatively large amounts of THP appear in fractions of density > 1.14 g/cm³ resulting in a significant barrier to further analysis of higher-density fractions. For comprehensive glyco-profiling of UC-uEV subfractions, this clearly represented an important obstacle. This issue has been well reported by others and has been most commonly been address by addition of DTT during key steps in the uEV isolation procedure although it
should be acknowledged that use of reducing agents may also result in loss of EV-associated protein domains due to cleavage of disulfide bonds (Fernández-Llama et al. 2010).

As a novel approach to elimination of THP from the gradients, we tested the potential for an alternative reducing agent, TCEP which has a track record in the field of proteomics but was also used with glycoproteins and glycopeptides before (Bai et al. 2013; Gong Chen et al. 2007; Miura et al. 2008; Rebecchi et al. 2011). TCEP-HCl is an odorless, crystalline, air-stable solid which is soluble in water and reacts rapidly (<5 min) with disulfides at r.t. in dilute (1 mM) solutions and has provides stronger reduction at pH between 1.5 to 8.5 compared to DTT (Han and Han 1994). Thus, uEVs isolates can be readily stored and manipulated in buffered saline solutions at r.t. without the necessity for working in a hood and without interfering with the reducing power of the compound.

It is important to acknowledge, however, that the use of TCEP as a reducing agent to eliminate THP contamination also carries a risk of modifying the structure of EV-associated proteins including the possibility of unwanted alterations to their carbohydrate attachments. Nevertheless, in our initial analyses of UC-uEV fractions generated by sucrose density gradients, the patterns of protein banding on silver stained Bis-Tris PAGE as well as the results of immunoblotting and, as demonstrated in the next Chapter, visualization by TEM did not indicate any major disruption to the resulting vesicles or their protein content. We concluded, therefore, that addition of TCEP represents a previously unreported and valid approach to consistently eliminating THP during the process of uEV subfractionation for downstream biomolecular analyses. An alternative method, reported by Musante et al. in 2012, involved the use of 1% CHAPS buffer with similar success in purification of THP-free uEVs but this compound was not tested in context of the project reported in this thesis (Musante et al. 2012).

5.4.3 Lectin-microarray-based glycoprofiling of subfractionated uEVs
Proof-of-principle experiments presented in this Chapter indicate that a gradient-based subfractionation approach with pre-labelling of the initial UC-uEV pellet by a lipid membrane-incorporated fluorescent dye and elimination of THP by addition of TCEP, appears to be well suited to the downstream application of LM. Given the small amount of material obtained for low-density uEV fractions, an important advantage of the LM platform proved to be its capacity to generate high quality data from limited samples. In keeping with
the results for PAGE and immunoblot, the preliminary LM results described here for pooled and individual UC-uEV fractions were indicative of specific density-dependent variabilities. Given the well documented sensitivity and specificity of the LM platform for detecting differences in glycosylation among samples of individual proteins, complex protein lysates, microbes, eukaryotic cells and subcellular particles, these results provided evidence that the overall surface glycoprofiles observed for UC-uEV in Chapter 3 and in Gerlach et al. could be further resolved through analysis of fractionated samples. We further hypothesise that accurate definition of the normal glycosylation signatures of specific uEV subfractions can increase the likelihood of subsequently identifying disease-specific alterations to uEV glycosylation.

The majority of studies published in this area discuss the fact that it remains difficult to separate EV subpopulations using protein based surface markers (D.-S. Choi et al. 2014; Colombo, Raposo, and Théry 2014; Graça Raposo and Stoorvogel 2013). However, glycosylation of proteins can vary based on the concept of microheterogeneity in specific glycosylation sites on the proteins as well a variances in glycosylation occurring in cells from diverse cell types and tissues (Moremen, Tiemeyer, and Nairn 2012; P. Van Den Steen et al. 1998). Thus accurate glyco-profiling of uEV surfaces could be of value for improvement of vesicle isolation techniques or as a complement to studies investigating uEV surface marker protein expression (e.g. by FCM or TEM approaches). For instance, a particular lectin panel might allow for affinity-based isolation of specific EV subpopulations based on their surface glycosylation with the potential to better characterize their biomolecular content using mass spectrometry or, in the case of nucleic acids, high-throughput sequencing approaches (Gonzales et al. 2009; Hogan et al. 2014; Ji et al. 2014; Miranda et al. 2014; Pisitkun, Shen, and Knepper 2004).

In the results presented here, LM glycoprofiling of fractionated samples from 2 healthy adults demonstrated several consistent trends which served as a basis for a more extensive study described in Chapter 6. Overall, for the fractionated samples, a broad range of lectin binding was observed as previously reported for unfractionated UC-uEVs (Echevarria et al. 2014; Gerlach et al. 2013). For example, a distinct grouping of GlcNAc-binding lectins such as LEL, DSA and WGA were found to bind strongly to uEV subfractions across the entire gradient (Hirabayashi et al. 2013). Similarly, RCA-I with known specificity for LacNAc
motifs was shown to bind highly to uEV surfaces independently of density. However, a striking trend within the LM profiles was a progressive increase in fluorescence intensities for the Man binding lectins NPA, GNA and HHA from low- to higher-density fractions. Higher density UC-uEV fractions also showed trends toward increase binding of Gal/GalNAc- and LacNAc-specific lectins as well as of lectins associated with binding of complex carbohydrate motifs. In contrast, a limited number of lectins, including SNA-I, MAA and STA, appeared to preferentially bind to uEVs from the low density fractions suggesting higher surface Neu5Ac and GlcNAc residues on vesicles from these fractions. Although of a preliminary nature, these trends were consistent with distinct uEV subpopulations across the gradient based on varying carbohydrate signatures.

The presence of distinct vesicles subtypes within sucrose density gradient fractions has been previously reported, most notably by Théry et al. who have described the following enrichment pattern in EVs from cultured cells and biological fluids: “true” exosomes at \(~1.15-1.19\) g/cm\(^3\), ER-derived vesicles at \(~1.18-1.25\) g/cm\(^3\) and Golgi-derived vesicles at \(~1.05-1.12\) g/cm\(^3\) (Théry et al. 2006a). Alternatively, Keller et al. referred to exosomes isolated from human urine in a range of \(1.05-1.15\) g/cm\(^3\) (based primarily on CD24 expression) and membrane blebs in a range from \(1.17-1.25\) g/cm\(^3\) (Keller et al. 2007). Certainly, our observations of higher AQP-2 and CD24 expression in low- to medium density fractions as well as partially differing glycoprofiles across the fractions would be consistent with such reported findings. Indeed, the high signals obtained for Man-binding lectins could be due to the presence of N-glycans of proteins with high-mannose structures that did not encounter much trimming and elongation by glycosyltransferases and glycosidases which occurs in the Golgi apparatus (Moremen, Tiemeyer, and Nairn 2012; Vagin, Kraut, and Sachs 2009).

### 5.5 Conclusion

In this Chapter, the strategy of subfractionating UC-uEVs from 200-400 ml urine samples of healthy adults was pursued with a view to: (a) determining the feasibility of subjecting uEV fractions to LM glycoprofiling and (b) generating preliminary data in support of the hypothesis that diverse uEV subtypes display differing surface glycans. By addressing specific technical challenges and carefully evaluating the quantity and quality of individual gradient fractions the approach was considered to be promising enough to proceed with the
more extensive study described in Chapter 6. Importantly, while novel in their own right, the observations made during this phase of the project were generally consistent with concepts and experimental findings reported by others in the field between 2004 and 2012.
Chapter Six
Results

Glyco-profiling of Urine Extracellular Vesicle Subfractions from Healthy Adults and Adults with Chronic Glomerular Disease
6 Results

6.1 Introduction

At the time this project was initiated, research related to EVs from biological fluids appeared to be in transition from a basic discovery phase towards more sophisticated projects. Specifically, there was a strong perceived potential for profiling of EV-associated biomolecules to reveal novel details about disease pathogenesis and to identify new biomarkers for diagnostic and prognostic purposes across a range of disease types (Cheruvanky et al. 2007; Miranda et al. 2010; Pisitkun, Shen, and Knepper 2004; Smalley et al. 2008; Douglas D. Taylor and Gercel-Taylor 2008; Welton et al. 2010; Hua Zhou et al. 2008). In the case of kidney disease, the revelation that urine is rich in EVs originating from all sections of the nephron had raised high expectations for uEV-based clinical studies to yield new generations of clinical assays for individual renal conditions or for the more general categories of CKD and AKI (van Balkom et al. 2011; Dimov, Jankovic Velickovic, and Stefanovic 2009; Goligorsky, Addabbo, and O’Riordan 2007; Gonzales et al. 2009; Hoorn et al. 2005; Pisitkun, Shen, and Knepper 2004). Today, proposed clinical applications for assays are based on detection/quantification of specific uEV proteins or nucleic acids included non-invasive diagnosis of genetic or non-genetic kidney disease, early prediction of severity of AKI or identification of patients at risk for CKD (Hogan et al. 2009; L.-L. Lv et al. 2014; Miranda et al. 2010; Pisitkun, Shen, and Knepper 2004; Sonoda et al. 2009; Hua Zhou et al. 2008). In the case of uEV glyco-profiling, which was the focus of this project, the known links between renal cell glycosylation characteristics and renal homeostasis, filtration, glomerular cell architecture and specific disease processes (as discussed in detail in Chapter 1), raised the possibilities that uEV surface glycans occur and those may altered in the setting of kidney disease and that the LM platform could provide an initial testing of this hypothesis (Andrews 1979; Del Boccio et al. 2012; Cha et al. 2008; Gerlach et al. 2013; Haraldsson, Nyström, and Deen 2008). Thus, the central goal of the experimental work described in this Chapter was to perform comparisons of UC-uEV isolates from healthy adults with those from adults with known kidney disease.

Despite the acknowledged potential for uEV research to facilitate the development of novel biomarker assays for kidney disease diagnosis and management, the field continues to face
substantial obstacles and challenges, some of which have been outlined in the preceding Chapters. These include: (a) The lack of high throughput technologies for isolating EVs of high purity and yield. (b) The relatively low amount of uEV material that can be extracted from the single-void urine samples which are typically obtained during patient clinical care (Merchant et al. 2010; Mitchell et al. 2009; H. Zhou, Yuen, et al. 2006). (c) The need for stringent, timely transport and processing of urine samples for uEV isolation (Musante et al. 2012; H. Zhou, Yuen, et al. 2006). (d) The complexity and heterogeneity of vesicle types and cell sources present within unfractionated uEV preparations (Mitchell et al. 2009; Pisitkun, Shen, and Knepper 2004). (e) The frequent and variable co-purification of non-vesicle associated proteins and nucleotides (Cvjetkovic, Lötvall, and Lässer 2014; Van Deun et al. 2014; Gerlach et al. 2013; Jeppesen et al. 2014). (f) The common occurrence of increased albumin and other soluble proteins in the urine of individuals with established kidney disease (Levey, Cattran, et al. 2009; Rood et al. 2010; Zubiri et al. 2014). (g) The lack of basic knowledge regarding uEV generation and excretion in the setting of abnormal kidney function. Because of these challenges, only a limited number of research groups have assembled the resources and expertise for large-scale clinical projects based on uEV isolation from patients with kidney disease and published studies before 2010 remain few (Sonoda et al. 2009; H. Zhou, Pisitkun, et al. 2006; Hua Zhou et al. 2008). Until today the primary approaches used have been the application of proteomics and RNA profiling techniques (S. Alvarez et al. 2013; H.-H. Chen et al. 2014; Kalani et al. 2013; Moon, Lee, et al. 2011; Peake et al. 2014; Ramirez-Alvarado et al. 2012). Analyses performed on subfractionated uEV isolates or at the level of visualized intact vesicles have remained infrequent due to the fact that most cell-based assay platforms are simply not applicable to EVs due to lack of resolution in nanoscale (Maas et al. 2015; Van der Pol et al. 2013). For the pilot study of uEV surface glycosylation described in this Chapter, we considered it important to subfractionate the UC-uEV isolates prior to analysis. We reasoned that this would allow us to better compare the yield and content of uEV samples from healthy subjects to those from patients with kidney disease and would enhance the potential for detecting glycosylation differences occurring within certain EV subpopulations that may have been otherwise masked by the complexity and heterogeneity of total uEV preparations (discussed in Chapter 5) (Bobrie et al. 2012; Kowal, Tkach, and Théry 2014; Mitchell et al. 2009; Théry, Ostrowski, and Segura 2009).
For the purposes of clinical practise, kidney disease is often broadly categorized in acute (referred to as AKI) and chronic (CKD), with the latter being subdivided into five stages (CKD-1 to CKD-5) (Eckardt et al. 2013; Zoccali, Kramer, and Jager 2010). Both AKI- and CKD-related research fields have included a large emphasis on the need for novel biomarkers with improved speed and accuracy in identifying altered kidney function and guiding appropriate therapy (Kerr et al. 2014; Siew, Ware, and Ikizler 2011). However, these broad terms do not fully convey the wide variety of aetiologies that may contribute to acute or chronic renal impairment (Bellomo, Kellum, and Ronco 2012; Remuzzi et al. 2013). Thus, interpretation of the molecular constituents of blood, urine or other biological samples among populations with reduced kidney function becomes more complicated by the diversity of underlying diseases and pathophysiological processes responsible for renal injury (Levey and Coresh 2012; Levey, Catran, et al. 2009).

The most common aetiologies for CKD among adults include diabetic kidney disease, hypertensive nephrosclerosis, glomerulonephritis (GN – of which there are multiple pathological subtypes), hereditary renal diseases (such as ADPKD and Alport syndrome), obstructive urological disease and renovascular disease (Eckardt et al. 2013; Jha et al. 2013; Mochizuki, Tsuchiya, and Nitta 2013; Pierides et al. 2013; Wouters et al. 2015). Although clinical history may suggest a likely aetiology in many patients, precise diagnosis of the cause of CKD may require a series of investigations including serological studies, radiological imaging and, in some cases, histological analysis of a kidney biopsy (Chadban and Atkins 2005; Levey and Coresh 2012; “Part 8. Recommendations for Clinical Performance Measures” 2002, “Part 9. Approach to Chronic Kidney Disease Using These Guidelines” 2002). Generally, kidney biopsy is reserved for the situation in which an accurate diagnosis cannot be made by other means and in which the treatment plan is likely to be dependent upon a biopsy result (Levey and Coresh 2012; “Part 9. Approach to Chronic Kidney Disease Using These Guidelines” 2002; Shenoy and Stannard 2014). In the time-frame available for the study described in this Chapter, it was clear to us from our experiences with healthy adult urine, that preparation and analysis of high quality, sucrose density gradient subfractionated uEVs from patients with kidney disease could only be performed on a limited number of samples. For this reason, it was elected to study uEVs from CKD patients falling into a single aetiological category - biopsy-proven GN. This category was selected because of its relatively high frequency among outpatients attending CKD clinic, its primary localization to a critical
functional part of the nephron (the glomerulus) and its known association with increased inflammatory activity – a process that is functionally linked to changes in glycosylation (Chadban and Atkins 2005; Marth and Grewal 2008). In addition, GN of various subtypes may be responsible for all stages of CKD severity and with abnormal proteinuria varying from mild to severe (Beck et al. 2013; Macanovic and Mathieson 2007).

Taken together, the considerations summarized above prompted us to further explore the similarities and differences between UC-uEV subfractions from clinically-obtained urine samples of a small cohort of adults affected by biopsy-proven GN and those from healthy adults. Using the techniques and approaches developed for Chapters 4 and 5, we aimed to generate novel data regarding differences between healthy- and GN-uEVs for vesicle yield, marker expression, size and surface glycosylation.
6.2 Hypothesis, aim and objectives

Results presented in Chapter 5 established fundamental techniques for reproducible subfractionation of UC-uEVs from urine samples of healthy adults using the reducing agent TCEP to eliminate THP contamination. In addition, the potential to analyse surface glycosylation of individual UC-uEV subfractions prepared by this approach was confirmed by LM. In this Chapter, subfractions of healthy urine UC-uEVs were further analysed at the level of individual vesicles using immunogold-labelled TEM and nanoparticle tracking analysis (NTA). Subsequently, comparisons were made between the yields, particle sizes and surface glycosylation profiles of selected UC-uEV subfractions from five healthy adults and five adults with CKD due to biopsy-proven GN.

6.2.1 Hypothesis
Chronic kidney disease due to GN is associated with altered UC-uEV size characteristics and surface glycosylation profile in comparison to the healthy state.

6.2.2 Aim
The overall aim of this Chapter was to determine whether uEV density, size and surface glycosylation characteristics are altered in the urine of adults with CKD due to biopsy-proven GN.

6.2.3 Objectives
1. Compare the density and size characteristics of UC-uEV subfractions generated from healthy adult urine using TCEP-sucrose density gradient centrifugation.
2. Generate glycoprofiles of UC-uEV subfractions from healthy adult urine using LM.
3. Compare protein yield, particle count and surface glycosylation profiles of UC-uEVs subfractions from adults with biopsy-proven GN to those of healthy controls.
6.3 Results

6.3.1 Analyses of TCEP-UC-uEV density subfractions from healthy adult urine samples
Using the approaches described in Chapter 5, TCEP-UC-uEV subfractions were derived from 300-400 ml urine samples of five healthy adult “donors”. The protein content of these isolates was examined by 4-12% Bis-Tris PAGE with silver staining (figure 6-1). As shown, fractions 3-12 for all five samples demonstrated minimal or complete lack of THP co-purification with otherwise rich protein band patterns across a wide range of molecular weights.

Figure 6-1: Silver stained 4-12% Bis-Tris PAGES of TCEP-UC-uEV sucrose density gradient (1.04-1.24 g/cm³) subfractions generated from urine samples of five healthy adult donors.
Protein aliquots from individual
For these same samples, BCA assay was applied to all fractions in order to estimate the total protein yields which were then expressed as µg/ml of urine (figure 6-2). In keeping with our observations for test samples in Chapter 5, the protein yields were lowest for fractions at the lower (F3, F4) and upper (F10, F11) ends of the density spectrum. Protein yields were greatest for F6 to F8 but, within these mid-range density fractions, there was also a greater degree of inter-individual variation.

![TCEP*HCl treated samples (n=5)](image)

**Figure 6-2:** Estimated exosomal protein content (µg/ml of urine) of TCEP-UC-uEV sucrose density gradient (1.04-1.24 g/cm³) subfractions generated from urine samples of five healthy adult donors. Horizontal bars and error bars represent mean ± SD.

Next, selected fractions from the individual TCEP-UC-uEV gradients were analysed by TEM to confirm the presence of intact EVs and to evaluate their shape, size and exosome marker expression, (figure 6-3). For these analyses, fractions F5 (1.08 g/cm³), F7 (1.14 g/cm³) and F9 (1.20 g/cm³) from three of the five healthy adult samples were subjected to TEM with and without immunogold labelling for CD24 and CD63. The primary reason for limiting the TEM analysis to these fractions was the relatively low overall EV associated protein yield as shown in figure 6-2.
Figure 6.3: Representative images from immunogold TEM analysis of fractions 5, 7, and 9 of Donor 1 TCEP-UC-uEV gradient.

Immunogold labelling was carried out with: A. Primary mouse anti-human CD24 followed by anti-mouse IgG conjugated gold-particle or B. Rabbit anti-human CD63 followed by anti-rabbit IgG conjugated gold-particle. “No-stain” images represent samples incubated with relevant secondary antibody conjugated gold-particle alone. Images were recorded at 75 kV.
Overall the presence of vesicles of expected size range, shape and density was verified in all tested fractions. Positive surface labelling for CD24 was observed in proportions of the vesicles present in all fractions. Vesicles from F5 and F7 appeared similar in size range and in the frequency and density of CD24 labelling. In contrast, vesicles in F9 appeared to be predominantly of larger size and to have lower frequency and density of CD24 labelling. Vesicles with surface labelling for the common exosomal marker CD63 were present in all selected fractions but were generally less frequent than CD24+ vesicles. Among the CD63+ vesicles, some contained only one or two bound gold particles while others had five or more bound particles. In contrast to CD24, no apparent difference was noted for the frequency or density of CD63+ vesicles across the fractions examined. When the numbers of bound particles per vesicle were quantified for 50-100 clearly-identified vesicles for CD63 and 17 clearly-identified vesicles for CD24 from the TEM images of F5, F7 and F9 from the three analysed samples (figure 6-4), there was a trend toward lower CD24 expression in F9 compared to the lower density fractions (p = 0.057) while CD63 particle counts were closely comparable across the fractions.

![Figure 6-4: Per-vesicle gold particle counts for A. anti-CD24 and B. anti-CD63 immunogold TEM analysis of fractions 5, 7, and 9 from TCEP-UC-uEV sucrose density gradient (1.04-1.24 g/cm³) subfractions generated from urine samples of healthy adult donors. Result represent mean ± SD particles per vesicle for n = 3 donors. For each the donor, gold particles were counted and averaged for 17 in case of CD24 or 50-100 in case of CD63 individual positively-labelled vesicles. Particle counts were compared between fractions by two-tailed, paired Student’s t-test (p-value for CD24 particle count for F9 vs F7 is shown in graph A).](image)

Taken together, these result allowed us to conclude that TCEP-density gradient separated UC-uEVs from healthy adult urine: (a) Provide good quality THP-depleted vesicular protein in all fractions albeit with limited yields at the lowest and highest density fractions. (b) Contain intact vesicles of heterogenous size (10-200 nm) and surface marker expression. (c) Include a greater proportion of larger, CD24 low-to-negative vesicles within higher density fractions.
Next, vesicle sizes from the representative gradient regions were investigated more accurately using nanoparticle tracking analysis (NTA). Nanoparticle tracking analysis allows for accurate particle sizing in nanoscale based on tracking of Brownian motion of particles in a liquid buffer system. As for TEM analysis, aliquots of fractions F5, F7 and F9 from donor 1-3 were subjected to NTA using the NanoSight® NS 500 System in collaboration with the laboratory of Dr. Ariele Prina-Mello at Trinity College Dublin (see Methods). Examples and summaries of the NTA results are summarized in figure 6-5 and table 6-1.

The histograms shown in figure 6-5 demonstrate that large numbers of particles were detectable in each of the fractions analysed with diameter ranging from approximately 50 to 400 nm. Profiles were comparable for each fraction from the three healthy donors. There were clear differences in the histogram patterns among the fractions with the appearance of progressively greater proportion of particles of diameter > 200 nm from F5 to F9.

Figure 6-5: Histogram representations of nanoparticle tracking analyses of uEVs from fractions 5, 7 and 9 of TCEP sucrose density gradients of UC-uEVs from 3 healthy adults (recorded with Nanosight® NS500).
As summarized table 6-1 particle diameters for these samples, expressed either as mean or mode, were lowest for F5 and highest in F9 in all samples with F7 particles diameters being intermediate between the two. A graphical presentation of the mean diameters (figure 6-6) confirms that the differences were statistically significant.

Table 6-1: Summary of numerical results of NTA (n=3, 1 technical repeat, recordings of 6 videos 30 sec).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Fraction</th>
<th>Mean</th>
<th>Mode</th>
<th>SD</th>
<th>D10</th>
<th>D50</th>
<th>D90</th>
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<tr>
<td>1</td>
<td>5</td>
<td>139.8±1.3</td>
<td>116±1.3</td>
<td>45.5±1.2</td>
<td>100±1.2</td>
<td>126±2.1</td>
<td>189±1.1</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>141.9±1.9</td>
<td>121±6.6</td>
<td>53.2±5.9</td>
<td>99±1.0</td>
<td>127±2.2</td>
<td>196±3.6</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>117.1±2.8</td>
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<td>42.1±5.4</td>
<td>80±1.5</td>
<td>105.3±2.2</td>
<td>164±3.6</td>
</tr>
<tr>
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<td>50.2±2.7</td>
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<td>146.7±1.7</td>
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<td>164.4±2.9</td>
<td>132±9.0</td>
<td>63.1±1.7</td>
<td>103±2.6</td>
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<td>139.7±4.0</td>
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<td>111±1.8</td>
<td>171.7±2.7</td>
<td>239±4.4</td>
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</table>

Abbreviations: SD = standard deviation; D10 = diameter of the first 10% of the total particles; D50 = diameter of the first 50% of the total particles; D90 = diameter of the first 90% of the total particles.

Figure 6-6: Graph of the mean particle diameters of TCEP-UC-uEV fractions 5, 7 and 9 from three healthy adults. Results are shown as mean ± SD of the average values for n = 3 samples per fraction. P values by two-tailed, paired Student’s t-test: F5 v. F7 = 0.03; F5 v. F9 = 0.0007; F7 v. F9 = 0.02.

In summary, the combined results for NTA and TEM analyses of selected fractions indicated a conclusion that uEVs of different sizes and subtypes can be at least partially separated by sucrose density gradient ultracentrifugation suggesting potential value of this approach for better resolving uEV surface glycosylation signatures and for comparing uEV glycosylation in health and disease.
6.3.2 Analysis of subfractionated TCEP-UC-uEVs from patients with glomerulonephritis

In the next study, urine samples (100 – 150 ml volume) were collected under informed consent from five adults with biopsy-proven GN attending an outpatient clinic for Nephrology follow-up. The demographic and clinical characteristics of these five subjects are summarized in 6-2.

Table 6-2: Clinical details of study subjects with biopsy-proven GN.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Gender</th>
<th>Age</th>
<th>Primary Diagnosis</th>
<th>eGFR</th>
<th>Proteinuria grade (0-4)</th>
<th>Medial Co-morbidities</th>
<th>Active Treatment</th>
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</thead>
<tbody>
<tr>
<td>uEV1</td>
<td>M</td>
<td>71</td>
<td>IgAN</td>
<td>41</td>
<td>3</td>
<td>Hypertension. Type 2 diabetes</td>
<td>Anti-hypertensives, Statin, Metformin, PPI, Aspirin</td>
</tr>
<tr>
<td>uEV2</td>
<td>M</td>
<td>53</td>
<td>ANCA-associated Vasculitis</td>
<td>51</td>
<td>2</td>
<td>Hypertension</td>
<td>Immunosuppressants, Anti-hypertensives, Bisphosphonate</td>
</tr>
<tr>
<td>uEV3</td>
<td>M</td>
<td>25</td>
<td>IgAN</td>
<td>80</td>
<td>1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>uEV4</td>
<td>F</td>
<td>30</td>
<td>Membranoproliferative Glomerulonephritis (recurrent in transplant)</td>
<td>30</td>
<td>4</td>
<td>Hypertension, Transplant</td>
<td>Immunosuppressants, Anti-hypertensives, Diuretics</td>
</tr>
<tr>
<td>uEV5</td>
<td>M</td>
<td>36</td>
<td>IgAN (recurrent in transplant)</td>
<td>21</td>
<td>4</td>
<td>Hypertension, Transplant</td>
<td>Immunosuppressants, Anti-hypertensives, Diuretic</td>
</tr>
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</table>

At the same time, urine samples of similar volume were also collected from a group of five new healthy adults with no history of kidney disease. For all samples, UC-uEVs were isolated and labelled with PKH26 then subjected to TCEP sucrose density gradient subfractionation as described in Chapters 3, 4 and 5. Proteins yields for healthy (H)- and GN-UC-uEV fractions are shown in figure 6-7.
Figure 6-7: Protein yields for fractions 3 to 11 (F3-F11) of PKH26-labelled TCEP-UC-uEV sucrose density gradients prepared from 5 healthy adults (H-uEV) and 5 adults with biopsy-proven glomerulonephritis (GN-uEV).

The protein yield of each fraction was estimated by BCA assay and expressed as µg/ml urine. * = p ≤ 0.05 for H-uEV vs. GN-uEV using two-tailed, unpaired Student’s t-test.

Of particular note, while detectable protein was present in all fractions from the GN-uEV samples with the exception of F3, the protein yields for the fractions of mid-level density (F6-8) were significantly lower than those from H-uEV samples. Thus, the presence of kidney disease was associated with a distinct alteration in the relative distribution of uEV protein across the density spectrum.

Next, NTA was applied to fractions 6 (1.11 g/cm³) and 9 (1.20 g/cm³) of three H-uEV and three GN-uEV subfractionated samples. Restriction of the analyses to two specific fractions and to three subjects from each group was necessary because of limitations to the amount of material available for this and other planned analysis approaches. The resulting histograms are shown in figures 6-8 (F6) and figure 6-9 (F9).
Figure 6-8: Histogram representations of nanoparticle tracking analyses of uEVs from fraction 6 (F6) of TCEP-sucrose density gradients of UC-uEVs from 3 healthy adults (H-uEV) and 3 subjects with biopsy-proven GN (GN-uEV). The portions of the histograms between 200 and 300 nm diameter are indicated by vertical dashed lines for illustrative purposes.

n=3, 1 technical replicate, 6 videos of 30 sec were recorded per graph.
Figure 6-9: Histogram representations of nanoparticle tracking analyses of uEVs from fraction 9 (F9) of TCEP-sucrose density gradients of UC-uEVs from 3 healthy adults (HuEV) and 3 subjects with biopsy-proven GN (GN-uEV). The portions of the histograms between 200 and 300 nm diameter are indicated by vertical dashed lines for illustrative purposes. n=3, 1 technical replicate, 6 videos of 30 sec were recorded per graph.

Analysis of F6 from H-uEVs revealed few particles of diameter >200 nm while GN-uEVs appeared to have a higher proportion of particles >200 nm. In contrast, for F9, particle diameters were larger than F6 for both H-uEVs and GN-uEVs with histogram shapes varying from a single dominant peak to double or multiple peaks. It was also noticed that particle numbers were 10- to 100-fold lower in these fractions of the GN-uEV samples compared to the H-uEV samples.

The results of these NTA analyses expressed in terms of mean and mode diameters are summarized in table 6-3 below with graphical representation of mean ± SD presented in figure 6-10.
Table 6-3: Summary of numerical results of NTA of sucrose density gradient fractions F6 and F9 from 3 healthy adults (H-uEV) and 3 adults with biopsy-proven GN (GN-uEV).

<table>
<thead>
<tr>
<th>Individual</th>
<th>Fraction</th>
<th>Mean</th>
<th>Mode</th>
<th>SD</th>
<th>D10</th>
<th>D50</th>
<th>D90</th>
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</thead>
<tbody>
<tr>
<td>H-uEV1</td>
<td>6</td>
<td>153.5±2.6</td>
<td>121±2.2</td>
<td>50.5±3.6</td>
<td>110±1.0</td>
<td>138.7±2.7</td>
<td>206±5.0</td>
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<tr>
<td>H-uEV2</td>
<td>6</td>
<td>136.5±5.6</td>
<td>110±5.7</td>
<td>54.2±9.5</td>
<td>86±3.1</td>
<td>126.7±3.7</td>
<td>190±11.4</td>
</tr>
<tr>
<td>H-uEV3</td>
<td>6</td>
<td>125.4±12.1</td>
<td>103±3.5</td>
<td>51.4±5.4</td>
<td>85±1.1</td>
<td>111.0±1.2</td>
<td>185±12.2</td>
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<tr>
<td>GN-uEV1</td>
<td>6</td>
<td>158.7±1.2</td>
<td>122±2.0</td>
<td>58.7±1.8</td>
<td>109±1.3</td>
<td>143.3±1.1</td>
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<tr>
<td>GN-uEV2</td>
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<td>124±3.2</td>
<td>59.8±4.0</td>
<td>110±3.0</td>
<td>153.0±2.9</td>
<td>240±5.5</td>
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<tr>
<td>GN-uEV3</td>
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<td>108±2.9</td>
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<td>144.0±9.4</td>
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<tr>
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<td>194±37.9</td>
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<td>101±9.3</td>
<td>205.0±21.9</td>
<td>292±8.6</td>
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<td>GN-uEV1</td>
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<td>204.4±3.2</td>
<td>198±3.2</td>
<td>43.8±2.2</td>
<td>155±3.5</td>
<td>200.7±2.2</td>
<td>255±4.8</td>
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<tr>
<td>GN-uEV2</td>
<td>9</td>
<td>187.6±2.1</td>
<td>195±15.9</td>
<td>60.1±1.5</td>
<td>111±2.4</td>
<td>187.3±2.2</td>
<td>257±4.3</td>
</tr>
<tr>
<td>GN-uEV4</td>
<td>9</td>
<td>252.2±3.7</td>
<td>246±1.1</td>
<td>64.0±1.4</td>
<td>177±8.4</td>
<td>247±2.0</td>
<td>328±5.1</td>
</tr>
</tbody>
</table>

Abbreviations: SD = standard deviation; D10 = diameter of the first 10% of the total particles; D50 = diameter of the first 50% of the total particles; D90 = diameter of the first 90% of the total particles.

Figure 6-10: Graphs of the mean particle diameters of sucrose density gradient fractions 6 and 9 from 3 healthy adults (H-uEV) and 3 adults with biopsy-proven GN (GN-uEV). Results represent mean ± SD. * = p < 0.05 for H-uEV vs. GN-uEV by two-tailed, unpaired Student’s t-test.
In summary, this limited NTA experiments of selected mid- and higher-density fractions from the two sets of gradients confirmed the presence of particles of expected sizes in the patient samples obtained from outpatient clinics while also illustrating the limited yield available for analysis. Particle sizing suggested that patient samples may be characterized by higher content of larger vesicles (>200 nm diameter) at lower density.
6.3.3 Lectin microarray profiling of 5 glomerulonephritis individuals vs. healthy controls

Finally, the PKH26-labelled density gradient fractions from H-uEV and GN-uEV isolates were glycoprofiled using LM as described earlier. The full, normalized results for this analysis are summarized in heat-map format in figure 6-11.

**Lectin microarrays: H-uEVs versus GN-uEVs**

Figure 6-11: Heat map of LM analysis of sucrose density gradient fractions of H-uEVs (n=5) and GN-uEVs (n≤5). Data shown represents colour-coded, normalized fluorescence intensities for a panel of 48 lectins and two controls (BSA and phosphate buffered saline (PBS)).
While fractions 3 to 11 were suitable for analysis for all five H-uEV isolates, the lower amounts of material obtained from the GN-uEV gradients (as shown in figure 6-7) resulted in limited numbers for individual fractions from the patient-derived samples (n = 2 to 5). Nonetheless, for five of the nine fractions from GN-uEV samples, 3 or more samples were suitable for analysis allowing for statistical comparison with the glycoprofiles of the equivalent H-uEV fractions (figure 6-11).

For H-uEVs, specific trends were observed for individual lectins across the density fractions. These included higher binding to lectins with affinity for high-mannose structures (GNA, HHA) in higher compared to lower density fractions (figure 6-12A) as well as higher binding to lectins with high affinity for GlcNAc (DSA, LEL) in lower compared to higher density fractions (figure 6-12B). In contrast, consistent medium-to-strong binding of lectins with affinity for LacNAc and GalNAc (RCA-I and SNA-II) was observed across all H-uEV fractions (figure 6-12C) in keeping with results presented in Chapter 5.

For these same lectins, fraction-by-fraction comparisons between H-uEV and GN-uEV samples most strikingly showed lower binding intensities for GNA and HHA (figure 6-12A) in the higher density fractions of GN patient-derived samples. That this was not part of a general reduction in surface glycosylation for these GN-uEV fractions was evidenced by the fact that binding intensities and trends for DSA and LEL as well as for RCA-I and SNA-II were no different to those of H-uEV samples (figure 6-12B & C).
Figure 6-12: Normalized fluorescence intensities from LM analyses of individual fractions (F3-F11) of sucrose density gradients from H-uEVs and GN-uEVs. Results are expressed as % of the maximum fluorescence intensity for each individual lectin for each group of samples. Results are presented as group means ± SD (columns with no error bars represent fractions with n < 2). Statistical analysis was performed for H-uEVs using one-way ANOVA following Tukey’s multiple comparison test (n=5) for significant results excluding Fraction 3 (see figure 6-13).
A. H-uEV

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<th>5</th>
<th>6</th>
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B. GN-uEV

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Figure 6-13: Statistical analysis of individual gradient fractions to figure 6-12 based on one way-ANOVA:

H-uEVs - GNA: p-value <0.0001; HHA: p-value: <0.0001, DSA: p-value: <0.0001, LEL p-value <0.0001, SNA-II p-value 0.6285, RCA-I: p-value 0.2369.

GN-uEVs - DSA: p-value = 0.0099, LEL p-value = 0.0053.

Tables indicate row vs. column fractions analysis results from Tukey’s test. All other lectins were not significant.

Within the limitations of sample number, a statistical analysis was performed to compare the binding intensities of H-uEVs and GN-uEVs for each lectin across the range of fractions. From this analysis, the results for five specific lectins were notable as they indicated between-group trends and statistically significant differences for multiple fractions. The results for these lectins (ECA, GNA, HHA, SBA, PNA) are summarized in table 6-4. As referred to already, lectins GNA and HHA with affinities for Man-containing moieties demonstrated
consistent lower binding to GN-uEV compared to H-uEV samples. In contrast, lectins ECA, PNA and SBA with affinities for various Gal-containing moieties demonstrated higher binding intensities to GN-uEV compared to H-uEV. In general, these lectins reflect binding to N- and O-linked glycan structures on proteins (PNA, GNA, HHA) and also likely carbohydrate components of glycolipids (ECA).

Thus, the results of this comparative LM analysis provided evidence of distinct surface glycosylation alterations to uEV subfractions in the setting of kidney disease due to GN. Although, these differences were more striking for higher density fractions, it was not possible to make a strong conclusion in this regard as a result of the lower yields of low density fractions from the patient cohort.
Table 6-4: Summary of comparative binding of five specific lectins to individual fractions of H-uEVs and GN-uEVs.

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<th>Binding Specificity</th>
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<th>HHA</th>
<th>ECA</th>
<th>PNA</th>
<th>SBA</th>
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<td>n=5 0.01 0.03 0.07 0.3 0.2</td>
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<td>p ≤ 0.01</td>
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<td>p = NS</td>
<td>p = ND</td>
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</table>

unpaired, two-tailed Student’s t-test
NS: not significant; ND: not determined; p: p-value; H- healthy; GN -glomerulonephritis

The final conclusions from this pilot study were as follows: (a) The TCEP-based sucrose density gradient method for purifying uEV fractions for LM and other analyses can be successfully applied to single-void urine samples collected from patients with kidney disease of varying severity in an outpatient setting. (b) The resulting samples from patients with biopsy-proven GN are characterized by lower protein yields and particle counts within fractions of mid-range density. (c) For at least some uEV subfractions, vesicle size may be larger in adults with kidney disease compared to healthy adults. (d) LM analyses of subfractionated uEVs from both healthy adults and adults with kidney disease reveal complex surface glycosylation profiles in all fractions along with density-associated trends for a limited number of specific lectins. (e) Higher density uEV subfractions may have altered surface expression of specific carbohydrate moieties in the setting of CKD due to GN.
6.4 Discussion

In this Chapter uEV populations originating from healthy adults and adults with GN were fractionated and further explored. We successfully showed: (a) The isolation of uEVs from healthy adult urine without visible interference of THP using TCEP. (b) A density-size relationship of adult uEVs based on results for low (F5), medium (F7) and high (F9) density fractions. (c) A decrease in the frequency of CD24+, but not CD63+ vesicles across the gradient. (d) Variations in uEV surface glyosylation profile across the gradient fractions. (e) A size differential between GN-uEVs and H-uEVs within medium-density fractions (F6). (f) Differences between GN-uEVs and H-uEVs in binding intensities for the 5 lectins GNA, HHA, ECA, SBA and PNA in some of the mid- to high-density fractions.

As discussed in Chapter 5, during the studies of uEVs derived from healthy adults we encountered technical difficulties such as THP co-precipitation during UC which could be overcome by a brief incubation period with reducing agents as reported by others (Fernández-Llama et al. 2010; Musante et al. 2012). In our hands, TCEP was simple to handle at r.t. and yielded THP-free UC-uEV protein profiles following fractionation using an 8.5-50% sucrose gradient. Thus, the TCEP method described in Chapter 5 and applied in Chapter 6, may be an appealing alternative to DTT for THP removal from uEV samples (Han and Han 1994). In addition, as TCEP has been previously reported for use with glycopeptides and glycoproteins, we considered it likely that the integrity of carbohydrates would be not be affected by its addition to the UC-uEV isolates (Gong Chen et al. 2007; Miura et al. 2008; Rebecchi et al. 2011).

The size characteristics of uEVs fractionated by sucrose density gradient have not been extensively documented. However, Hogan et al. characterised uEV subpopulations purified and subfractionated from 270 ml urine samples in 5-30% D2O density gradients. (Hogan et al. 2014) The authors identified 3 major vesicle populations with discrete appearances based on microscopic studies: large exosome-like vesicles (median diameter 93 nm with punched soccer ball appearance refractive index: 1.3436 s.d.±0.00124), PKD-exosome like vesicles (median diameter 79.4 nm, refractive index: 1.3539 s.d.±0.000831) as well as glomerular membrane vesicles (GMV, median diameter: 72.1 nm, refractive index: 1.3625 s.d.±0.000911) (Hogan et al. 2014). Differences between those vesicles were confirmed by immunoblotting using antibodies against polycystin-1 (PC-1) and podocin. Interestingly, PC-
1 present in PKD exosome-like vesicles was reported as heavily N-glycosylated protein by the same group in 2009 (Hogan et al. 2009). However, separation of uEVs using D$_2$O density gradients did yield in a rather heterogeneous GMV fraction with vesicles populations that stained positive as well as negative for podocalyxin/podocin marker in immunogold TEM. Proteomic data on the GMV fraction indicated that a high proportion of protein was derived from glomerular cells. Amongst those proteins were some which are known to be associated with hereditary glomerular diseases including CD2-associated protein, CD151, integrin α3 and podocin.

Our own results included some similar observations. Using a sucrose density gradient technique of 8.5-50% in water with uEVs derived from urine of healthy adults, we observed two specific uEV populations within the gradient. Those populations were identified by using the molecules CD24 and CD63 for uEV surface staining. Expression of CD24 within human renal cortex has been reported to be primarily associated with parietal epithelial cells in Bowman’s capsule and proximal tubule often described as renal progenitor cells with regenerative potential when co-localized with CD133. Cells expressing CD24 but not CD133 reside along the human nephron in proximal tubule, collecting duct and in very low amounts in the thick ascending limb region (Lindgren et al. 2011; Smeets et al. 2013). The more traditional exosome marker CD63 was recently discussed to be present in the tetraspanin web promoting exosome biogenesis in vesicles and has been documented by TEM to be present on EVs from a variety of different cell lines (Colombo et al. 2013; Van Deun et al. 2014; Stoorvogel 2015). However neither CD24 nor CD63 can be considered to be exclusive markers for exosomes and they may also be present on other subtypes of EV. Staining of uEVs associated with distinct densities (F5, F7, F9) showed trends for lower CD24 surface staining from low to medium to high density fractions whereas CD63 staining remained constant. Although no combined staining was applied, it is likely that there are both CD24$^+/CD63^+$ and CD24$^+/CD63^-$ vesicles present with the latter being more common within the lower density fractions.

Size characteristics of UC-uEV subfractions imaged by TEM along with anti-CD24 immunogold labelling suggested larger vesicle size with increasing density which was confirmed by NTA. Thus the combination of CD24 expression and size may help to distinguish different uEV sub-populations. Little literature exists regarding the distribution of
CD24 among uEVs following gradient separation and, to our knowledge, no previous studies have addressed the size characteristics of CD24+ EVs. However, our results are in line with the work of Keller and Oosthyuzen in which CD24+ uEVs were primarily found in the gradient region 1.05-1.16 g/cm³ (Keller et al. 2007; Oosthuyzen et al. 2013). Of interest, van der Vlist et al. studied the density/size relationships in EV subfractions derived from CD4+ T cell lines after stimulation with anti-CD3/anti-CD28 antibodies. The authors described an increase in vesicle size from 103 ± 50 nm at 1.14 g/cm³ to 166 ± 77 nm at 1.17 g/cm³ (Van der Vlist et al. 2012). Moreover, three distinct EV populations were observed per density region by flow cytometry. We similarly noticed an increase in EV size from low to high density consistent with the presence of distinct vesicle populations that are at least partially separated by density gradient centrifugation. Our comparative results for H-UC-uEV and GN-UC-uEV subfractions, while limited by sample size and yield, indicated that a similar density/size relationship is present in the setting of kidney disease. Furthermore, we observed trends toward increased average vesicle size within individual fractions of uEVs from patients with GN compared to healthy controls. Therefore, we believe that further analysis of size/density relationships among uEV subfractions merit further analysis as an indicator of kidney health – particularly if technologies for EV isolation, fractionation and analysis evolve to allow for greater efficiency and cost-effectiveness. It should be noted that the study by Hogan et al. on GMVs derived from D₂O sucrose density gradients reports a decrease in median vesicle diameter across gradient fractions using microscopic studies. While differences in isolation methodology may explain this discrepancy, our NTA analysis sizing results are based on a dynamic light scattering by particles moving by Brownian motion within a liquid medium and may provide more accurate readings than simple calibration-based microscopy of fixed preparations.

The overall aim in this project was to generate lectin-based glycoprofiles of uEVs from healthy adults. In the past, lectins were frequently used as tissue staining markers for particular regions within the kidney with detection by immunofluorescence or immunohistochemical techniques (Faraggiana et al. 1982; Ravidà et al. 2015). Based on our results with LM in this Chapter, it is possible that lectin affinities could be harnessed to capture uEV subfractions with differing surface glycosylation characteristics and that this approach could enrich for uEV subfractions with differing densities and sizes. Indeed, isolation of uEVs using the lectin STL coupled to beads was recently demonstrated by
Echevarria et al. (Echevarria et al. 2014). Lectin-based affinity capture may also have the power to enrich EVs derived from particular nephron regions from total uEV isolates.

In this study, lectin glycoprofiles of fractionated uEVs from healthy adults revealed three major trends: 1. Lectins for which staining intensity increased from low to high density fractions [GNA and HHA (distinct Man)]. 2. Lectins for which staining intensity decreased from low to high density fractions [LEL (GlcNAc(β1→4)GlcNAc) and DSA (GlcNAc)]. 3. Lectins with positive staining which remained consistent in intensity across the gradient fractions [RCA-I (LacNAc) and SNA-II (GalNAc)]. Combining these results with the density/size relationships discussed above, we suggest that two broad sub-categories of uEV glycosylation could be considered: (a) uEVs of low to medium density and predominant size range of 50-200 nm with higher surface GlcNAc, lower surface Man and higher surface levels of CD24. (b) uEVs of higher density with predominant size range of 50-400 nm and displaying higher surface levels of Man compared to GlcNAc and low to no CD24. The “double peaks” we observed in several of the F9 samples by NTA is in keeping with this categorization. It should be emphasized, however, that such glycosylation-based subcategories are likely to incorporate EVs from multiple individual cell types.

Our pilot study to compare glycoprofiles from five H-UC-uEV and five GN-UC-uEVs samples confirmed the feasibility of performing such clinical studies using outpatient clinic “spot” urine samples while also highlighting some of the technical challenges and limitations. While partially limited by EV yield for some of the samples, the LM results obtained indicated a broad similarity between healthy and diseased. However, among the higher density fractions, significant differences were observed in binding intensities of five lectins. This included higher binding of lectins with specificities for Gal/GalNAc and α-Gal as well as lower binding of lectins with specificities for Man structures in GN-UC-uEVs. These differences were observed despite the heterogeneity of primary diagnosis among the GN group suggesting that they represent an alteration common to multiple forms of GN (and potentially other CKD causes). The decrease in high-mannose structures in GN-uEVs is of specific interest as apoptotic vesicles from tumor cell lines have been reported to express high levels of Man structures associated with clearance by macrophages (Bilyy et al. 2012). Whether renal disease is associated with altered urinary excretion of apoptotic vesicles is not currently known but may be an important topic for future research studies. Alterations to uEV
biomolecules in association with kidney disease have been reported previously and are summarized in Chapter 1, table 1-1. As a good example, Hogan et al. performed proteomic analysis of GMVs ($\eta=1.3625$) from adults with membranous and membranoproliferative glomerular disease and found them to be enriched with 5657 proteins compared to the healthy control fractions with 1830 proteins (Hogan et al. 2014). Altered glycosylation has also been reported in the setting of kidney disease – most strikingly in the study of Ravida et al., in which lectin-based analyses were used to demonstrated significant changes to total and glycosylated proteins from renal tissue lysates of diabetic rats (Ravidà et al. 2015). Thus, more investigation is required to shed light on the pathological changes that occur in cell surface glycosylation during kidney disease and to determine whether these changes can be effectively detected and quantified in patient uEV samples.

6.5 Conclusion

In this chapter we have demonstrated that uEVs can be isolated with high purity using 8.5-50% sucrose density gradient centrifugation combined with TCEP as reducing agent. Analysis of the densities, sizes and surface glycoprofiles of these fractions indicated two, partially overlapping, categories of uEV. In particular, differences in surface levels of Man and GlcNAc as well as the heavily glycosylated protein CD24 merit further investigation as markers of different vesicle subtypes in urine. We also revealed five lectins with known specificites for Man, Gal/GalNAc and $\alpha$-Gal that were differentially bound by higher density uEVs of patients with GN compared to those of healthy adults. Thus, surface glycosylation of higher density uEVs may be altered in the setting of GN and, possibly, other forms of CKD.
Chapter Seven
Final Discussion and Reflection
7 Discussion

Throughout this study we focussed on the carbohydrate coat of uEVs. Among the significant issues addressed were the establishment of a suitable isolation method and the potential for uEV-associated carbohydrates to serve as an indicator of kidney health status. In this final Chapter of the thesis, I would like to emphazise and reflect upon six specific findings that derived from the project: (1) uEVs can be enriched to high quality using UC. (2) Isolated uEVs are highly glycosylated and bear distinct glycosylation profiles compared to co-precipitated/co-concentrated proteins as demonstrated for THP. (3) Sucrose density gradient centrifugation in regular water is not sufficient to fully purify uEVs from THP. (4) The reducing agent TCEP provides an effective and practical alternative to DTT for removal of THP during UC-based uEV isolation. (5) Sucrose density gradient-separated uEV fractions demonstrate differences in protein content, size and surface glycosylation. (6) In our pilot study comparing UC-uEV fractions from 5 individuals with GN to those from healthy control samples, significant differences were observed for certain lectins which could be further explored as biomarkers.

Research in the field of EVs has been rapidly developing over the past several years. Nonetheless, the search for a highly-effective isolation method is on-going. The most consistently reliable methods appear to be UC followed by density gradient UC or centrifugal concentration combined with SEC (Lobb et al. 2015). However, capturing of uEVs using centrifugal concentration combined with immuno-affinity may become more widely adopted in the future due to increasing interest in subpopulations of EVs (Kowal, Tkach, and Théry 2014). Our own findings showed that centrifugal concentration as a one-step isolation method for uEVs leads to co-concentration of abundant non-vesicular proteins such as albumin and, thus, lowers the overall quality and limits the use of the resulting uEV preparations (Gerlach et al. 2013). Indeed, similar observations have been reported by other researches (Lobb et al. 2015; Merchant et al. 2010; Rood et al. 2010). It is possible that this problem can be overcome with appropriate protein depletion kits as shown by Zubiri et al., although this approach would also add significantly to the time and expense of the method (Zubiri et al. 2014). Our studies also showed that UC, while time-consuming, enriched cup-shaped vesicles that contain a wide diversity of specific glycoproteins. This was demonstrated, for example, by the band patterns of Fuc-containing glycoproteins observed when UC-uEV proteins were blotted with lectin AAA. Ultracentrifugation has been considered to be a “gold standard”
method for some time (Théry et al. 2006a). However, recently some groups have shown, directly or indirectly, that UC does not enrich all vesicles from culture supernatants (Cvjetkovic, Lötvall, and Lässer 2014; Musante et al. 2013; Nordin et al. 2015). Thus, it may prove difficult or even impossible to narrow down the available isolation approaches to one specific method that is optimal for all projects and downstream applications.

The results presented in this thesis confirm that uEVs display a broad range of carbohydrates on their surfaces. This observation was made in only two published reports prior to 2010, both involving non-urine-derived EVs. Mahal et al. studied the glycoprofiles of T cell-derived EVs and compared these to self-prepared cell membrane vesicles and to the HIV virus. In this study, the EVs and membrane preparations we shown to have distinct lectin-binding profiles (Krishnamoorthy et al. 2009). In the second study, glycosylation of apoptotic vesicles was shown to be relevant to macrophage uptake (Bilyy et al. 2012). Several studies have also reported on the presence of specific glycosylated proteins in uEVs such as CD24, AQP-2 and PC-1 (Hogan et al. 2009; Keller et al. 2007; Pisitkun, Shen, and Knepper 2004). Thus, our interest in glycosylation of human uEVs was stimulated by these studies as well by the general dearth of published research on the broader characteristics of uEV surface glycosylation and its relevance to kidney disease. In total, three distinct methods used in our laboratory indicated carbohydrates to be plentiful components of the uEV biomolecular repertoire. Initially, we used lectin immunoblotting as a screen for carbohydrate content in uEVs. Subsequently, the more dynamic techniques of lectin-based FCM and LM, were used to generate a comprehensive glycoprofile of intact uEVs from healthy individuals (Gerlach et al. 2013). Those glycoprofiles were found to be more specific than comparable profiles for purified THP from the same urine samples. Similarly, Staubach et al. reported that glycosylation of THP and uEVs from healthy individuals and galactosemia patients differed. Specifically, they described an increase in complex N-glycan structures for uEVs derived from patient samples (Staubach et al. 2012). We analyzed uEVs from ADPKD patients provided by our collaborators at Mayo Clinic, USA and found evidence for differences in Gal/GalNAc, Man and Fuc structures compared to their age-matched controls indicated by differential binding of AIA/Jac(Gal), PA-I(Gal), NPA(Man(α1→6)-), RCA-I(Gal(β1→4)GlcNAc), AAL(Fuc(α1→6)-)), GSL-IB4(α-Gal) in LMs (Gerlach et al. 2013). Subsequently, Saraswat et al. reported that uEVs contain glycopeptides with predominantly complex and high-mannose N-glycan structures (Saraswat et al. 2015). In addition, Echevarria
et al. generated glycoprofiles of uEVs and urine supernatants using LMs and showed that uEV-derived profiles were distinct from those derived from urine supernatants in healthy people (Echevarria et al. 2014). Interestingly, this work identified LEL(GlcNAc(β1→4)GlcNAc), STL(GlcNAc), RCA(Gal(β1→4)GlcNAc) and WGA(GlcNAc, NeuAc) as strong binders to uEVs. Accordingly, we found that LEL(GlcNAc(β1→4)GlcNAc), WGA(GlcNAc, NeuAc), DSA(GlcNAc) and RCA-I(Gal(β1→4)GlcNAc) were the strongest binders for uEVs from healthy individuals. Thus, our work is one of the first comprehensive studies to confirm that uEVs display a large amount of carbohydrate on their surfaces and to provide insight into the most highly-expressed glycan structures.

It has been well established that uEVs derive from epithelial cells of the entire urinary tract (Pisitkun, Johnstone, and Knepper 2006). Recent reports suggest that distinct EVs are released by individual cell types (Bobrie and Théry 2013; Bobrie et al. 2012; Théry, Ostrowski, and Segura 2009; Trajkovic et al. 2008). In 2009, Mitchell et al. discussed the heterogeneity of uEV isolates and proposed that the overlapping properties of the various EV subtypes present may lead to loss of valuable information if they are not further separated (Mitchell et al. 2009). This report stimulated us to sub-fractionate uEVs for our glycoprofiling project using sucrose density gradient UC with a view to increasing the resolution of our LM profiles. Our initial findings with this approach showed that, while THP does not interfere with gradient separation, it cannot be fully removed from the uEVs by this method and results in a significant contamination of multiple fractions that varies among different healthy individuals. In particular, THP tended to appear at densities > 1.14 g/cm³. In contrast, to our results using simple water-based gradients, Hogan et al. observed that uEVs can be purged of THP by applying a D₂O-gradient (Hogan et al. 2009). We believe that the apparent superiority of this approach reflects the corresponding density of THP being above that of the 5-30% D₂O gradient, allowing THP to accumulate more consistently at the bottom of the D₂O-gradient. As the use of D₂O-gradients was not an available option for us and the use of DTT as reducing agents in relatively large volume gradients was not desirable, we evaluated TCEP an easily handled, efficient reducing reagent to remove THP from the initial UC pellet. Of note, both TCEP and DTT have been used by others in regard to alkylation of glycopeptides prior to trypsinization for application in mass spectrometry (Gong Chen et al. 2007). We found that TCEP does not need to be heated, that it resulted in reliable removal of THP at pH 7.4 and
that it is provided substantially greater ease of use compared to DTT. To our knowledge, this is the first study performed using TCEP as a reducing agent to remove THP during the course of uEV isolation. Importantly, the use of TCEP during sucrose density gradient fractionation of UC-uEVs did not interfere with downstream glycoprofiling by LM. The glycoprofiles of TCEP-treated UC-uEV fractions showed similar lectin binding trends as non-reduced uEVs including high binding of LEL(GlcNAc(β1→4)GlcNAc), DSA(GlcNAc), WGA(NeuAc/GlcNAc) and RCA-I(Gal(β1→4)GlcNAc). Other possibilities for THP removal include treatment of UC-uEVs with DTT itself as well as the detergent CHAPS, although neither has been specifically reported in the context of lectin-based studies (Fernández-Llama et al. 2010; Musante et al. 2012).

The scope of our study included an exploration of the relative shape, size and glycoprofiles of density gradient-generated UC-uEV fractions. In this regard, we found that uEVs within low density fractions isolated from healthy individuals were associated with high expression of CD24. Vesicles from these fractions showed a smaller mean diameter than those from higher density fractions in which CD24 expression was lower. Strikingly, some vesicles showed asymmetric expression of CD24 under immunogold TEM. Recently, it was reported that epithelial vesicles released from basal or apical membranes have distinct differences (van Niel et al. 2001). Apical membranes of epithelial cells may be heavily loaded with mucins or other glycoproteins that play specific roles in epithelial luminal functions. Thus, asymmetric trapping of CD24 within uEVs of specific size and density may represent a signature of vesicles released from the luminal surface of specific CD24+ epithelial cells along the nephron. Our TEM images did not indicate soccer ball-like vesicles as recently reported by Hogan et al. and, again, it is possible that this reflects differences in the gradient-based approaches used for uEV fractionation (Hogan et al. 2014). We did, however, observe multiple differences in vesicle types between densities of 1.08-1.14 g/cm³ and 1.20 g/cm³. The timeline of this study did not allow for further characterisation of uEVs with distinct appearances from individual density fractions. However, it would be possible and of specific interest to apply multi-label immunogold TEM to correlate uEV size and density with the expression of cell type-specific glycoproteins or with binding of nephrone segment-specific lectins in individual fractions. Interestingly, tip vesiculation was reported from podocyte surfaces and those EVs appear in a size ranges of about 120-125 nm, a size range also observed in our uEV samples (Hara et al. 2010). A limitation of our study was that we were
not able to perform a comprehensive screen for exosome-specific and cell-type specific proteins across the fractions as a result of low uEV yields due to limited sample volumes. We did observe that the classical exosome marker CD63 was expressed by uEVs in all fractions of the gradient, although the expression level was low compared to vesicles from MCF-7 breast cancer cells as recently shown by Van Deun et al. (Van Deun et al. 2014). Recent studies have suggested that protein aggregates may increase with higher density ranges raising a concern about spurious results for glycoprofiles in these fractions (Aalberts et al. 2012). However, our glycoprofiles appeared to be as complex and diverse in high density fractions as they were in the lower density fractions. Furthermore, the presence of intact vesicles and particles of a size range consistent with EVs was confirmed by TEM and NTA in the higher density fraction F9 (1.20 g/cm³) of multiple uEV isolated. The corresponding glycoprofiles indicated stronger lectin binding of LEL (GlcNAc(β1→4)GlcNAc), DSA (GlcNAc), WGA (GlcNAc/NeuAc) to low density fractions of TCEP-HCl treated H-uEVs whereas PA-I(Gal), sWGA(GlcNAc), NPA(Man(α1→6)-), GNA(Man(α1→3))-), HHA (Man(α1→3)Man(α1→6)-), VVA-B4 (GalNAc), ECA (α-Gal) indicates stronger binding to high density fractions of to TCEP-HCl treated H-uEVs.

The lectin binding profiles that we observed using FCM and LM platforms suggested interactions with a wide range glycoproteins, GPI-anchored proteins and glycolipids. The extent to which the individual subtypes of glycoconjugates contribute to these lectin signatures remains to be determined and this would likely require larger uEV sample amounts and additional analysis approaches. Taking into account the results of other published glycomic studies on EVs, it is likely that glycoproteins make up the largest contribution to uEV surface glycosylation (Saraswat et al. 2015; Staubach et al. 2012). In this regard, carbohydrate-mediated interactions between uEV surface glycoproteins may play an important role in their binding to and uptake by target cells (Escrevente et al. 2011). Consistent with this, proteins with known carbohydrate affinities including Neu5Ac-binding Ig-type lectins (SIGLECs) and galectins have been shown to modulate EV adherence to cell surfaces (Barrès et al. 2010; Christianson et al. 2013). Also, the proteoglycan, heparin sulfate, has been identified as being involved in uptake of EVs by target cells (Saunderson et al. 2014). N-linked glycans have also been reported to play key roles in the packaging of specific bio-molecular cargoes into exosomes (Y. Liang et al. 2014) and the lectin profiles we observed in our studies of unfractionated and fractionated UC-uEVs are consistent with a
large component of N-linked glycans among the surface glycome components.

The extent of our lectin-based data on glycosylation of fractionated UC-uEVs in the setting of kidney disease was relativey limited, consisting of a pilot experiment comparing samples from small groups of adults with known diagnosis of GN and with no history of kidney disease (healthy controls). For these samples, we separated uEVs using our optimized sucrose density gradient approach and eventually generated LM glycoprofiles for as many of the subfractions as possible. Although our ability to profile uEVs from lower density fractions was compromised by limited amount of material recovered, the results indicated that 5 of 48 lectins differed significantly between GN and controls for some of the medium to high density fractions. The specific carbohydrate structures that are suggested by these data as being altered in the setting of GN are: Man(α1→3), Man(α1→3)Man(α1→6), GalNAc, Gal(β1→3)GalNAc, Gal(β1→3)GlcNAc oligomers. It should be acknowledged that the overall results of this experiment are consistent with the conclusion that uEV glycosylation changes between GN and healthy controls are relatively subtle and possibly difficult to detect due to the heterogeneity of EV subtypes and cell sources present even following subfractionation. Nonetheless, these preliminary results, as well as our findings for a small cohort of ADPKD patients, suggest that it may be of value to explore uEV surface carbohydrates further as a biomarker source in the setting of different forms of kidney disease. In keeping with this, glycosylation of uEVs or antibodies has already been shown to have clinical significance in galactosemia and IgAN (Amore et al. 2001; Staubach et al. 2012). Two advancements that could substantially increase the potential for uEV glycosylation screening to be more comprehensively evaluated for clinical applications would be the development of a consistent, rapid turn-around method of isolation of high quality EVs from urine and the development of a multi-colour cytometry platform capable of accurately distinguishing EV subtypes and phenotyping for multiple surface markers at the single vesicle level.

Our findings emphasize that accurate analysis of uEVs continues to be challenging work. Enrichment of uEVs for multiple down-stream applications is a time-consuming and resource-heavy process regardless of the isolation protocol. Although THP can be removed successfully using reducing agents, it could be preferable to use more specific depletion approaches for this and other contaminating proteins in projects involving large number of samples (Zubiri et al. 2014). For large-scale biomarker studies, the protocols used for uEV
isolation, storage, labelling and downstream analyses must be planned with care in advance and must be appropriate for the anticipated urine volumes – which are likely to be relatively low (50 – 200 ml) if “spot” urine samples from large numbers of subjects are to be collected. It should be recognized that analyses of crude, unfractionated uEV preparations may result in a failure to identify key disease-associated changes to specific EV subtypes or alterations to EVs derived from specific renal cell types. Based on our experiences during the project described in this thesis, we suggest that UC continues to be the method of choice for initial separation of uEVs from all other urine components. We also believe that further resolution of candidate biomarker signatures may currently require uEV sub-fractionation based on density, affinity (lectin or antibodies), charge (anion-exchange) or size (size-exclusion). Those initial comprehensive studies may be further simplified once one or more biomarkers of choice are identified.

Our results also indicate that uEVs from medium to higher density fractions may be distinctly different in healthy and diseased states. Based on this finding, we would emphasize that uEVs outside the typical size range for true exosomes (possibly representing membrane shed vesicles, tip vesicles, apoptosomes or other vesicles) may be of specific importance for identification of pathological changes or prediction of disease severity and response to treatment. Finally, it may be of scientific as well as clinical interest to determine whether uptake by renal epithelial cells of uEV populations derived from low, medium and high density subfractions result in distinct functional effects and whether uEV surface glycosylation characteristics play a role. Given the complexity of the uEV repertoire during health and the potential for variances in uEV quantity, source and bio-molecular content during different forms of kidney disease, it seems likely that research in this area is in its infancy and that the biological relevance of uEVs is largely unexplored. Our work described in this thesis has advanced knowledge of an aspect of EV biology that has been under-investigated to date and, we hope, will stimulate greater interest in the glycosylation characteristics of individual vesicle subtypes as well as in the development of new strategies and technologies for accurately profiling EV-associated glycans and their functional effects.
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9 Appendix

9.1 Galway University Hospitals - Patient Information Leaflet

Principal Investigator’s Name: Professor Matthew Griffin

Project Title: A Study of Glycosylation Signatures of Urinary Exosomal Proteins in Glomerulonephritis.

Telephone No. of Principal Investigator: 091-524222 or 091-751131

You are being invited to take part in a clinical research study carried out at Galway University Hospitals. Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP. Take time to ask questions – do not feel rushed or under any obligation to make a hasty judgement. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you – this process is known as Informed Consent.

You are not obliged to take part in this study and failure to participate will have no effect on your future care.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care you will receive from the medical staff.

Why is this study being done?

This study is being done to find new information about why some people with a form of kidney disease called glomerulonephritis respond to treatment differently to others. It is also being done to test new ways of analysing urine samples form people with glomerulonephritis that may help to guide treatment for these people. The study will use new laboratory techniques to compare protein and carbohydrate (sugar) molecules contained in the urine of healthy people with that of people who have been diagnosed with glomerulonephritis. A special feature of the study is to analyse tiny particles called exosomes which are present in normal urine and are shed form the cells within the kidney. The study investigators hope to prove that analysis of protein and carbohydrate molecules contained in these exosomes will
provide a new way of learning about kidney health during treatment for glomerulonephritis. If they are correct this study could be used to develop convenient and safe new tests for diagnosing and treating people with glomerulonephritis. You are being asked to take part because you have been diagnosed with a form of glomerulonephritis and will be receiving treatment for this at Galway University Hospitals.

**Who is organising and funding this study?**

The study is organised by Professor Griffin, who is a kidney specialist at Galway University Hospitals, and by other doctors at this hospital who specialise in the care of people with kidney disease. The funding for the study comes from a grant to Professor Griffin from the Health Research Board. The study will also involve a scientist, Professor Lokesh Joshi, at the National University of Ireland, Galway who is an expert in the analysis of proteins and carbohydrates.

**How will it be carried out?**

People who are attending the Nephrology (kidney disease) outpatient clinics at Galway University Hospitals (GUH) or who are being cared for by the Nephrology inpatient service at GUH will be asked by a nurse or doctor to take part in the study if they have recently been diagnosed with a form of glomerulonephritis. In all, 40 people with glomerulonephritis will be asked to take part during a period of 3 years. Each person who agrees to participate in the study will provide a sample of urine on the day of entering the study either at the Nephrology outpatient clinic or in one of the Galway University Hospitals. The urine sample will be taken to Professor Griffin and Prof. Joshi’s research laboratory at NUI Galway and will be analysed by purification of exosomes either immediately or after first being frozen. The laboratory tests carried out on protein and carbohydrate molecules from the urine exosomes will be compared with those from a group of healthy volunteers. Each person who agrees to participate in the study will then be asked to provide a urine samples during outpatient follow-up visits approximately 4, 8, 12, 24, 36 and 52 weeks after the first sample. These urine samples will be transported and analysed in the same way and the results will be used to find out how urine exosome proteins and carbohydrate molecules change in the urine during treatment. The maximum number of samples that each person in the study will provide will be 7.
Procedures/ What will happen to me if I agree to take part?

You will first be asked by a doctor or nurse to sign a form indicating that you agree to take part. After this you will be given some simple instructions for collecting a sample of your urine in a clean container. When you return the sample to the doctor or nurse it will be saved for transport to the research laboratory later that day. After giving the first urine sample you will not have to do anything for the study on that day. At follow-up visits to the Nephrology outpatient clinic at approximately 4, 8, 12, 24, 36 and 52 weeks later you be asked to provide another urine sample in the same way. You will not have to come to the outpatient clinic just for the purpose of providing a sample for the study. Instead the samples will be requested on days when you are being seen for medical follow-up by one of the doctors on the Nephrology team. Each sample will be stored for testing in Professor Griffin and Prof. Joshi’s research laboratory at NUI Galway.

What alternative treatments are available to me?

The study will have no effect on your treatment.

Risks & Discomforts

There is no risk to taking part in the study but there will be a small inconvenience if you have to give an extra urine sample during your clinic visits.

Benefits of this Study

You will not benefit directly from taking part in this study but the researchers may learn more about glomerulonephritis. This could help to develop new tests or treatments for glomerulonephritis in the future.

Compensation

There is no compensation for participating in this study.

Will there be any additional costs involved?

There will be no additional costs.
Confidentiality

Some information related to your medical condition will be recorded from your medical chart by one of the doctors or nurses involved in the study. Also your urine sample will be stored in Professor Griffin and Prof. Joshi’s research laboratory at NUI Galway. To avoid people who are not involved in the study learning about your medical condition, your name and personal details will be replaced on all study documents and samples by a numbered code known only to the study doctors and nurses. Written documents, computer files and urine samples will be kept in locked cabinets or password-protected computers for a total of 10 years. If you agree to it now, the samples and medical information may be used for other research projects or shared anonymously with other researchers at NUI Galway or elsewhere. After 10 years the written documents and remaining samples from the study will be destroyed by incineration.

Project Duration

The project will continue until a total of 40 people have provided suitable urine samples and have been followed for 1 year. This is expected to take about 3 years from the start of the study.

What if something goes wrong as a result of my participation in this study?

It is very unlikely that anything will go wrong as a result of your participation in this study but if you have a concern you should contact Professor Griffin at the numbers provided on this information sheet.

Your responsibility as a participant

Your responsibility as a participant in the study is to let us know if you do not understand any part of the study procedure.

Our responsibility to you as investigators

We are responsible for explaining the study carefully to you, for answering any questions or concerns you have about the study at any time, for protecting the confidentiality of your medical information and for making sure that your medical care is not affected in any way by your participation.

If you require further information

For additional information now or any future time please contact:

Name: Prof. Matthew Griffin
Address: Consultant Nephrologist, Merlin Park Hospital, Merlin Park, Galway
Phone No: 091-524222 or 091-751131
9.2 Consent form

**Full title of Project:** A Study of Glycosylation Signatures of Urinary Exosomal Proteins in Glomerulonephritis.

**Name, position and contact address of Chief/Principal Investigator:** Professor Matthew Griffin, Consultant Nephrologist, Merlin Park Hospital, Merlin Park, Galway.

<table>
<thead>
<tr>
<th></th>
<th>Please initial box</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>I am an <strong>adult</strong> taking part in this study.</td>
</tr>
<tr>
<td>2.</td>
<td>I confirm that I have read and understand the information sheet for the above study and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved.</td>
</tr>
<tr>
<td>3.</td>
<td>I agree to donate up to a total of 7 tissue samples (urine) for this research project. I understand that giving samples for this research is <strong>voluntary</strong> and that I am free to withdraw my approval at any time without my medical treatment being affected.</td>
</tr>
<tr>
<td>4.</td>
<td>I agree to take part in the above study.</td>
</tr>
<tr>
<td>5.</td>
<td>I give permission for my samples and information collected about me to be stored for possible future research related to this study (including DNA or genetic studies) but only if the research is approved by a Research Ethics Committee. <strong>Please tick box</strong></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>6.</td>
<td>I give permission for research personnel to look at my medical records to obtain information. I have been assured that information about me will be kept confidential.</td>
</tr>
</tbody>
</table>

_________________________  ___________________________  ___________________________
Name of Participant          Date                        Signature

_________________________  ___________________________  ___________________________
Name of Researcher/          Date                        Signature

Person Taking Consent
### 9.2.1 Equipment

**General laboratory equipment:**

<table>
<thead>
<tr>
<th>Device</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge 5810 R (rotor A-4-81)</td>
<td>Eppendorf AG, Germany</td>
</tr>
<tr>
<td>Balance AE240 (fine scale)</td>
<td>Mettler, Switzerland</td>
</tr>
<tr>
<td>Biorad PowerPac 3000</td>
<td>Biorad, USA</td>
</tr>
<tr>
<td>Centrifuge 5417 C/R (rotor FA-45-30-11)</td>
<td>Eppendorf AG, Germany</td>
</tr>
<tr>
<td>Digital refractometer HI96801</td>
<td>Hannah instruments, US</td>
</tr>
<tr>
<td>Heatblock TECHNE DRI-BLOCK®DIB-2A</td>
<td>Techne (Cambridge) Ltd., UK</td>
</tr>
<tr>
<td>Heatplate</td>
<td>Fisher Scientific, USA</td>
</tr>
<tr>
<td>Kodak Image Station 4000 MM Pro®</td>
<td>Carestream, Woodbridge, CT, USA</td>
</tr>
<tr>
<td>LEEC compact incubator model: K2N</td>
<td>LEEC limited, UK</td>
</tr>
<tr>
<td>Minicentrifuge spectrafuge Mini C1301</td>
<td>Hermle Z100 M, Labnet, Woodbridge, CT USA</td>
</tr>
<tr>
<td>pH-meter pH510 EUTECH</td>
<td>Eutech Instruments Pte Ltd., Singapore</td>
</tr>
<tr>
<td>PJ4000 (large scale)</td>
<td>Mettler, Switzerland</td>
</tr>
<tr>
<td>Plate reader SpectraMax M5e</td>
<td>Molecular Devices, UK</td>
</tr>
<tr>
<td>Rotator shaker Luckham Multimix Major</td>
<td>Mason technology, Ireland</td>
</tr>
<tr>
<td>Shaker model IKA® Schüttler MTS4 S14 orbital</td>
<td>IKA Labortechnik, Germany; distributor: Stuart Limited advances applied technologies, UK</td>
</tr>
<tr>
<td>Thermo-shaker PHMT</td>
<td>Grant-bio, UK</td>
</tr>
<tr>
<td>Trans-Blot®SD Semi-Dry Electrophoretic Transfer Cell</td>
<td>Bio-Rad, USA</td>
</tr>
<tr>
<td>Vortex model IKA-VIBRO-FIX VF2</td>
<td>IKA Labortechnik, Germany; distributor: Stuart Limited advances applied technologies, UK</td>
</tr>
<tr>
<td>Wet transfer cell</td>
<td>Bio-Rad, Germany</td>
</tr>
<tr>
<td>Wheel rotator SB2</td>
<td>Stuart®, UK</td>
</tr>
<tr>
<td>XCell Surelock™MiniCell</td>
<td>Invitrogen, life technologies™</td>
</tr>
</tbody>
</table>
### Specialized laboratory material

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well Microtest-plate Vee-shape</td>
<td>Sarstedt, Germany</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Blotting paper extra thick (15 cm x 15 cm)</td>
<td>Sigma-Aldrich</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Centrifugal filters Amicon® Ultra, 0.5 ml, MWCO100 kDa</td>
<td>Merck Millipore Ltd., Ireland</td>
<td>Fisher Scientific, Ireland</td>
</tr>
<tr>
<td>Collection jars 150ml</td>
<td>Sarstedt, Germany</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Copper grids, formvar coated 200 mesh</td>
<td>Agar Scientific Ltd UK</td>
<td>Agar Scientific, UK</td>
</tr>
<tr>
<td>Falcon tubes</td>
<td>Sarstedt, Germany</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Gold grids, formvar coated 200 mesh</td>
<td>Agar Scientific Ltd UK</td>
<td>Agar Scientific, UK</td>
</tr>
<tr>
<td>Microtest-Plate 96 well, F</td>
<td>Sarstedt, Germany</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>NalgeneTM Oak Ridge high-speed centrifuge tubes</td>
<td>Nalgene, Thermo Scientific, Dublin Ireland</td>
<td>Fisher Scientific, Ireland</td>
</tr>
<tr>
<td>Parafilm M</td>
<td>BEMIS, US</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Polyallomer tubes, 36ml</td>
<td>Kendro, Ashville, USA,</td>
<td>Fisher scientific, Ireland</td>
</tr>
<tr>
<td>PVDF membrane (Immobilon®-PSQ Transfer membrane, 0.22 µm pore size</td>
<td>Millipore™, US</td>
<td>Fisher Scientific, Ireland</td>
</tr>
<tr>
<td>Serological pipette 10 ml, 25 ml</td>
<td>Sarstedt, Germany</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Tubes 5 ml (FACS tubes)</td>
<td>Sarstedt, Germany</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Ultra-clear thinwall tube, 13.2 ml</td>
<td>Beckman Coulter, USA</td>
<td>Beckman Coulter, Ireland</td>
</tr>
</tbody>
</table>

### Specialized laboratory equipments and software:

- **Beckman Centrifuge J2-21**
  - Beckman Coulter, US (rotor JA-17 fixed angle rotor)
- **CanoScan LiDE90**
  - Canon Corporation Ireland, Saggart, Ireland
- **Concentrator 5301 (rotor F45-48-11)**
  - Eppendorf, Germany
- **eight-well gasket slide**
  - Agilent Technologies, Cork, Ireland
- **FACSCanto® cytometer**
  - Becton Dickinson (BD) Biosciences, (San Jose, CA, USA)
- **Hitachi H100,000 at 80 kV**
  - Hitachi HTI, Leixlip, Ireland
- **Hitachi H7500 TEM at 75 kV**
  - Hitachi HTI, Leixlip, Ireland
Humidity chamber  
Incubation cassette system  
Microarray Scanner System and Agilent G2545A Hybridization Oven  
NanoSight NS 500 system (laser 405 nm long pass filter at 430 nm)  
Nexterion® H NHS functionalized glass slides  
Perkin-Elmer Scanarray HT (543 nm or 633 nm channel, 90 % laser power, 70 % PMT)  
Scienion S3 non-contact spotter  
Sorvall 100SE Ultra Centrifuge  
Wheaton Coplin staining jars

**Software**

**Software**
- Agilent software  
- Flowjo® version 7.6.4  
- GenePix Pro v6.1.0.4  
- Hierarchical Clustering Explorer v3.0  
- Kodak imager software  
- Minitab® 16 version 16.1.1  
- NTA version 2.3 build version 2.3.5.0033.7-Beta 7 proprietary *.gal file, Excel  
- Scanner software Canon  
- Scienion printer software

**Manufacturer**
- Agilent, Ireland  
- TreeStar, Inc., Ashland, OR, USA  
- Molecular Devices, Sunnyvale, CA, USA  
- Malvern, UK  
- Minitab, Inc., State College, PA, USA  
- Malvern, UK  
- Microsoft, WA, USA  
- Canon Corporation Ireland, Saggart, Ireland  
- Scienion, Germany

**Chemicals**

**Chemical**
- 4% (w/v) aldehyde sulfate beads 4 µm  
- 4-12 % Bis-Tris NuPAGE  
- 6-Aminocaproic acid  
- Acetic acid  
- Acetic acid 96% puriss p. A.  
- Bovine albumin fraction V

**Supplier**
- Molecular probes, US  
- Life technologies, US  
- Sigma-Aldrich, China  
- Sigma-Aldrich, Germany  
- Sigma-Aldrich, Germany  
- Pentex, US
Bromo phenol Blue  
Calcium chloride dehydrate  
Complete, EDTA-free Protease inhibitor cocktail tablets in EASYpack  
DL-Dithiothreitol  
Ethanol, absolute analytical grade  
Glutaraldehyde solution, Grade I, 25% in H2O, specially purified use as an electron microscopy fixative  
Glycerol 86-88%  
Glycine  
HEPES  
Hydrocholoric acid min 32% puriss p.a.  
Magnesium chloride  
Manganese chloride dehydrate  
Methanol, 215 super purity solvent  
Methylcellulose  
MOPS buffer 20x  
Nonfat dried milk powder  
Oxalic acid dehydrate  
Paraformaldehyde – powder, 95%  
Phosphotungstic acid solution 10% (w/v)  
Pierce TM BCA protein assay kit  
Pierce® Silver Stain kit  
Pierce™ BCA protein assay kit  
PKH26 Red Fluorescent Cell Linker Mini Kit for General Membrane Labeling  
Potassium dihydrogen phosphate  
Seeblue® Plus2 Pre-Stained Standard  
SIGMA-FAST BICP/NBT  
Sodium phosphate dibasic dihydrate  
Sucrose  
Super Signal® Molecular Weight Protein Ladder  
Super Signal® West Pico Chemiluminescent substrate- ECL reagent  
LKB-Produckter AB  
Bromma, Sweden  
Sigma-Aldrich, Germany  
Roche Diagnostics, West Sussex, UK  
Sigma-Aldrich, Canada  
Fisher Scientific, Ireland  
Sigma-Aldrich, UK  
Riedel de Haën, Germany  
Sigma-Aldrich, Belgium  
Sigma-Aldrich, US  
Sigma-Aldrich, Germany  
Sigma-Aldrich, Germany  
Merck, Germany  
ROMIL Ltd., UK  
Sigma-Aldrich, Japan  
Life technologies, US  
Sigma-Aldrich, US  
Sigma-Aldrich, Japan  
Sigma-Aldrich, Germany  
Sigma-Aldrich, UK  
Thermo Scientific, US  
Thermo Scientific, US  
Thermo Scientific, US  
Sigma-Aldrich, US  
Riedel de Haën, Germany  
Life technologies, US  
Sigma-Aldrich, US  
Sigma-Aldrich, Germany  
BDH Anala® Laboratories, UK  
Fisher Scientific, Ireland  
Thermo Scientific, US
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP*HCl)  
Trishydroxymethylamine  
Tween® 20 for molecular biology  
Uranylacetate

9.2.3 Antibodies

**Antibody**

- **Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in goat**
- **Anti-Rabbit IgG (whole molecule)-Peroxidase antibody produced in goat**
- **Anti-water channel aquaporin-2 (AQP-2) developed in rabbit**
- Asialo Fetuin AF647 labelled
- Avidin alkaline phosphatase
- BD Pharmingen™ APC Streptavidin
- Biotin conjugated Anguilla anguilla Lectin (Fresh Water Eel)-AAA
- Biotin conjugated Maackia amurensis Lectin – MAA
- Biotin conjugated sambucus nigra (Elderberry Bark) – SNA-I
- Biotinylated GSL-I isoelectin B4
- CD24
- Goat anti-mouse IgG (whole molecule)-conjugated gold (11 nm)
- Goat anti-rabbit IgG (whole molecule)-conjugated gold (5-9 nm)
- Goat F(ab’)2 anti-mouse IgG (Fc) domain conjugated FITC
- Lectin kit I, biotinylated
- Lectin kit II, biotinylated
- Lectin kit III, biotinylated
- Streptavidin-PE-Cy7
- THP (H-135) prim. antibody for immunoblotting

**Supplier**  
**Code**  
Sigma-Aldrich  
A05278-1ML
Sigma-Aldrich  
A0545-1ML
Sigma-Aldrich  
A7310-.05ML
In house with labelling  
None
Sigma-Aldrich  
A7294-1ML
BD Biosciences  
554067
EY laboratories  
BA-4901-1
EY Laboratories  
BA-7801-2
EY Laboratories  
BA-6802-1
Vector laboratories  
B-1205
Prof. Peter Altevogt  
None
Sigma-Aldrich  
G7652-.4ML
Sigma-Aldrich  
G7277-.4ML
Beckman Coulter  
IM1619
Vector Laboratories  
BK-1000
Vector Laboratories  
BK-2000
Vector Laboratories  
BK-3000
BD Biosciences  
557598
Santa Cruz Biotechnology  
sc-20631
9.2.4 Lectin set according to microarray studies

9.2.5 The table was modified from the published paper J.Q. Gerlach et al.. (Gerlach et al. 2013)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Species (Common name)</th>
<th>Major Ligand(s)</th>
<th>Origin</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARA, Jacalin</td>
<td>Arctocarpus integrifolia (Jack fruit lectin)</td>
<td>Gal (sialylation independent)</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>RPA-I</td>
<td>Robinia pseudoacacia (Black locust lectin)</td>
<td>Gal, GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>PA-I</td>
<td>Pseudomonas aeruginosa (Pseudomonas lectin)</td>
<td>Gal, Gal derivatives</td>
<td>Bacteria</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>SNA-II</td>
<td>Sambucus nigra (Sambucus lectin)</td>
<td>Gal, GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>SAJ</td>
<td>Sapindus japonica (Paspodree lectin)</td>
<td>βGalNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>DPA</td>
<td>Dolichos biflorus (Horse gram lectin)</td>
<td>GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>APP</td>
<td>Aeglopsidium podagricae (Ground elder lectin)</td>
<td>GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>SBA</td>
<td>Glycine max (Soy bean lectin)</td>
<td>GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>VVA-B4</td>
<td>Vicia villosa (Hairy vetch lectin)</td>
<td>GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>BPA</td>
<td>Bauhinia purpurea (Camels foot tree lectin)</td>
<td>GaINAc/Gal</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>WFA</td>
<td>Wisteria floribunda (Japanese wisteria lectin)</td>
<td>GaINAc/Sulfated GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>ACA</td>
<td>Amaranthus caudatus (Amaranth)</td>
<td>Sialylated Gal[1→3]GaINAc</td>
<td>Plant</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>ABL</td>
<td>Agaricus bisporus (Edible mushroom lectin)</td>
<td>Gal[1→3]GaINAc, GalNAc</td>
<td>Fungi</td>
<td>EY Labs</td>
</tr>
<tr>
<td>PNA</td>
<td>Araehis hypogea (Peanut lectin)</td>
<td>Gal[1→3]GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>GSL-II</td>
<td>Griffonia simplicifolia (Griffonia/Sambucus lectin II)</td>
<td>GlcNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>aWGA</td>
<td>Triticum vulgaris (Succinyl WGA)</td>
<td>GlcNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>DSA</td>
<td>Datura stramonium (Jimson weed lectin)</td>
<td>GlcNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>STA</td>
<td>Solanum tuberosum (Potato lectin)</td>
<td>GlcNAc oligomers</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>LEL</td>
<td>Lycopersicum esculentum (Tomato lectin)</td>
<td>GlcNAc[1→4]GlcNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>NPA</td>
<td>Narcissus pseudonarcissus (Daffodil lectin)</td>
<td>Man[1→6]-</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>GNA</td>
<td>Galanthus nivalis (Snowdrop lectin)</td>
<td>Man[1→3]-</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>CanA</td>
<td>Cannaralia ensiformis (Jack bean lectin)</td>
<td>Man</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>Lch-B</td>
<td>Lens culinaris (Lentil isolecitin B)</td>
<td>Man, fucose dependent</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>PSA</td>
<td>Psaum sativum (Pea lectin)</td>
<td>Man, fucose dependent</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>WGA</td>
<td>Triticum vulgaris (Wheat germ agglutinin)</td>
<td>NeuAc/GlcNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>MAA</td>
<td>Maackia amurensis (Maackia agglutinin)</td>
<td>Sialic acid[2→3]Gal</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>SNA-I</td>
<td>Sambucus nigra (Sambucus lectin I)</td>
<td>Sialic acid[2→6]Gal, GaINAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>PHA-L</td>
<td>Phaseolus vulgaris (Kidney bean lectin)</td>
<td>TriTetra- antennary</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>PCA</td>
<td>Phaseolus cocineus (Scarlet runner bean lectin)</td>
<td>GlcNAc in complex oligos</td>
<td>Plant</td>
<td>Sigma Aldrich</td>
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<tr>
<td>PHA-E</td>
<td>Phaseolus vulgaris (Kidney bean erythroagglutinin)</td>
<td>Biantennary, bisecting</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>RCA-1/20</td>
<td>Ricinus communis (Castor bean lectin)</td>
<td>Gal[1→4]GlcNAc</td>
<td>Plant</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>OPA</td>
<td>Cicer arietinum (Chickpea lectin)</td>
<td>Complex glycopeptides</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>CAA</td>
<td>Caragana arborescens (Pea tree lectin)</td>
<td>Gal[1→4]GlcNAc</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>ECA</td>
<td>Erythrina cristagalli (Cock’s comb/coral tree lectin)</td>
<td>Gal[1→4]GlcNAc oligomers</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>AAL</td>
<td>Anemia aurantia (Orange peel fungus lectin)</td>
<td>Fuc[1→6]</td>
<td>Fungi</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>LTA</td>
<td>Lotus tetragonolobus (Lotus lectin)</td>
<td>Fuc[1→3]</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>UE-A-I</td>
<td>Ulex europeae (Gorse lectin I)</td>
<td>Fuc[1→2]</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>EEA</td>
<td>Euonymus europaeus (Spindle tree lectin)</td>
<td>α-Gal</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>GSL-I-B4</td>
<td>Griffonia simplicifolia (Griffonia/Sambucus lectin I)</td>
<td>α-Gal</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>MPA</td>
<td>Macleura ponifera (Osage orange lectin)</td>
<td>α-Gal</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>VRA</td>
<td>Vigna radiata (Mung bean lectin)</td>
<td>α-Gal</td>
<td>Plant</td>
<td>EY Labs</td>
</tr>
<tr>
<td>MOA</td>
<td>Merasius oreades (Fairy ring mushroom lectin)</td>
<td>α-Gal</td>
<td>Fungi</td>
<td>EY Labs</td>
</tr>
</tbody>
</table>
9.2.6 Buffers

General buffers:

HEPES, PBS, sodium phosphate buffers have been prepared as described in current protocols appendix 2A.

**Phosphate buffered saline (PBS):** 8 g sodium chloride (0.137 M), 0.2 g potassium chloride (2.7 mM), 1.778 g sodium dihydrogen phosphate (0.01 M used as dihydrate), 0.19 g potassium dihydrogen phosphate (1.4 mM) fill up to 800 ml adjust pH to 7.4 with sodium hydroxide if needed and dilute to 1 liter.

**Tris buffered saline (TBS):** 20 mM Tris, 180 mM sodium chloride add water til 800 ml mark then adjust pH to 7.5 with HCl and fill up to 1 liter.

**Tris buffered saline supplemented with Tween 20 (TBST):** 20 mM Tris, 180 mM sodium chloride add water til 800 ml mark then adjust pH to 7.5 with hydrochloric acid and fill up to 1 liter, 0.05% (v/v) Tween 20.

**Membrane blocking buffer:**

1.5% (w/v) dried fat milk in TBST

**Antibody binding buffer:**

TBST with BSA 3% (w/v) or 5% (w/v)

**HEPES:**

20 mM HEPES in water ad 0.5 L, pH 7.4

**Sodium phosphate buffer (0.1 M, pH 7.4):**

Solution 1: 27.6 g NaH$_2$PO$_4$ $\cdot$H$_2$O per liter (0.2 M final) in water
Solution 2: 53.65 g Na$_2$HPO$_4$·7H$_2$O per liter (0.2 M) in water

Mix 19 ml solution 1 and 81 ml solution 2 and fill up to 200 ml to receive pH 7.4.

As described in general buffers, media and stock solutions current protocols appendix 2A.

**Cathodic buffer:** 0.3 M amino caproic acid, 0.03 M Tris, 1.875 ml of 20% (w/v) sodium dodecyl sulfate ad 1 L dH$_2$O.
**Anodic buffer:** 0.3 M Tris, 0.1 M glycine, 1.875 ml of 20% (w/v) sodium dodecyl sulfate solution ad 1 L with dH₂O.

**Reducing buffer 5x:** 0.5 M Tris pH 6.8 (2.13 ml), glycerol 100% 5 ml, sodium dodecyl sulfate 1 g, 2.56 ml β-mercaptoethanol, bromophenol blue traces. The mixture was diluted up to 20 ml with double deionized H₂O until completely dissolved. The buffer was stored at 4°C.

### 9.3 Supplementary data for Chapter 4

**Titration of PKH26 CF-uEVs binding to latex beads**

Figure 9-1: Titration of CF-uEVs binding to latex beads for FCM assays. Work was carried out by Susan Gallogly and Dr. Shirley Hanley.
Figure 9-2: Titration of THP binding to latex beads for FCM assays. Work was carried out by Susan Gallogly and Dr. Shirley Hanley.
Figure 9-3: Titration of the CD24 antibody
Figure 9-4 Titration of lectins for assays with uEVs. Corresponding to the final working concentration table. Solid line tined uEV or protein bound to beads, complex line unspecific lectin binding to a non binding control, complex binding tined 5 µg lectin unspecific binding to a non binding control, dotted lines lectin titrations 5 µg-20 µg/ml, dashed line unspecific binding control of streptavidin- conjugated dye to the protein or uEVs., dotted tined line is the optimized working concentration.