ISEV Meeting Presentations

Melbourne, Australia, 19 May 2023: INOVIQ Limited (ASX:IIQ) (**INOVIQ** or the **Company**) advises that as previously communicated to the ASX on 19 April 2023, the Company will present new data further confirming the effectiveness of its proprietary exosome isolation technology, EXO-NET®, at the Annual Meeting of the International Society for Extracellular Vesicles (ISEV) in Seattle, USA from May 18-21, 2023.

The oral presentation and five poster presentations will be delivered by INOVIQ and its collaborators, including the University of Queensland and Johns Hopkins University, at ISEV2023 from Thursday, 18 May 2023 to Saturday, 20 May 2023. ISEV's Annual Meeting is the leading global exosome scientific conference for INOVIQ to showcase these important advances to key opinion leaders in the extracellular vesicle field worldwide.

INOVIQ CEO, Dr Leearne Hinch said: *"The presentations highlight the broad utility of EXO-NET for fast and efficient exosome isolation and biomarker discovery across multiple biofluids including plasma, serum, saliva and cell culture media.*

Data are presented supporting the reproducibility and efficiency of EXO-NET for discovery of early and accurate biomarkers for use in development of INOVIQ's next-generation EXO-Ovarian Cancer Test for early detection of ovarian cancer when it can be cured."

The oral abstract and posters are appended to this release.

Authorised for release by Company Secretary, Mark Edwards.

- ENDS -

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ABOUT INOVIQ LTD

INOVIQ Ltd (ASX:IIQ) (**INOVIQ**) is developing and commercialising next-generation exosome capture tools and precision diagnostics to improve the diagnosis and treatment of cancer and other diseases. The Company has commercialised the EXO-NET pan-exosome capture tool for research purposes and the hTERT test as an adjunct to urine cytology testing for bladder cancer. Our cancer diagnostic pipeline includes blood tests in development for earlier detection and monitoring of ovarian, breast and other cancers. For more information on INOVIQ, see www.inoviq.com.

FORWARDING LOOKING STATEMENTS

This announcement contains certain 'forward-looking statements' within the meaning of the securities laws of applicable jurisdictions. Forward-looking statements can generally be identified by the use of forward-looking words such as 'may', 'should', 'expect', 'anticipate', 'estimate', 'scheduled' or 'continue' or the negative version of them or comparable terminology. Any forecasts or other forward-looking statements contained in this announcement are subject to known and unknown risks and uncertainties and may involve significant elements of subjective judgment and assumptions as to future events which may or may not be correct. There are usually differences between forecast

and actual results because events and actual circumstances frequently do not occur as forecast and these differences may be material. The Company does not give any representation, assurance or guarantee that the occurrence of the events expressed or implied in any forward-looking statements in this announcement will actually occur and you are cautioned not to place undue reliance on forward-looking statements.

ISEV PRESENTATIONS

Oral Presentation

Dynamic changes in the miRNA and protein content of circulating extracellular vesicles associated with ovarian cancer progression.

Presentation Number:	OT02.5
Session Date:	Thursday May 18, 2023
Presentation Time:	12:30- 12:45 (GMT-7)
Presenter:	Assoc Prof. Carlos Salomon from University of Queensland

OT02.5 | Dynamic changes in the miRNA and protein content of circulating extracellular vesicles associated with ovarian cancer progression

Dominic Guanzon[1]; Andrew Lai[2]; Carlos Palma[3]; Ramin Khanabdali[4]; Aase Handberg[5]; Lewis Perrin[6]; John Hooper[6]; Jim Coward[7]; Terry K. Morgan[8]; Gregory E. Rice[9]; Carlos Salomon[10]

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Introduction: Circulating changes in the content and bioactivity of extracellular vesicles (EVs) has been associated with oncogenic transformation of ovarian cancer (OVCA). Thus, characterisation of EV protein and nucleic acids is an essential step for understanding changes to predict cancer outcome. The objective of this study was to describe changes in the EV-associated proteins and miRNAs that change with the progression of the disease and determine biological processes that are disturbed in OVCA.

Methods: A cohort of 97 patients were included in this study from healthy controls (n = 20), benign (n = 20), high grade epithelial ovarian cancer stage I (n = 20), stage II (n = 20), stage III (n = 20), and stage IV (n = 20). EV were isolated using EXO-NET (INOVIQ LTDA, Australia) and characterised by Nanoparticle tracking analysis, protein abundance (CD63, CD9, Alix, TSG101 and CD81) and morphology using NanoSight, Western blot and electron microscopy, respectively. In addition, a Targeted Multiple Reaction Monitoring proteomic approach was designed to evaluate the top 20 proteins associated with EV (exocarta) in our preparations. EV-associated miRNA and protein profile was determined by small RNA sequencing and Mass Spectrometry SWATH Analysis, respectively. Generalised additive modelling was used to model protein and miRNAs abundance as a function of progression of OVCA, while pathway analysis was performed using Ingenuity.

Results: Using an EV capture technology (EXO-NET) around 20% of the total circulating particles was isolated, and an enrichment of proteins CD63, CD9, Alix, TSG101 and CD81 compared to total plasma was observed. A total of 18 out of the top 20 proteins associated with EVs in the Exocarta were identified in our preparations. Of the total proteins identified (1517) within EV, 33% (599) changed in abundance as function of the progression of OVCA ($p < 0.05$). Our modelling analysis identified a total of 20 clusters with different trends, in which 116 proteins (including Carboxypeptidase E, Adenosine deaminase, and Sex hormone-binding globulin) increased with the progression of ovarian cancer. We identified 703 miRNAs within EV, and 24% (171) changed with the progression of OVCA. A total of 10 clusters with different trends were identified, in which 7 miRNAs (miR-503-5p miR-181d-5p, miR-548ay-5p, miR-548ad-5p, miR-3157-5p, miR-135a-5p, miR-6815-5p) continuously increased with the progression of OVCA. Bioinformatic analysis showed that the top functions associated with the proteins and miRNAs with EV across progression of OVCA are inflammation, lipid metabolism, transport, and binding of tumour cells.

Summary/Conclusion: The EV proteome and miRNA profile across OVCA demonstrates dramatic changes associated with the progression of the disease. Such information is important to understand the physiology of OVCA and the development of biomarkers to differentiate women with early stages of OVCA and determine the response to chemotherapy.

Funding: NHMRC, MRFF, INOVIQ.

Poster Presentations

The details of the five presentations are as follows:

1. **Reproducibility and efficiency of an extracellular vesicle capture technology for the detection of ovarian cancer**
   Presentation/Poster Number: PT01-02
   Session Date: Thursday May 18, 2023
   Presentation Time: 16:45- 18:45 (GMT-7)
   Presenter: Dr Andrew Lai from University of Queensland

2. **High-throughput isolation and enrichment of extracellular vesicles using an immunoaffinity magnetic bead-based matrix**
   Presentation/Poster Number: PF15.04
   Session Date: Friday May 19, 2023
   Presentation Time: 16:00- 18:00 (GMT-7)
   Presenter: Dr Ramin Khanabdali from INOVIQ

3. **Tumor derived extracellular vesicles enrichment using a novel immunoaffinity magnetic bead-based matrix**
   Presentation/Poster Number: PF15.03
   Session Date: Friday May 19, 2023
   Presentation Time: 16:00- 18:00 (GMT-7)
   Presenter: Dr Greg Rice from INOVIQ

4. **Enrichment of extracellular vesicles from human plasma with molecular net matrix-coated magnetic beads (EXO-NET®)**
   Presentation/Poster Number: PT10-10
   Session Date: Friday May 19, 2023
   Presentation Time: 16:00- 18:00 (GMT-7)
   Presenter: Dr Olesia Gololobova from Johns Hopkins University

5. **EXO-NET enriched salivary small extracellular vesicles in periodontitis**
   Presentation/Poster Number: PS07.12
   Session Date: Saturday May 20, 2023
   Presentation Time: 16:50- 18:50 (GMT-7)
   Presenter: Dr Ramin Khanabdali from INOVIQ

Reproducibility and efficiency of an extracellular vesicle capture technology for the detection of ovarian cancer

Andrew Lai[1], Dominic Guanzon[1], Carlos Palma[2], Ramin Khanabdali[2], Lewis Perrin[3], John Hooper[3], Jim Coward[3], Gregory Rice[2], Carlos Salomon[1]

1 Translational Extracellular Vesicles in Obstetrics and Gynae-Oncology Group, University of Queensland Centre for Clinical Research, The University of Queensland, St Lucia, QLD, Australia
2 Inoviq Limited, Notting Hill, Australia
3 Mater Research Institute-University of Queensland, Translational Research Institute, Woolloongabba, Australia

INTRODUCTION

Ovarian cancer (OVCA) continues to present significant challenges despite numerous advances in treatments over recent decades. Epidemiological studies reveal a marginal impact on the disease's course, primarily due to the absence of reliable early detection methods. Current extracellular vesicle (EV) isolation techniques are laborious and often struggle with maintaining quality control.

In this study, we assess the performance and reproducibility of EXO-NET®, an innovative EV capture technology, for characterizing a range of EV biomarkers associated with ovarian cancer.

EXPERIMENTAL METHODS

Figure 1: EXO-NET® enrichment of EVs from biological fluids (ie plasma)

- To evaluate the efficiency of EV capture by EXO-NET®, commercially available EVs were spiked into PBS, and both particle size and concentration were monitored before and after EXO-NET® treatment.
- Titration experiments were conducted using increasing volumes of plasma (50 to 500 µL) added to a fixed volume of EXO-NET®.
- To assess the reproducibility of the isolation procedure, EXO-NET® was employed to repeatedly isolate a pooled sample (n=6) from multiple aliquots, and the inter-assay coefficient of variation (CV) was calculated. The intra-assay CV was determined from the analysis of 529 serum/plasma samples (ovarian cancer + control) that were measured in duplicate.

RESULTS

- We found that EXO-NET® was able to capture 47% and 20% of the total sEV (**Figure 2A**) and mEV (**Figure 2B**) respectively.
- Titration experiments using increasing volumes of plasma (50 - 500 µL) combined with a fixed volume of EXO-NET® reveal the optimal sample:EXO-NET® ratio as 200 µL plasma: 30 µL EXO-NET® (**Figure 3, 4**).
- The isolation process is reproducible, with a inter-assay coefficient variation of <10 % (**Table 1**).
- Western blot analysis targeting CD9 demonstrates that the recovery of CD9 is EXO-NET dependent, with the highest intensity observed at 10x (30 µL). CD9 was weakly detected in samples isolated using size-exclusion chromatography and ultracentrifugation (**Figure 5**).

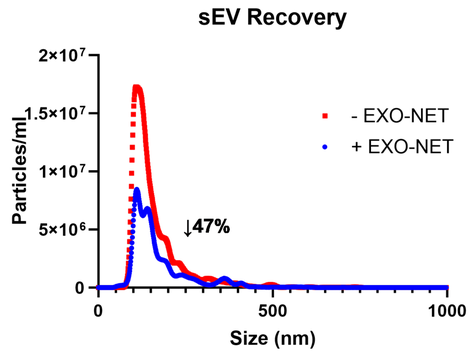

Figure 2A: Assessment of small EV (sEV) recovery. Purified sEVs were diluted in PBS prior to EV isolation. Nanoparticle tracking analysis was used to evaluate particle size and concentration in the supernatant both before and after EXO-NET treatment.

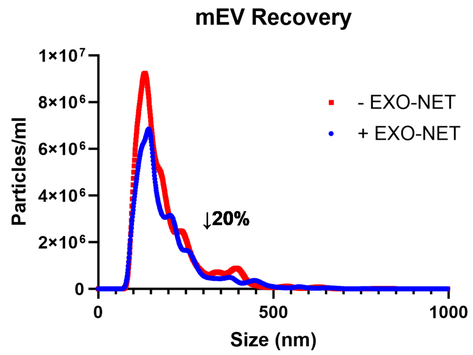

Figure 2B: Determination of medium EV (mEV) recovery. Purified mEVs were diluted in PBS prior to EV isolation. Nanoparticle tracking analysis was used to evaluate particle size and concentration in the supernatant both before and after EXO-NET® treatment.

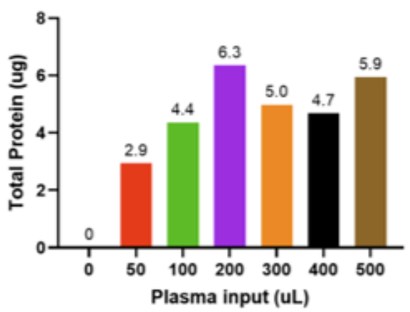

Figure 3: Optimisation of plasma volume for exosome isolation using EXO-NET® (30 ul): a titration study from 50 to 500 ul plasma followed by protein extraction with RIPA buffer and quantification via BCA assay.

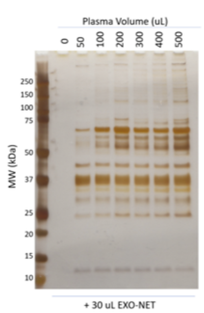

Figure 4: Protein profile of isolated EVs. Lysates obtained from EVs isolated from increasing plasma volumes were separated using SDS-PAGE and stained using silver.

RESULTS (CONT.)

	Inter-Assay CV		Intra-Assay CV
	Repeat	Repeat 2	
Pooled rep #1	148.437	160.855	
Pooled rep #2	192.833	185.532	
Pooled rep #3	210.189	196.44	
Pooled rep #4	198.804	186.911	
Pooled rep #5	171.365	184.153	
Pooled rep #6	171.675	187.006	
	Average	182.85	
	SD	17.19877	Intra-assay CV (n=529 samples, duplicates)
	% CV	**9%**	**8%**

Table 1: Reproducibility of EV enrichment. The inter-assay coefficient of variation was determined by analyzing the protein concentrations of the positive control, which consisted of pooled plasma repeatedly isolated using the EXO-NET® method. Meanwhile, the intra-assay CV was calculated by taking the average of the CVs obtained for each sample across two independent protein assays

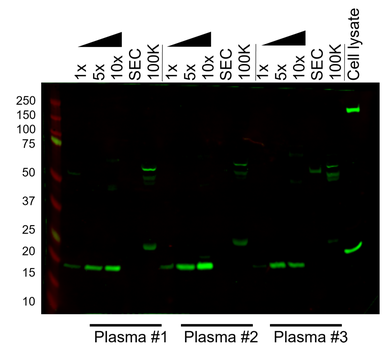

Figure 5: Comparison of EXO-NET with other isolation techniques. The figure contrasts the use of 200 µL of plasma and increasing volumes of EXO-NET® (1x = 3 µL, 5x = 15 µL, 10x = 30 µL) to size-exclusion chromatography (SEC) and ultracentrifugation methods for EV isolation.

CONCLUSIONS

- EXO-NET® can be reliably employed for the isolation of Extracellular Vesicles (EVs) with consistent results.
- It may be necessary to conduct titration experiments to ascertain the optimal sample to EXO-NET® ratio.

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High-throughput isolation and enrichment of extracellular vesicles using an immunoaffinity magnetic bead-based matrix

Ramin Khanabdali[1], Carlos Palma[1], Sara Nikseresht[1], Siena Barton[1], Khairul Ansari[1], Sukhdeep Spall[2,3], Laura F Dagley[2,3], Susan Belzer[1] and Gregory Rice[1]

[1]INOVIQ Limited, Notting Hill, VIC, 3168, Australia; [2]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia; [3] Department of Medical Biology, University of Melbourne, Melbourne, Victoria, Australia

INTRODUCTION

Extracellular vesicles (EVs) have significant potential for both diagnostic and therapeutic applications. The lack of standardized methods for efficient and high-throughput isolation and analysis of specific subpopulations of EVs, however, has limited their use as routine diagnostic applications. Here we present a novel bead-based immunoaffinity system (EXO-NET®) that captures a highly enriched subpopulation of EVs. EXO-NET is 3-D monoclonal antibody matrix constructed on magnetic bead-based immunoaffinity for isolation and enrichment of EVs.

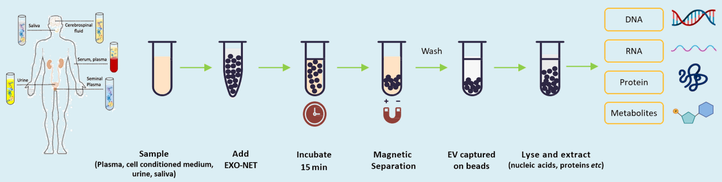
AIMS

The aims of this study were:

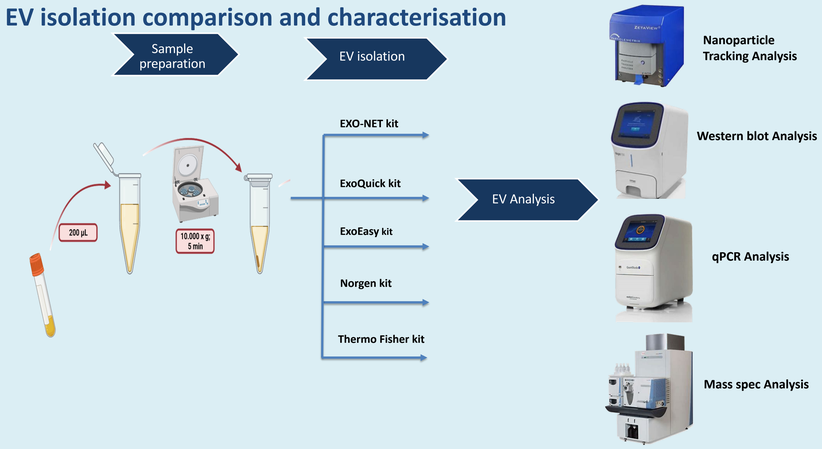
(i) To isolate and characterise EVs from different biofluids including plasma, saliva and cell conditioned medium using EXO-NET

(ii) To evaluate and compare EXO-NET with different EV isolation kits in term of EV yield and downstream analysis.

METHODS



EV isolation comparison and characterisation



EXO-NET EV isolation from different biofluids

EXO-NET Plasma-EV Isolation and Characterisation

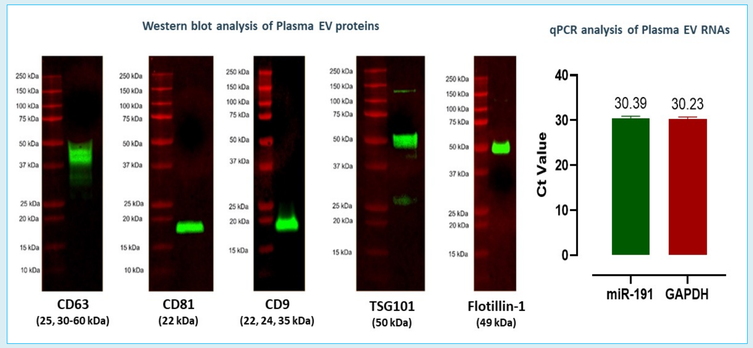


Figure 1 Western blot analysis confirmed the presence of CD63, CD81, CD9, TSG101 and Flotillin-1. qPCR analysis demonstrated that EXO-NET isolated EVs had high yield of mRNA (GAPDH) and microRNA (miR-191).

EXO-NET Cell Conditioned Medium-EV Isolation and Characterisation

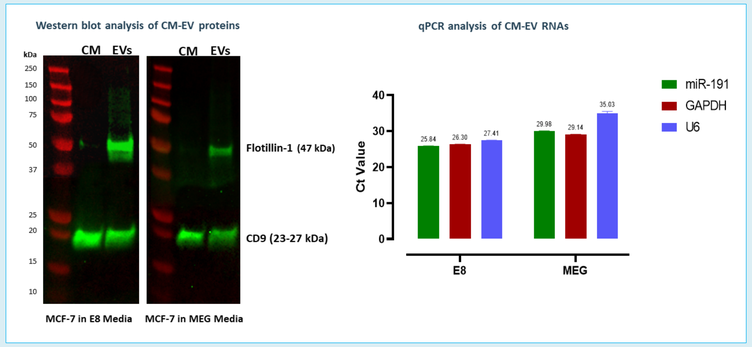


Figure 2 Western blot analysis confirmed the presence of CD9 and Flotillin-1 in EXO-NET isolated EVs from MCF-7 cell conditioned media (CM), grown in both E8 and MEG. qPCR analysis further demonstrated that EXO-NET-isolated EVs had higher yield of mRNA (GAPDH) and microRNA (U6 and miR191) in E8-CM than MEG-CM .

EXO-NET Saliva-EV Isolation and Characterisation

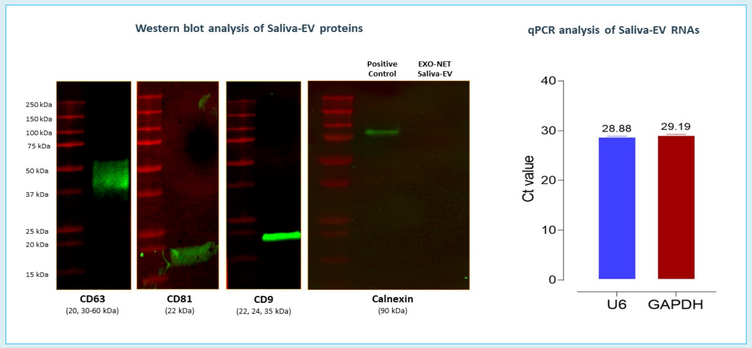


Figure 3 Western blot analysis confirmed the presence of CD63, CD81 and CD9. Calnexin (EVs negative marker) was not detectable. qPCR analysis demonstrated that EXO-NET isolated EVs had high yield of mRNA (GAPDH) and microRNA (U6) in isolated EVs from saliva.

EV Isolation Comparison Analysis

ZetaView Nanoparticle Comparison Analysis

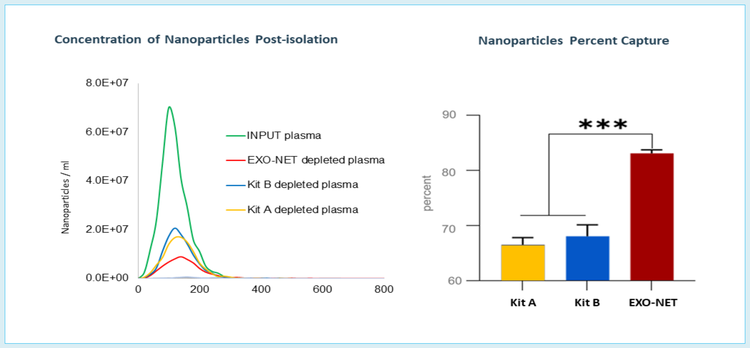


Figure 4 ZetaView analysis demonstrated that EXO-NET captured more EVs (~80%) than two other EV isolation kits. EVs were isolated from 500 µL pooled normal human plasma by EXO-NET and two other bead-based kits (Kit A and Kit B) according to manufacturers' instructions. The depleted and input plasma was analysed using ZetaView system.

qPCR Analysis-RNAs and microRNAs Comparison

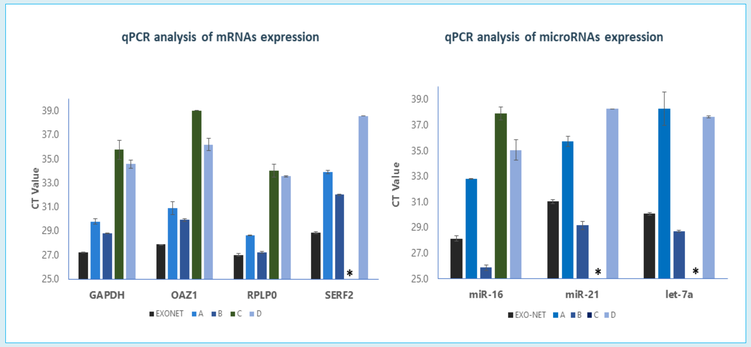


Figure 5 EVs were isolated from pooled normal human plasma by EXO-NET and 4 commercial kits according to manufacturers' instructions. Isolated EVs were lysed and total RNA were extracted for qPCR analysis to measure both microRNAs and mRNAs. EXO-NET delivers equivalent or higher recovery of both microRNAs and mRNAs compared to other 4 commercial EV isolation kits.

Mass Spectrometry Analysis-Protein Comparison

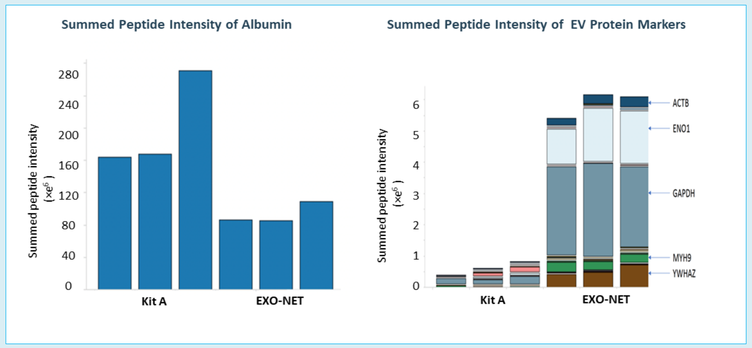


Figure 6 Mass spectrometry analysis showed that albumin peptide intensity in Kit A was 2-fold higher than that EXO-NET isolated EVs. The EV peptides intensity in Kit A was 6 to 10-fold less than that observed in EXO-NET isolated EVs. These data are consistent with EXO-NET delivering a higher enriched preparation of EVs than other EV isolation kit.

CONCLUSIONS

These results establish that EXO-NET provides an effective solution for the isolation of a surface-epitope defined subpopulation of EVs and the downstream analysis of both protein and nucleic acid biomarkers. The EXO-NET kit is a novel method for the rapid, efficient and scalable enrichment and purification of EVs that reduces contaminants which may confound downstream analysis.

Tumor-derived extracellular vesicles enrichment using a novel immunoaffinity magnetic bead-based matrix

Ramin Khanabdali[1], Sara Nikseresht[1], Khairul Ansari[1], and Gregory Rice[1,2]

(1) INOVIQ Limited, Notting Hill, VIC, 3168, Australia(2) Centre for Clinical Research, The University of Queensland, Brisbane, QLD 4006, Australia

INTRODUCTION

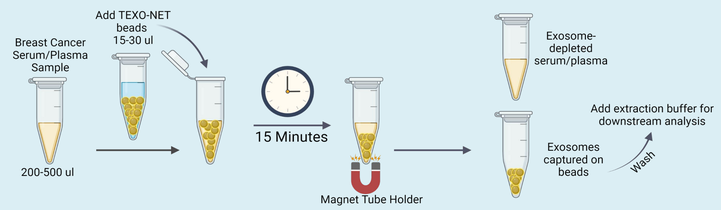
Neoplastic transformation is often associated with aberrant glycosylation. Changes in cell-surface sialylated glycans promote tumor growth, metastasis and immune evasion. Preferential incorporation of glycolylneuraminic acid (Neu5Gc) in tumor-derived glycoconjugates is a feature of many cancer biomarkers. Extracellular vesicles (EVs) released by cancer cells express tumor-specific epitopes on their surface, including Neu5Gc-glycans. SubB2M is a site-directed mutation of the B subunit of subtilase cytotoxin that has enhanced affinity for Neu5Gc. As Nue5Gc in not synthesized by human somatic cells, SubB2M may be of utility in discriminating between cancer-derived and non-cancer EVs.

AIMS

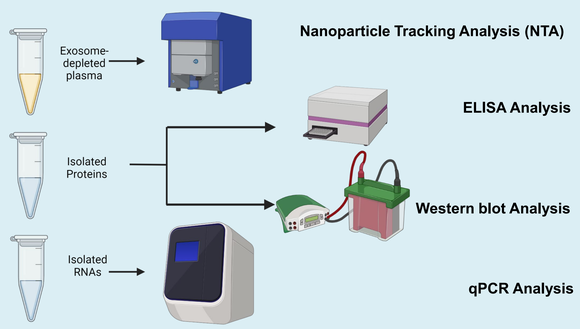
The aim of this study was to evaluate the utility of SubB2M immobilized on paramagnetic nanoparticles (TEXO-NET) to isolate Neu5Gc enriched subpopulation of tumor-derived EVs for diagnostic application.

METHODS

TEXO-NET EVs isolation workflow



Characterization of TEXO-NET Isolated EVs:



RESULTS

TEXO-NET isolated plasma EVs

ZetaView nanoparticle tracking analysis and percent capture of EVs

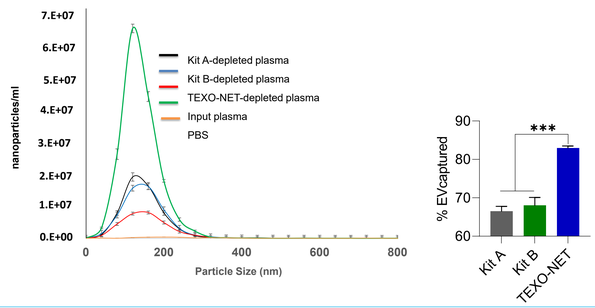


Figure 1. EVs were isolated from pooled breast cancer plasma (500 µL) using 30 µL of TEXO-NET (n=3) or according to manufactures' instructions (Kit A and Kit B). **Panel A:** Plasma nanoparticle concentration before (green) and after EV isolation using TEXO-NET (red), Kit A (black) and Kit B (blue), as quantified by ZetaView analysis. **Panel B**. Percent of nanoparticle captured. TEXO-NET captured a significantly greater percentage of nanoparticles (83%) from breast cancer plasma compared with two other magnetic bead-based EV isolation kits, 67% and 68%, respectively.

Western blot analysis of EV-associated CA 15-3 protein from serum

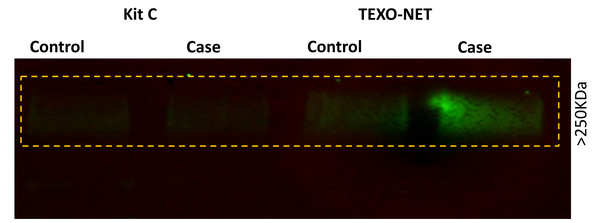


Figure 4. EVs were isolated from 500 µL pooled breast cancer serum and match controls using TEXO-NET and a pan EV isolation kit (Kit C). Captured EVs were lysed and total protein extracted and quantified. Western blot was perform using normalized protein concentration for detection of CA 15-3. CA15-3 signal intensity was greater in TEXO-NET isolated EV than in Kit C. TEXO-NET associated CA15-3 signal discriminated between case and control samples.

TEXO-NET capture of EVs is blocked by pre-incubation with Neu5Gc

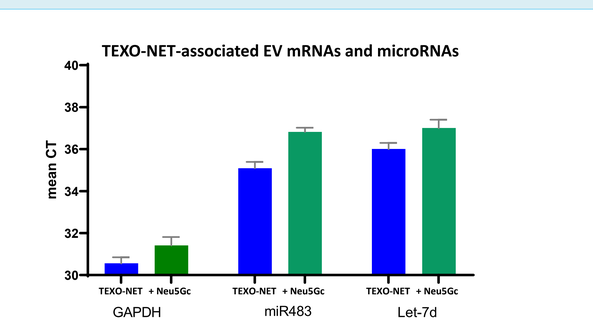


Figure 2. Mean CT values for EV-associated GAPDH, miR483 and Let-7d. TEXO-NET was preincubated with or without 1 mM Neu5C or Neu5Ac for 15 min, washed with PBS four times and then 30 uL of the beads were used to capture EVs from 500 uL of normal human Plasma. The captured EVs were subjected to RNA extraction and qPCR analysis.

CA-15-3 ELISA and protein concentration of EV-associated CA15-3

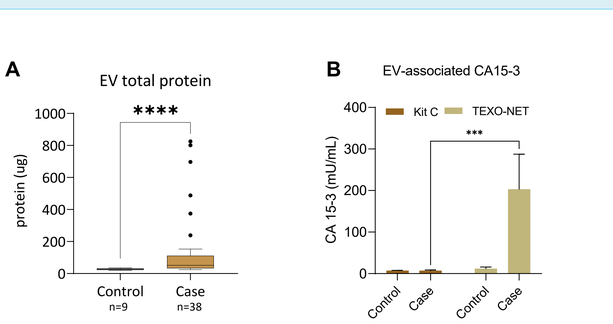


Figure 5. EVs were isolated from 500 µL pooled breast cancer serum and match controls by TEXO-NET and a pan EV isolation kit (Kit C). Panel A: Total EV protein isolated from control and breast cancer serum. Panel B: EV-associated CA15-3 quantified by ELISA. Significantly higher concentrations of CA 15-3 were recovered in TEXO-NET isolated EVs than Kit C. No significant difference between case and control CA15-3 concentration was observed in EV isolated using Kit C.

Neu5Gc releases EVs from TEXO-NET

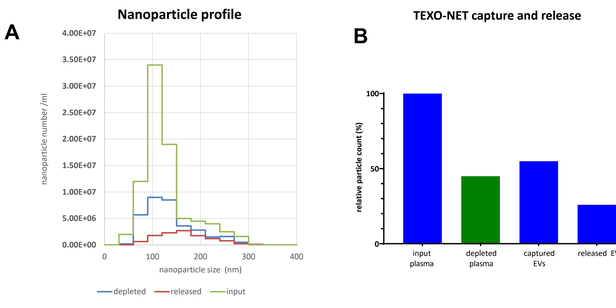


Figure 3. Neu5Gc release of EVs from TEXO-NET. Plasma (500uL) was incubated with 30uL TEXO-NET from 15 min. Panel A: The concentration and size of EVs present in plasma before and after incubation with TEXO-NET and following incubation with 1 mM Neu5Gc for 1 min were determined by ZetaView analysis. Panel B: Percent of EVs captured and released from TEXO-NET.

Neu5Gc releases EVs from TEXO-NET

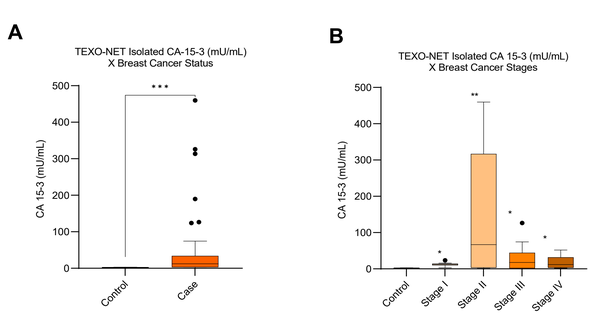


Figure 6. EV-associated CA15-3 isolated from serum obtained from healthy women and women with breast cancer (Stages I-IV). EVs were isolated from 200 µL of serum using 15µL of TEXO-NET. Panel A: EV-associated CA15-3 was significantly greater in case (n=39) than control samples (n=9, p <0.001). (B) EV-associated CA15-3 varied significant by disease stage (p< 0.05, Kruskal-Wallis test, control n=8, stage 1 n=9, stage 2 n=10 stage 3 n=10, stage 4 n=9).

CONCLUSIONS

The data obtained confirm that TEXO-NET captures a subpopulation of EVs that is enriched with tumor-specific biomarkers and can be used in the downstream analysis and may be of utility in the identification of informative diagnostics biomarkers of tumor onset, progression, triage to treatment, and treatment response.

Enrichment of extracellular vesicles from human plasma with molecular net matrix-coated magnetic beads (EXO-NET®)

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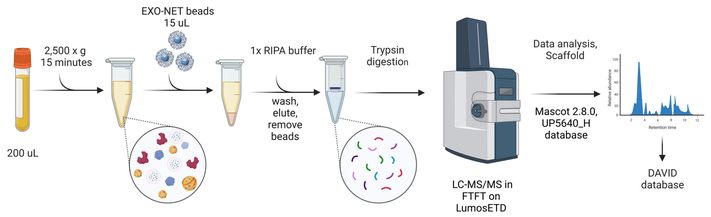
Introduction

Separating extracellular vesicles (EVs) from human plasma can be challenging. EVs are at low concentration in plasma compared with, e.g., lipoprotein particles, which further overlap in size and density with EVs. To separate EVs, methods such as differential centrifugation, ultrafiltration, size exclusion chromatography (SEC), and combinations thereof are time-consuming and may be unsuitable for clinical settings. There is an urgent need for a fast, robust, and simple method for enriching vesicles in a one-step process for downstream analysis. Here we present proteomics data from blood plasma-derived EVs separated using an affinity matrix magnetic nanoparticle technology (EXO-NET, INOVIQ Ltd).

Methods



EVs were separated from 200 µL of a healthy donor plasma pool using EXO-NET, following the manufacturer's instructions. On-bead lysis with RIPA buffer was used to recover EV-associated proteins. The samples were then treated with chemicals (DTT and Iodoacetomide) and purified using SP3 beads. The purified samples were enzymatically digested using trypsin on SP3 beads. The resulting peptides were purified using Oasis u-HLB plates and analyzed using LC-MS/MS (liquid chromatography-tandem mass spectrometry) on a LumosETD instrument. The instrument settings included a high-resolution scan for precursor ions and a lower-resolution scan for fragment ions. We used data-dependent acquisition (DDA). The acquired MS2 spectra were then searched against a human protein database (UP5640 _H.sapiens) using the Mascot software. The output files from Mascot were compiled in Scaffold for data analysis.

The DAVID knowledgebase (version 2022q3) was used as a reference for further interpretation and annotation of the identified proteins.

Results

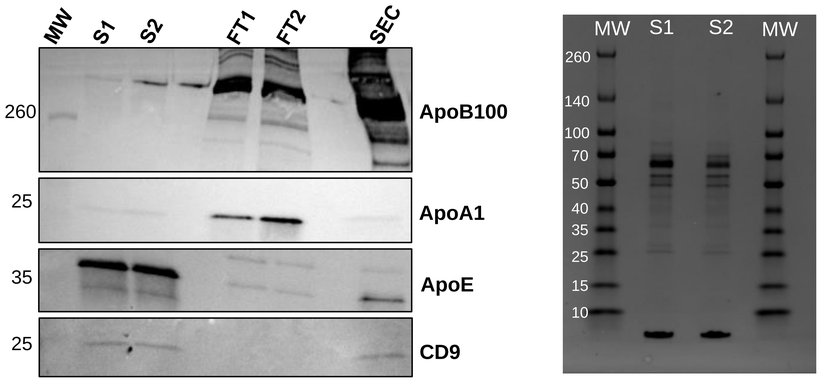


Fig.1: (LEFT) Equal volumes of two independent EXO-NET-separated samples (S1, S2), corresponding flow-through samples (FT1, FT2), and size-exclusion chromatography (SEC) fractions were analyzed by SDS-PAGE and Western blot for EV marker CD9, LDL marker ApoB100, HDL marker ApoA1, and lipoprotein marker ApoE. **(Right)** Image of Coomassie blue-stained EXO-NET-separated samples.

Protein	Protein ID
angiogenin	ANG
cholesteryl ester transfer protein	CETP
coagulation factor XI	F11
complement factor H related 5	CFHR5
cystatin C	CST3
EGF containing fibulin extracellular matrix protein 1	EFEMP1
fetuin B	FETUB
insulin like growth factor 2	IGF2
insulin like growth factor binding protein 2	IGFBP2
insulin like growth factor binding protein 4	IGFBP4
insulin like growth factor binding protein 5	IGFBP5
joining chain of multimeric IgA and IgM	JCHAIN
keratin 71	KRT71
keratin 72	KRT72
keratin 82	KRT82
mannosidase alpha class 1A member 1	MAN1A1
multimerin 2	MMRN2
plakophilin 1	PKP1
SAA2-SAA4 readthrough	SAA2-SAA4
ST6 beta-galactoside alpha-2,6-sialyltransferase 1	ST6GAL1
suprabasin	SBSN
tissue factor pathway inhibitor	TFPI
transforming growth factor beta induced	TGFBI
tsukushi, small leucine rich proteoglycan	TSKU

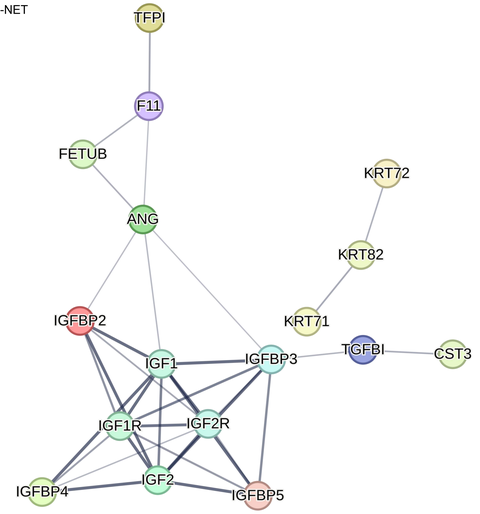


Fig.2: **(Left)** 199 proteins were analyzed and compared with proteins reported in Vesiclepedia for plasma EVs only [3]. 25 proteins were identified in the experiment which are not present in the database **(Right).** Network of new proteins analyzed in STRING [4] **(Left).**

Results

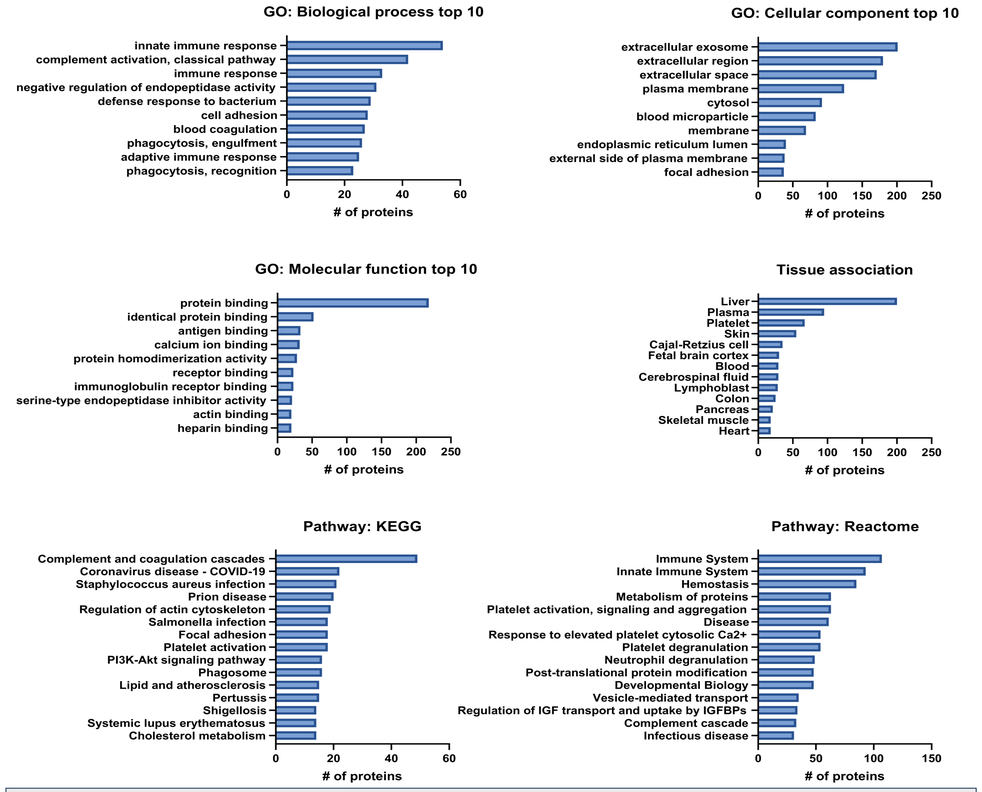


Fig.3: Protein annotation result based on DAVID knowledgebase: Gene ontology (GO), tissue expression (Up_Tissue), and pathway analysis (KEGG and Reactome).

Reference

1. B.T. Sherman, et all. DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Research*. 2022. doi:10.1093/nar/gkac194
2. D.W. Huang, et all. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc.* 2009;4(1):44-57.
3. H. Kalra, et al. Vesiclepedia: A compendium for extracellular vesicles with continuous community annotation. *PLoS Biology*. 2019; 12, e1001450.
4. D. Szklarczyk, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019;47(D1):D607-D613. doi: 10.1093/nar/gky1131

Conclusions

The EXO-NET platform may provide a simple and rapid enrichment EVs from human plasma, especially for clinical settings, offering minimal co-isolation of lipoproteins and a one-step process.

EXO-NET enriched salivary extracellular vesicles in periodontitis

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INTRODUCTION

Salivary extracellular vesicles (EVs) have emerged as promising candidates for the diagnosis of periodontitis, a common chronic inflammatory oral disease that is associated with microbial dysbiosis and host immune response. Information on the isolation and characterization methods of saliva EVs, as well as the use of EV cytokines cargo as biomarkers for periodontitis, however, remains limited.

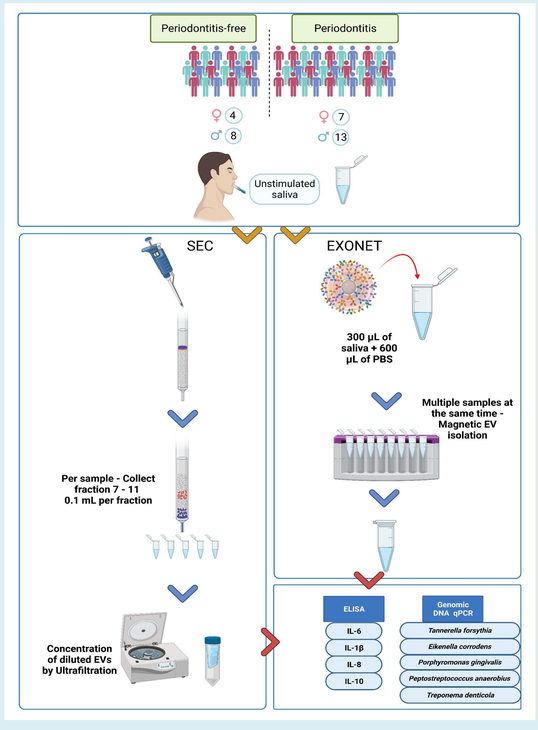
AIMS

The aims of this study were:

(i) to compare the enrichment of salivary EVs from 12 periodontitis-free patients using size exclusion chromatography (SEC) and bead-based immunoaffinity capture (EXO-NET®).

(ii) To assess the differential expression of EXO-NET enriched EVs associated inflammatory cytokines between individuals with (n=20) and without periodontitis (n=12).

METHODS



RESULTS

EXO-NET Saliva-EV Characterisation

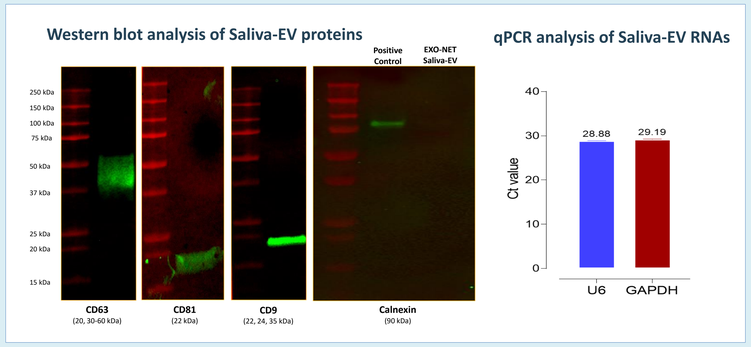


Figure 1. EXO-NET saliva EV isolation and characterisation. Western blot analysis confirmed the presence of CD63, CD81 and CD9. Calnexin (EVs negative marker) was not detectable. qPCR analysis demonstrated that EXO-NET isolated EVs had high yield of mRNA (GAPDH) and microRNA (U6) in isolated EVs from saliva.

Periodontal pathogen detection in EVs by SEC and EXO-NET

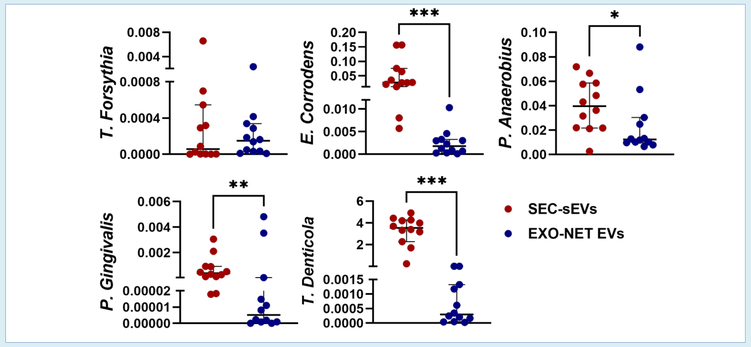


Figure 3. The expression of bacterial genes in EVs isolated from saliva using SEC and EXO-NET. Gene expression related to *Eikenella corrodens, Porphyromonas gingivalis, Peptostreptococcus anaerobius* and *Treponema denticola was significantly lower in* EXO-NET compared with SEC EVs (median ± 95% CI. *p < 0 .05, **p < 0 .002, ***p < 0 .0001). No differences were observed in expression for *Tannerella forsythia* between both methods.

Saliva EV isolation and characterisation by EXO-NET vs SEC

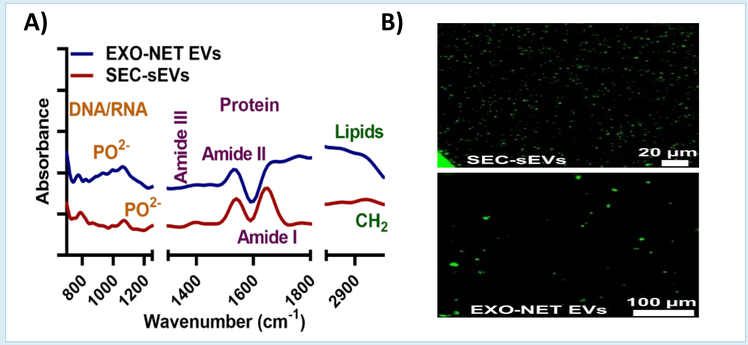


Figure 2. Biomolecular characterisation of EV isolated from saliva using EXO-NET and SEC. (A) Using Fourier transform infrared spectroscopy, EXO-NET isolated EVs displayed higher DNA/RNA PO^{2-} bonds at 1000-1200 cm^{-1}; protein of Amide I, II, III at 1650, 1550, and 1350 cm^{-1}, respectively; and lipid of CH2 around 1420, 2880, and 2920 cm^{-1}. (B) Scanning laser confocal microscope imaging of isolated saliva EV by both EXO-NET and SEC.

Inflammatory cytokines detection in EXO-NET enriched EVs for Periodontitis

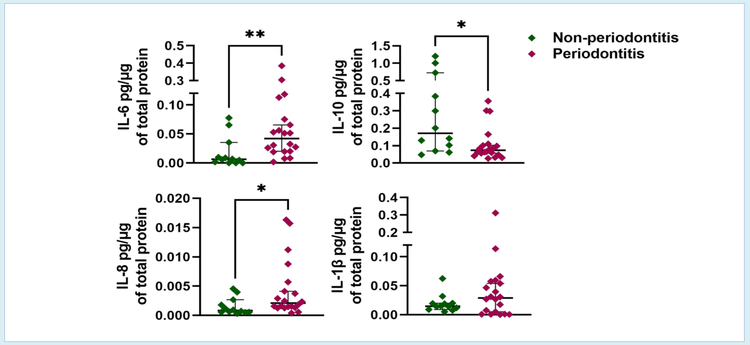


Figure 4. Evaluation of different inflammation-related cytokines between periodontitis and non-periodontitis in saliva isolated EV by EXO-NET. EVs isolated from patients with periodontitis had significantly higher expression of IL-6, IL-10 and IL-8 compared with non-periodontitis controls. No differences were observed between non-periodontitis and periodontitis group for IL-1β. (median ± 95% CI. *p < 0 .05, **p < 0 .002)

CONCLUSIONS

EXO-NET isolated EVs from periodontitis patients showed higher expression of pro-inflammatory cytokines compared to SEC method. These results indicate that EXO-NET is a fast and reliable method for enriching salivary EVs without contamination from bacteria-derived EVs or DNA. EXO-NET can be used as a high throughput method for biomarker discovery in periodontitis.