Title

Syncytiotrophoblast 5'-tRNA fragments are placental endocrine signals contributing to sterile inflammation in preeclampsia

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23 Abstract

24 The relationship between placental pathology and the maternal syndrome of
25 preeclampsia is incompletely characterised. Mismatch between placental nutrient
26 supply and fetal demands induces stress in the syncytiotrophoblast, the layer of
27 placenta in direct contact with maternal blood. Such stress alters the content and
28 increases the release of extracellular vesicles into the maternal circulation. 5'-tRNA
29 fragments (5'-tRF) constitute the majority of short RNA in these vesicles. Here we
30 characterise 5'-tRF profiles in syncytiotrophoblast vesicles in preeclampsia, reporting
31 differential expression of >900 fragments. We detected preeclampsia-dysregulated
32 5'-tRF in peripheral plasma, where we identified a placentally-derived load. We
33 demonstrated these 5'-tRF induce sterile inflammation in macrophages and
34 monocytes. Our findings suggest syncytiotrophoblast-derived 5'-tRF may contribute
35 to distant vascular inflammation, previously described in preeclampsia. This is the
36 first demonstration of endocrine actions of vesicle-bound 5'-tRF, which may have
37 relevance in other disciplines studying their functions, such as immunology and
38 cancer biology.

39 Introduction

40 Preeclampsia is a complex placental syndrome, with multiple causes and a variable
41 phenotype. Clinically it is characterised by maternal sterile inflammation and
42 endothelial dysfunction[1,2]. A point of convergence in the disorder is stress in the
43 syncytiotrophoblast, the interface between maternal and fetal circulations[3]. The link
44 between syncytiotrophoblast stress and maternal symptoms, likely through blood-
45 borne factors, is incompletely characterised[4]. Angiogenic proteins (soluble fms-like
46 tyrosine kinase [sFlt-1] and placental growth factor [PlGF]) are important
47 syncytiotrophoblast stress signals which contribute to the preeclamptic syndrome[5].
48 Excess sFlt-1 sensitises endothelial cells to pro-inflammatory cytokines[6]. These
49 biomarkers have also been successfully applied to the clinical diagnosis of
50 preeclampsia[7]. Altered extracellular vesicle [EV] release from the
51 syncytiotrophoblast, whilst also a key contributor in the pathophysiology of
52 preeclampsia, remains underexplored[8].

53

54 The healthy syncytiotrophoblast releases EV into the maternal circulation in
55 increasing quantities with advancing gestation[9]. These lipid bilayer-bound particles
56 are decorated with surface proteins and shuttle their contents to distant cells[10,11].
57 Cellular stress increases EV release; this is reflected in preeclampsia where
58 circulating placental EV are more abundant[12,13]. Placental EV cargoes also change
59 in preeclampsia; for example nitric oxide synthase is reduced and neprilysin (a
60 metalloprotease causing hypertension) is increased[14,15].

61

62 We recently reported 5'-transfer RNA fragments [5'-tRF] as the predominant short
63 RNA species in healthy syncytiotrophoblast EV[16]. 5'-tRF form when mature tRNA

molecules are cleaved by a number of stress-induced ribonucleases including angiogenin[17,18]. They can be exported as EV cargo[19]. 5'-tRF expression profiles can be complex; at least 417 tRNA genes can be cleaved at multiple loci[20]. 5'-tRF are also multifaceted signalling molecules, regulating transcription, translation and epigenetic inheritance[21–24]. 5'-tRF have mostly been investigated in cancer biology and immunology where they are described as intracellular, autocrine and paracrine signals[19,25,26].

5'-tRF are stress signals; syncytiotrophoblast stress is a key feature of preeclampsia. We hypothesised that syncytiotrophoblast 5'-tRF release may change in preeclampsia. We used placental perfusion as a source of syncytiotrophoblast EV to show that 5'-tRF expression in preeclampsia differs from normal pregnancy. Our in-vivo work demonstrates circulating placentally-derived 5'-tRF. Cell culture studies determined preeclampsia-dysregulated 5'-tRF trigger sterile inflammation in macrophages. Together these findings suggest 5'-tRF are placental endocrine signals, linking syncytiotrophoblast stress with maternal inflammation in preeclampsia.

81 Results

82 **The preeclamptic syncytiotrophoblast exports 5'-tRF in EV**

83 We isolated small extracellular vesicles (SEV) and medium-large EV (MLEV),
84 alongside placental biopsies from women with early-onset preeclampsia (n=8) and
85 matched healthy controls (n=6, Supplementary Table 1) using dual-lobe placental
86 perfusion[16]. We performed single-end short RNA sequencing (size selecting <50
87 nucleotides) using the Illumina HiSeq 4000 platform[27]. Sequence length distribution
88 plots confirmed the majority of reads in MLEV and SEV from both preeclampsia and
89 normal placentas were 30-34 nucleotides long (Figure 1A). In contrast a peak at 22
90 nucleotides was greater in the placental samples from both groups. The majority of
91 fragments in MLEV and SEV mapped to tRNA species, rather than to the microRNA
92 and ribosomal RNA species seen in placental tissues (Figure 1B). Coverage plots
93 demonstrated that 5'-tRF constitute almost all tRNA reads in EV in preeclampsia as
94 well as normal pregnancies (Figure 1C). Enrichment of 5'-tRF (but not 3' fragments)
95 in EV compared to cells of origin is consistent with studies in immune cells[19,26].

96

97 **5'-tRF are differentially expressed in preeclamptic syncytiotrophoblast EV**

98 We used a bespoke bioinformatics pipeline to investigate differential expression of
99 tRNA fragments. To minimise data loss, we assigned each unique RNA fragment an
100 identifier, then annotated fragments after differential expression analysis (see details
101 in Methods). We identified 983 differentially expressed short RNA fragments in
102 preeclampsia MLEV compared to controls; 182 mapped to 5'-tRF using GtRNAdb
103 2.0[28]. No fragments were found to be differentially expressed in preeclampsia SEV.
104 This is likely consistent with the different biogenesis of smaller EV, which are
105 released constitutively via the endosomal pathway[29].

106

107 The 12 most abundant differentially expressed fragments are shown in Table 1 and
108 Figure 2A. Using the sum of preeclampsia MLEV normalised counts as a
109 denominator, these 12 fragments account for 64% of the differentially expressed
110 counts. 5% of these counts were accounted for by the 626 least abundant fragments.
111 Thus, a small number of abundant fragments represented the majority of the signal
112 in an otherwise complex dysregulated short RNA profile in preeclampsia MLEV.
113
114 Comparing to human small RNA databases, among the 12 most abundant
115 differentially expressed fragments, 3 were identified as 5'-tRF and 4 as piwi-
116 interacting RNA. The remaining 5 had no directly matched reference sequences in
117 human small RNA databases and are referred to as "unmatched". Review of
118 fragment sequences demonstrated overlap; indeed all 12 showed only one or two
119 nucleotide insertions or deletions differentiating them from known 5'-tRF (differences
120 highlighted in Table 1). A complex profile of differential expression in preeclampsia
121 MLEV was thus dominated by minor variants of a handful of abundant 5'-tRNA
122 fragments.
123
124 We sought target 5'-tRF which were differentially expressed in preeclampsia EV to
125 validate our findings *ex-vivo* (in placental perfusion samples) and *in-vivo* (in plasma).
126 5'-tRF are known to be expressed in other circulating EV, most notably from immune
127 cells. The most abundant EV in plasma are blood-cell derived[30]. Hence, we used
128 publicly available blood-cell datasets to quantify possible target 5'-tRF likely to be
129 within contaminating EV in plasma (Supplementary Table 2). Using these data we
130 selected three targets for validation which were: abundant in syncytiotrophoblast EV

131 (above 90th centile PE expression amongst 983 differentially expressed fragments),
132 upregulated in preeclampsia and of low relative abundance in potentially
133 contaminating EV source cells. The relative abundance of these three targets in
134 STBEV using RNAseq is shown in Figure 2B (5'-tRF targets named A, B and C for
135 brevity but full sequences and tRNA derivations shown in Supplementary Table 2).
136
137 We used a custom small RNA assay with stem-looped reverse transcription primers
138 and a specific minor-groove binding TaqMan™ probe to compare relative 5'-tRF
139 expression in placental EV obtained by perfusion using quantitative real-time
140 polymerase chain reaction. Results validated target 5'-tRF upregulation and were
141 consistent with RNAseq findings, showing a median of 1.47 fold upregulation in
142 preeclampsia (Figure 2C).
143
144 **Plasma EV 5'-tRF are upregulated in pregnancy and placentally-derived**
145 Total EV RNA were isolated from maternal plasma using membrane-affinity columns
146 (EV size profiles for plasma compared to perfusion MLEV are shown in
147 Supplementary Figure 1; extensive characterisation of plasma membrane-affinity EV
148 has previously been published[31]). Target 5'-tRF were significantly more abundant in
149 pregnant peripheral plasma EV than in non-pregnant matched control samples
150 (median 2.8 fold higher in pregnancy, $p<0.05$ for all three) (Figure 3A). The
151 difference between these sample groups suggested a pregnancy-specific load of 5'-
152 tRF in plasma EV. The high abundance of these 5'-tRF in non-pregnant samples
153 confirms they were not unique to pregnancy (median Cq values in non-pregnant
154 samples: A 24.0, B 27.9, C 25.9). We then acquired paired plasma samples
155 simultaneously from the uterine and peripheral veins of women without preeclampsia

156 undergoing elective caesarean section for an indication unrelated to preeclampsia
157 (e.g. breech presentation). The uterine vein directly receives blood from the
158 placenta, thus placentally-derived molecules are typically more abundant in these
159 samples (Figure 3B)[32,33]. All three 5'-tRF were more abundant in uterine vein
160 samples (median 1.4 fold, $p<0.05$ for all three) (Figure 3C). These data support a
161 placentally-derived load of 5'-tRF in circulating plasma EV, consistent with our
162 hypothesis that syncytiotrophoblast 5'-tRF are endocrine signals.
163
164 **EV-bound preeclampsia-upregulated 5'-tRF promote macrophage, but not**
165 **monocyte activation**
166 5'-tRF can influence cellular function through a wide-range of regulatory mechanisms
167 at the level of transcription, translation and the epigenome[24]. Vascular inflammation
168 is a key feature of preeclampsia[34]. Tissue-resident macrophages are known to be
169 activated in preeclampsia[35]; recent data suggest syncytiotrophoblast EV contribute
170 to this[36,37]. We treated monocytes and macrophages with the most abundant 5'-tRF
171 upregulated in preeclampsia (labelled A – full sequence in Supplementary Table 2),
172 contained within otherwise undecorated lipid vesicles (in order to distinguish the
173 effect of one tRF from the accompanying RNA, lipids and proteins in perfusion-
174 derived EV). We used a scrambled version of the most abundant STBEV 5'-tRF
175 sequence as a negative control, following validation against untreated cells
176 (Supplementary Figure 2). Macrophages were activated to a type 1 phenotype after
177 12 hours treatment with tRF-A, increasing expression of pro-inflammatory cytokines
178 (Figure 4A). This pro-inflammatory 5'-tRF action was confined to macrophages, with
179 no changes observed when the same experiment was repeated in undifferentiated
180 monocytes (Figure 4B).

181

182 **EV-bound 5'-tRF may indirectly activate endothelial cells, reducing expression**

183 **of endothelial nitric oxide synthase**

184 Endothelial damage is considered a common factor in the wide-ranging maternal

185 organ dysfunction of preeclampsia[1,2]. Vascular macrophages are known to modulate

186 adjacent endothelial cell function[38,39]. We treated human umbilical vein endothelial

187 cells with vesicle-bound tRF-A and with the supernatants from activated

188 macrophages (cells which lie adjacent to endothelial cells in the maternal vessel

189 wall). tRF-A activated endothelial cells (quantified through increased expression of

190 adhesion molecules) indirectly through macrophage stimulation, but not directly

191 (Figure 4C).

192

193 The primary presenting feature of preeclampsia is high blood pressure. Immediate

194 blood pressure regulation involves constitutive release of nitric oxide from endothelial

195 cells, which maintains a state of relative vasodilation. In preeclampsia, circulating

196 nitrite levels are reduced, though the mechanism for this remains unclear[40]. tRF-A

197 reduces expression of endothelial nitric oxide synthase in endothelial cells, indirectly

198 via macrophage activation (Figure 4C). Thus aberrant placental EV 5'-tRF release in

199 preeclampsia may contribute to the maternal endothelial dysfunction and

200 hypertension which characterise the disease.

201 Discussion

202 5'-tRF have gained significant interest as signalling molecules in diverse disciplines:
203 from human biology to drosophila, yeasts and plants[41–43]. Most studies have used
204 cell culture as a model system. Work in breast cancer has identified intracellular 5'-
205 tRF release promoting metastasis[25]. Mycobacterium infection in human
206 macrophages has been shown to trigger local 5'-tRF release in EV, resulting in Toll-
207 like receptor 7 activation in neighbouring cells[26]. T cell activation can be inhibited by
208 blocking released 5'-tRF, suggesting autocrine and paracrine actions[19]. Pregnancy
209 provides a unique opportunity to investigate 5'-tRF at a whole-organ level. The
210 placenta is expelled with the fetus during parturition and can be studied intact *ex-*
211 *vivo*. Both our sequencing and qPCR data demonstrate 5'-tRF release from the
212 syncytiotrophoblast in extracellular vesicles, in both healthy and preeclamptic
213 pregnancies. Moreover, routine opening of the abdomen for caesarean section offers
214 additional *in-vivo* evidence to further assess discoveries from placental perfusion. By
215 sampling uterine and peripheral veins simultaneously, we have conclusively
216 demonstrated a 5'-tRF load in maternal plasma which is placentally-derived.
217 Together with functional data in macrophages which are found distant to the
218 syncytiotrophoblast, this provides compelling evidence for placental endocrine
219 signalling with 5'-tRF. This principle might be conserved across other organ systems
220 and species, with far-reaching implications for short RNA biology.
221
222 Mature tRNA molecules are cleaved by a number of ribonucleases, most notably
223 angiogenin, in response to various cellular stressors[18]. Preeclampsia is a
224 heterogeneous syndrome, unified by stress in the syncytiotrophoblast[4]. A change to
225 the 5'-tRF expression profile in syncytiotrophoblast EV is thus consistent with

226 existing models for preeclampsia pathophysiology. We note reports that angiogenin-
227 cleaved 5'-tRF have a 2-3 cyclic phosphate residue at their 3' end, meaning they are
228 not captured by conventional RNA sequencing[44]. Our approaches would only detect
229 3'-hydroxyl groups, suggesting syncytiotrophoblast 5'-tRF may be a product of other
230 ribonucleases.
231
232 There is evidence for a variety of regulatory functions for 5'-tRF[24]. This includes
233 binding argonaute proteins to regulate mRNA stability and affecting components of
234 the ribosomal machinery to impact translation. We found 5'-tRF-A directly activated
235 macrophages, but not monocytes or umbilical vein endothelial cells. We propose this
236 difference may be accounted for by phagocytosis of EV by macrophages, trafficking
237 to the endosomal compartment (usually free of nucleic acids) and activation of Toll-
238 like receptor 7 or 8[45]. This hypothesis is supported by published work demonstrated
239 a lack of macrophage response to unencapsulated 5'-tRF and warrants further
240 investigation[26]. Macrophages are not typically in direct contact with blood; however
241 in preeclampsia the endothelial barrier is disrupted[1]. Syncytiotrophoblast vesicles
242 contribute to this endothelial damage[46]. We suggest that circulating EV-bound 5'-tRF
243 might directly reach macrophages in the vessel walls in preeclampsia, where their
244 functional effect could contribute to the well-described sterile inflammation of the
245 disease. The close relationship between vascular macrophages and neighbouring
246 endothelial cells may indirectly implicate 5'-tRF in potentiation of the hypertension
247 and diffuse organ dysfunction which typify preeclampsia[38]. We found no direct effect
248 of the most abundant preeclampsia-dysregulated 5'-tRF on human umbilical vein
249 endothelial cells, yet syncytiotrophoblast EV are known to change endothelial cell
250 function[46]. This discrepancy may be attributed to the absence of other EV RNA and

251 proteins which are necessary to trigger or potentiate this effect; it may reflect our
252 decision to use umbilical vein endothelium as a model. We speculate that the direct
253 endocrine role for 5'-tRF in preeclampsia may extend beyond macrophages other to
254 cells in direct contact with maternal blood that are involved in maternal disease, such
255 as liver sinusoidal endothelium and pericytes. Investigating these alternative targets
256 is the topic of ongoing work in our laboratory.
257
258 High-profile studies have recently highlighted the importance of circulating RNA in
259 preeclampsia, reporting both transcriptomic and miRNA dysregulation[47–49]. The
260 placenta is both necessary and sufficient to cause preeclampsia, yet the majority of
261 cell-free plasma/serum RNA is non-placental[50]. By using placental perfusion as our
262 experimental model, we eliminated the confounding non-placental RNA in blood,
263 finding the majority of short RNA released into the maternal circulation are 5'-tRNA
264 fragments. Previous studies investigating syncytiotrophoblast EV short RNA have
265 used lower fidelity models to source EV (e.g. explants) and taken bioinformatic
266 approaches which disregard 5'-tRF data, despite reporting their presence[13,51]. Our
267 ongoing work also focuses on the optimisation of techniques to isolate placental EV
268 5'-tRF from complex biofluids such as plasma, with attention to their clinical
269 relevance. Other endocrine products of syncytiotrophoblast stress such as sFlt-1 and
270 PlGF have proved highly valuable as diagnostic markers in preeclampsia,
271 transforming the diagnostic approach at the bedside[7,52]. However, the relatively poor
272 positive predictive value of angiogenic markers makes the search for other
273 biomarkers of the stressed syncytiotrophoblast clinically important.
274

275 In summary, we demonstrate differential release of syncytiotrophoblast 5'-tRF
276 fragments into the maternal circulation in preeclampsia can trigger sterile
277 inflammation in potentially distant macrophages. We propose that 5'-tRF form
278 another piece of the jigsaw, contributing to an explanation for the transmission of
279 preeclampsia from a single organ (the placenta) to multiple distant diseased cells in
280 the unwell mother.

281 Methods

282 **1. Sample collection and storage**

283 This project was approved by the Central Oxfordshire Research Ethics Committee
284 (07/H0607/74 and 07/H0606/148). Participants provided informed written consent.
285 Preeclampsia was defined using the ISSHP classification. Placentas were obtained
286 at the time of caesarean section and perfused within 10 minutes of delivery. Uterine
287 vein samples were taken during caesarean section, just prior to uterine incision,
288 using a 21-gauge needle and syringe. Peripheral blood samples were taken from the
289 antecubital fossa using 21-gauge needle with vacutainer system into 4.5 ml sodium
290 citrate vacutainer tubes (BD Diagnostics, UK). Non-pregnant samples were from
291 females of reproductive age. Plasma was obtained by centrifugation at 1500 *g* for 15
292 minutes. Samples were processed within 30 minutes of collection, aliquoted and
293 stored immediately at -80°C.

294

295 **2. Placental perfusion**

296 Placentas from 8 women with early-onset preeclampsia and 6 normal pregnancies
297 were perfused using a well-established dual-lobe perfusion technique[53]. Maternal
298 perfusate was centrifuged at 10 000 *g* for 30 minutes to isolate medium-large
299 extracellular vesicles (MLEV). The supernatant was centrifuged at 150 000 *g* for 2
300 hours to isolate small extracellular vesicles (SEV). Biopsies of placental tissue were
301 taken from the maternal surface of a non-perfused lobe. EV were characterised
302 using Nanoparticle Tracking Analysis, transmission electron microscopy and
303 Western blotting as described previously[53].

304

305 **3. RNA sequencing**

306 RNA was isolated from MLEV, SEV and placental tissue using Total RNA
307 Purification Plus Kit (Norgen Biotek Corporation, Canada). After confirming RNA
308 quantity and integrity using Bioanalyser (Agilent Technologies, Germany), the same
309 amount of input RNA was loaded for library preparation using the NEBNext Multiplex
310 Small RNA library preparation kit (New England Biolabs, USA). Libraries were size-
311 selected for fragments 15-50 bp by gel electrophoresis; fragment size and
312 concentration was confirmed using High Sensitivity D1000 ScreenTape (Agilent,
313 UK). Single-end sequencing by synthesis was undertaken using an Illumina HiSeq
314 4000 machine (Illumina, USA).
315
316 Sequence reads were analyzed using sRNAnalyzer[54]. Briefly, sequencing adaptors
317 and low-quality reads were removed using Cutadapt[55]. All identical reads in
318 sequence reads were collapsed, thus generating a set of unique reads (referred to
319 as fragment ID in this study). The number of sequence reads attributed to a fragment
320 ID were defined as the raw expression level of such a fragment ID. The fragment IDs
321 were mapped to the human small RNA databases allowing 2 mismatches. The
322 human small RNA databases comprised miRNA, piRNA, snoRNA, rRNA, and tRNA.
323 The miRNA, piRNA, and snoRNA reference sequences were obtained from the
324 study by Wu et al[54]. The rRNA and tRNA reference sequences were obtained from
325 SILVA database[56] and GtRNAdb 2.0[28], respectively. The normalised expression
326 level for each fragment ID within a class of RNA was used for downstream
327 differential expression analyses. The normalised expression level for a fragment ID
328 in a RNA class was calculated by dividing the raw expression level by a per million
329 scaling factor of total reads of that RNA class, expressed as reads per million (RPM).
330 Compared to normal pregnancy placentas, differentially expressed fragment IDs in

331 the MLEV of preeclampsia placentas were determined using the following criteria: i)
332 At least one MLEV sample had expression level of greater than 100 RPM, ii) P-value
333 was required to be less than 0.05 based on Mann Whitney *U* test after Benjamini-
334 Hochberg correction. The in-house bioinformatics pipeline was written in Perl and R
335 languages for counting sequence reads, tag annotations, and differential expression
336 analysis. Sequence read archive (SRA) data for blood cell datasets were
337 downloaded from NCBI, with SRA identifiers shown in Supplementary Table 3[57].
338
339 **4. Plasma EV RNA isolation**
340 Plasma aliquots were thawed at 37°C and centrifuged at 3000 *g* for 5 minutes to
341 remove cryoprecipitates. For membrane-based affinity isolation of total vesicular
342 RNA, 500 µL plasma was loaded onto exoRNeasy™ Midi columns and
343 manufacturer's instructions followed (Qiagen, Hilden, Germany). RNA was stored in
344 aliquots at -80°C.
345
346 **5. RT-qPCR detection**
347 Stem-loop primers were designed for specific short RNA target sequences identified
348 from RNA-sequencing analysis. Reverse transcription reactions and qPCR detection
349 were undertaken using Custom TaqMan™ Small RNA Assays (Applied Biosystems,
350 USA) following manufacturer's protocols. Custom assay linearity and specificity were
351 verified.
352
353 Two qPCR instruments were used: QuantStudio™ 7 for plasma and QuantStudio™
354 6 for cell culture assays (Applied Biosystems, USA). Quantification cycles (Cq) were

355 determined automatically by instrument-associated software using standard settings.
356 Relative expression was determined by following the PFAFFL approach.
357
358 **6. Cell culture**
359 THP-1 cells (ATCC, Manassas, VA) were seeded onto 24-well Nunc plates at 50 000
360 cells per well in RPMI-1640 medium supplemented with 10% Fetal Calf Serum. Cells
361 were grown with 6.2 ng/mL phorbol 12-myristate-13-acetate (PMA) for 24 hours to
362 differentiate into macrophages. Transfection experiments were performed using RNA
363 oligonucleotides (IDT, San Diego, CA) with a 5'-P modification; the sequence for
364 tRF-A is shown in Supplementary Table 2. The scramble RNA control was the most
365 abundant STBEV tRF without 5'-P modification and U nucleotides replaced with A
366 (sequence 5'-GCAAAGGAGGAACAGAGGAAGAAAACACGCCA-3'). 9.2uM RNA
367 was packaged into lipid vesicles using N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-
368 trimethylammonium methylsulfate (DOTAP), following manufacturer's instructions
369 (Roche, Basel, Switzerland). Human Umbilical Vein Endothelial Cells were
370 purchased and seeded onto 24-well Nunc plates at 25 000 cells per well in
371 Endothelial Cell Growth Medium 2 (PromoCell, Heidelberg, Germany). Macrophage
372 supernatant experiments were conducted by preparing 2X Growth Medium and
373 mixing 50:50 with THP-1 supernatant. Cellular RNA was isolated using RNeasy™
374 Plus Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany).
375 Target mRNA expression was quantified using the following TaqMan™ Assays
376 (Applied Biosystems, USA): Hs02758991_g1, Hs00164932_m1, Hs01003372_m1,
377 Hs01574665_m1, Hs00174128_m1, Hs01555410_m1, Hs01011518_m1.
378
379 **7. Data presentation**

All data within this manuscript are derived from distinct samples. Figure 3B was created using BioRender.com (Toronto, Canada). Statistical analyses and figures were generated using RStudio (RStudio, USA) and Prism9 (GraphPad, USA). Normality of data was assessed and two-tailed Mann-Whitney *U* or t-tests used accordingly in Figures 3A and 4. One-tailed Wilcoxon matched pairs signed rank tests were used in Figure 3C given paired samples were from the same patient, and only a reduction in peripheral venous samples was anticipated.

Data availability

Raw data and bioinformatic pipelines will be made available upon reasonable request to the corresponding author.

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540

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544

545 Author contributions

546 Study was conceived and designed by WRC, CR and MV. Bioinformatic analyses

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548 YMDL, CR and MV. Experimental work was performed and manuscript written by

549 WRC. All authors edited and approved the final manuscript.

550

551 Competing interests

552 YMDL holds equity in DRA, Insighta Grail/Illumina and Take2. PJ holds equity in

553 Illumina. PJ is a consultant to Take2. PJ is a Director of DRA and KingMed Future.

554 YMDL, PJ, and LJ receives royalties from Illumina, LabCorp, Grail, DRA, Xcelom

555 and Take2.

556

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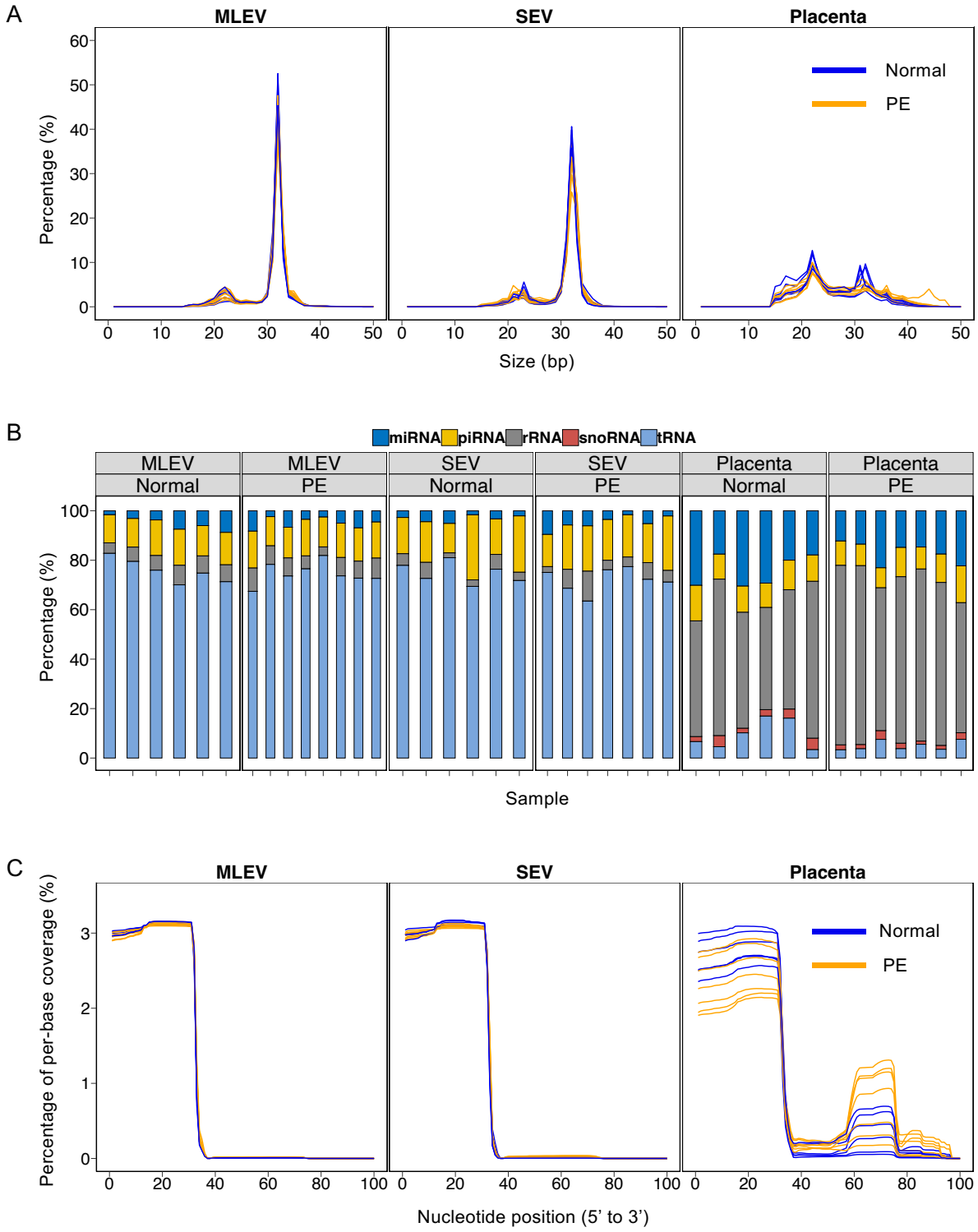
1 Figures and Tables

Figure 1



2

Figure 1

Assessment of reads from medium-large extracellular vesicles (MLEV), small extracellular vesicles (SEV) and placental samples obtained from placentas in normal pregnancy (Normal) and early-onset preeclampsia (PE).

A: Sequence length distribution plots showing size in base pairs (bp) for short RNA fragments after removal of adaptors and low-quality reads. B: Mapping of short RNA fragments. C: Coverage plots showing percentage of per-base coverage for mapped tRNA fragments.

Figure 2

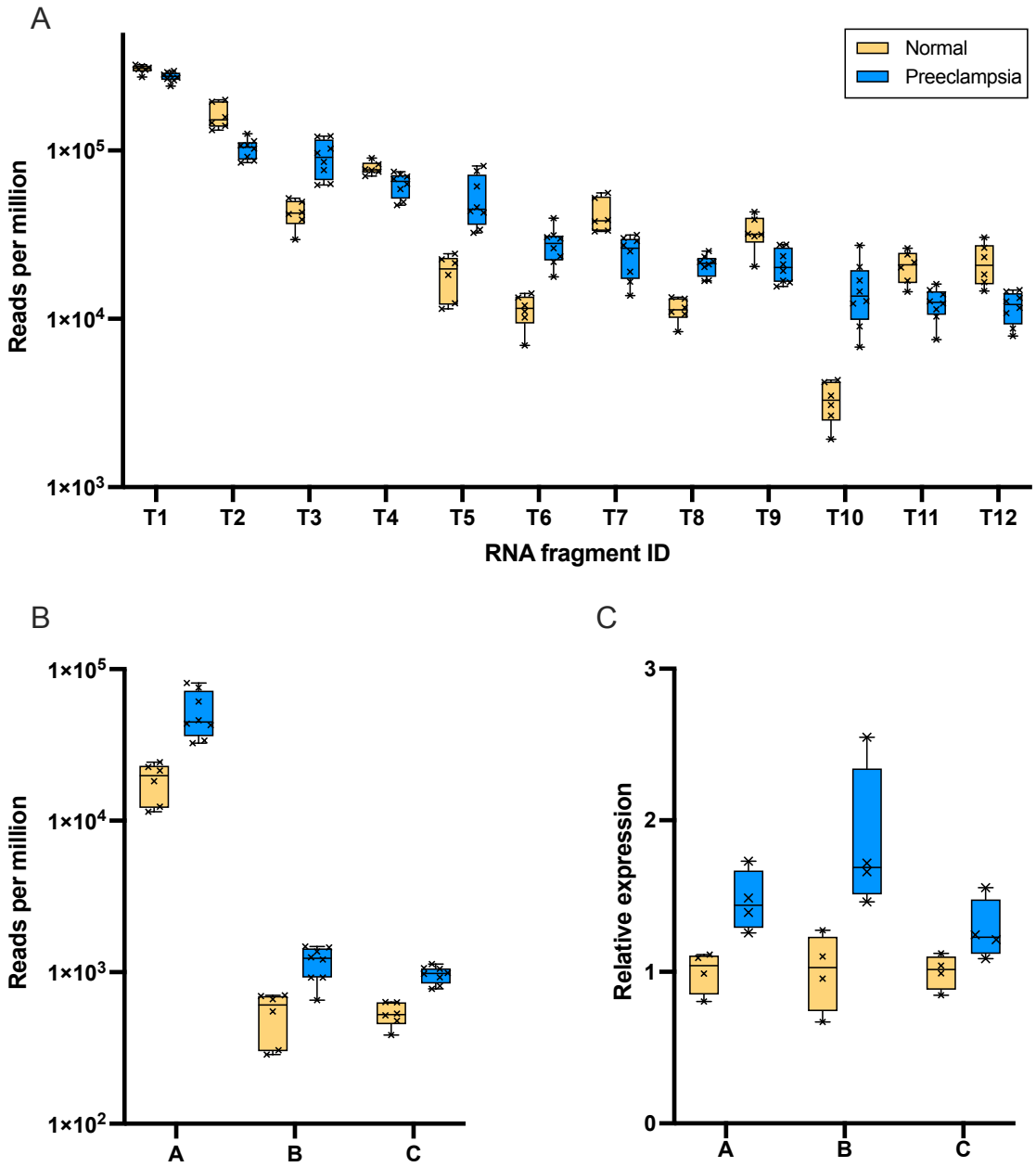


Figure 2

Expression in early-onset preeclampsia and normal pregnancy syncytiotrophoblast EV obtained by placental perfusion:

A: twelve most abundant differentially expressed short RNA fragments quantified using RNAseq (n=6 normal, n=8 preeclampsia).

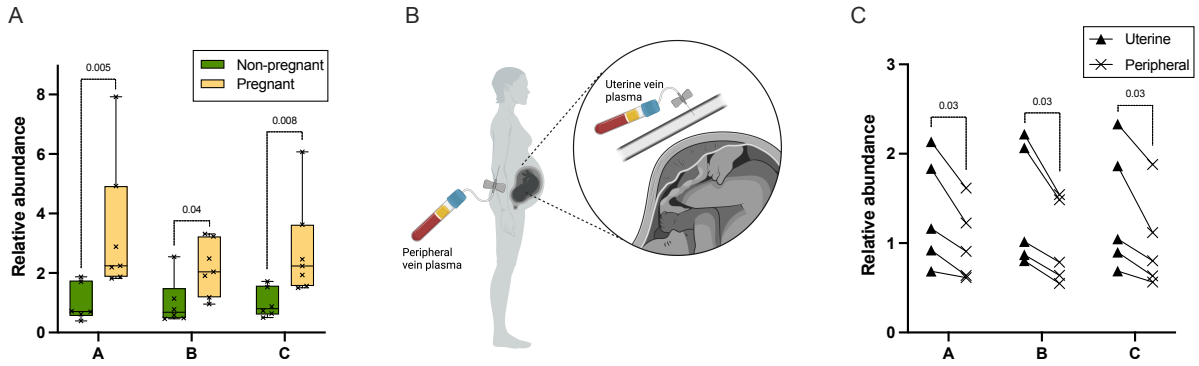
B: three selected target tRF quantified using RNAseq (labelled A, B, C for brevity; full sequences in Supplementary Table 2; n=6 normal, n=8 preeclampsia).

19 C: three target tRF quantified using stem-loop qPCR (normalised to reference gene

20 TBP, n=4 per group).

21 Boxes show median/interquartile range; whiskers show max/min.

22 Figure 3



23

24 **Figure 3**

25 Detection of target tRF in plasma EV

26 A: Expression of three target tRF using qPCR in total EV isolated from

27 pregnant and non-pregnant peripheral venous plasma (labelled A, B, C for brevity;

28 full sequences in Supplementary Table 2); n=7 pregnant, n=6 non-pregnant; boxes

29 show median/interquartile range; whiskers show max/min.

30 B: Diagram demonstrating sampling rationale for uterine and peripheral venous

31 blood

32 C: Expression of three target tRF using qPCR in total EV isolated from uterine and

33 peripheral venous plasma; n=5 paired samples from the same women taken

34 simultaneously.

Figure 4

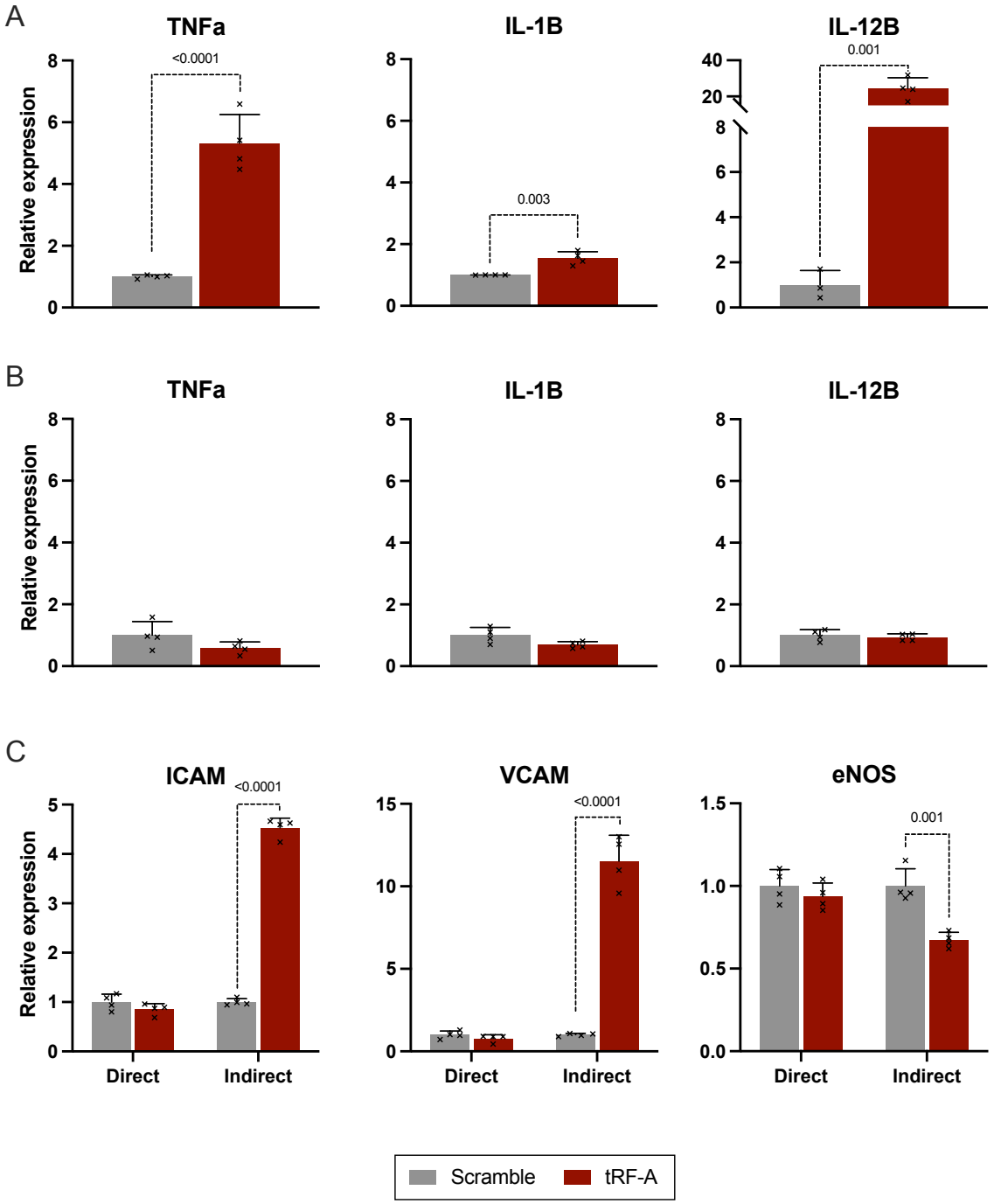


Figure 4

tRF actions relevant to preeclampsia.

A: Cytokine expression by THP-1 derived macrophages after treatment with vesicle-encapsulated RNA after 12 hours (labelled tRF-A and Scramble for brevity; full sequences in Supplementary Table 2 and Methods).

41 B: Cytokine expression by THP-1 monocytes (undifferentiated) after treatment with
42 vesicle-encapsulated RNA after 12 hours.
43 C: mRNA expression in human umbilical vein endothelial cells treated with vesicle-
44 encapsulated RNA (Direct), or the cell culture medium from macrophages pre-
45 treated with vesicle-encapsulated RNA (Indirect) after 6 hours.
46 All data represent n=4 biological repeats per group.

47 Table 1

ID	Sequence	Length	GtRNAdb mapping	Median PE count	Median N count	Log2Fold Change	Adjusted p value	% PE DE reads
T1	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCT	32	tRF-Gly-GCC	276174	307639	0.16	0.0335	24.5
T2	GCATTG_TGGTTCAGTGGTAGAATTCTCGCCT	31	Unmatch	104785	152208	0.66	0.0058	9.2
T3	TCCCT_GTGGTCTAGTGGTTAGGATTCGGCGCT	32	Unmatch	91141	42460	-1.10	0.0058	8.2
T4	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCC	32	tRF-Gly-GCC	65470	76778	0.32	0.0150	5.6
T5	TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGCT	33	tRF-Glu-CTC	44786	19826	-1.50	0.0054	4.7
T6	TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGC_	32	piR-hsa-5938	28094	11533	-1.29	0.0085	2.5
T7	GCATTGTGGTTCAGTGGTAGAATTCTCGCCC_	31	Unmatch	26253	38285	0.80	0.0058	2.2
T8	TCCCTG_TGGTCTAGTGGTTAGGATTCGGCGCC	32	Unmatch	21370	11323	-0.88	0.0058	1.9
T9	GCATTGTGGTTCAGTGGTAGAATTCTCGCC__	30	Unmatch	20168	31843	0.65	0.0305	1.9
T10	TCCCTGGTGGTCTAGTGGTTAGGATTCGGCG__	31	piR-hsa-5938	13644	3284	-2.19	0.0085	1.3
T11	GCCCGGCTAGCTCAGTCGGTAGAGCATGAGAC	32	piR-hsa-27622	12516	20917	0.73	0.0220	1.1
T12	GTTTCCGTAGTGTAGTGGTTATCACGTTCGC__	31	piR-hsa-28877	12154	20820	0.87	0.0128	1.1

48

49 **Table 1**

50 Descriptors of twelve most abundant differentially expressed short RNA fragments in
51 preeclampsia EV. Normal pregnancy (N), early-onset preeclampsia (PE),
52 differentially expressed (DE). Nucleotide differences from GtRNAdb annotated tRNA
53 fragments are highlighted in grey.