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Proteomics Clinical Applications

DATASET BRIEF

Lipopolysaccharide-regulated secretion of soluble and vesicle-based proteins from a panel of colorectal cancer cell lines

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Abstract

Purpose: To mimic the perioperative microenvironment where bacterial products get in contact with colorectal cancer (CRC) cells and study its impact on protein release, we exposed six CRC cell lines to lipopolysaccharide (LPS) and investigated the effect on the secretome using in-depth mass spectrometry-based proteomics.

Experimental design: Cancer cell secretome was harvested in bio-duplicate after LPS treatment, and separated in EV and soluble secretome (SS) fractions. Gel-fractionated proteins were analysed by label-free nano-liquid chromatography coupled to tandem mass spectrometry. NF-κB activation, triggered upon LPS treatment, was evaluated.

Results: We report a CRC secretome dataset of 5601 proteins. Comparison of all LPS-treated cells with controls revealed 37 proteins with altered abundance in the SS, including RPS25; and 13 in EVs, including HMGB1. Comparing controls and LPS-treated samples per cell line, revealed 564 significant differential proteins with fold-change >3. The LPS-induced release of RPS25 was validated by western blot.

Conclusions and clinical relevance: Bacterial endotoxin has minor impact on the global CRC cell line secretome, yet it may alter protein release in a cell line-specific manner. This modulation might play a role in orchestrating the development of a permissive environment for CRC liver metastasis, especially through EV-communication.

KEYWORDS
colorectal cancer, exosome, extracellular vesicles, lipopolysaccharide, NF-κB, secretome

1 | MANUSCRIPT

Colorectal cancer (CRC), among the major cancer types in the Western world [1], is responsible for 10% of cancer related deaths, often due to its late detection when the tumor has already spread out to distant sites [2].

Abbreviations: CRC, Colorectal cancer; LPS, lipopolysaccharide; NF-kB, Nuclear factor KB; EV, extracellular vesicle; SS, Soluble secretome; MS, Mass spectrometry; LC, Liquid chromatography; GO, Gene ontology

Surgery is the mainstay curative treatment for CRC [3]. Nevertheless, between 15% and 30% of stage II and III patients develop hepatic metastases within 5 years [4,5]. Paradoxically, tumor surgery may contribute to the metastatic process, due to inflammatory mediator release in response to surgery, which promotes the metastatic ability of cancer cells [6–8]. During surgery, bacterial products, such as endotoxins as lipopolysaccharide (LPS), from commensal bacteria translocate across the bowel wall, and reach the systemic circulation [9,10]. The released endotoxins can subsequently induce an inflammatory response [11,12] through to the activation of the Nuclear Factor-κB (NF-κB) pathway [13]. Inflammation is known to promote malignant transformation [14], but its direct effect on cancer cells and contribution to metastases development remains to be elucidated.

The aim of our study is to unravel the role of LPS in CRC protein secretion by mass spectrometry-based proteomic profiling of secreted proteins in vitro. The secretome includes classically secreted proteins as well as proteins released via extracellular vesicles (EVs), including exosomes [15]. Exosomes are a subset of EVs derived from endosomes which are secreted by almost all cell types [16,17] and involved in intercellular communication [18,19]. Importantly, exosomes are secreted by CRC cells [20] and their cargo has been implicated in preconditioning to distant site CRC liver metastasis [21,22].

To mimic the bacterial exposure to CRC cells using an in vitro system, we treated six CRC cell lines (CaCo-2, COLO-205, HCT116, HT-29, LoVo and SW480) with LPS and studied the changes in secreted proteins, either via EVs or as soluble proteins [23–25]. The conditioned medium (secretome) was harvested in bio-duplicates as previously described [26], after 18 h in serum-free media (with or without 1 μg/mL LPS), and centrifuged twice to remove dead cells (500 x g) and cell debris (2000 x g). This LPS dose was chosen according to literature and previous results (data not shown) that indicated a response from CRC cells with no changes in cell viability [27]. Secretomes were then concentrated using a 4 Kd MWCO centrifugal concentrator. Next, we isolated the EVs by ultracentrifugation [28]. In brief, the concentrated cancer cell secretome was centrifuged twice at 20,000 g for 30 min, using SW40Ti Rotor (Beckman Coulter) and at 100,000 g for 1 h. The pellet consisted of the EVs, and the supernatant the soluble secretome (SS). The SSs were concentrated using a 4 kD-cutoff centrifugal filter to a volume of 200 μL.

EVs and SS proteins were processed for proteomics analysis using our label-free GeLC-MS/MS workflow that was bench-marked for secretome analysis [26]. An overview of the study design including 48 samples that were gel fractionated in five bands and analyzed using 240 nanoLC-MS/MS runs is provided in Figure 1A. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE [29] partner repository with the dataset identifier PXD011611.

We ensured equal protein loading at different levels of the experiment. To this end, a similar number of cells were seeded for each condition and equal amounts of medium were added and collected for whole secretome harvesting. Moreover, the same sample input was used for EV isolation from each CRC cell line and similar protein input was loaded onto the acrylamide gels. These precautions resulted in a proteome dataset with similar numbers of identified proteins per sample (Figure S1) and similar numbers of summed raw protein counts across samples per fraction, underscoring the consistency of the protein loads and nanoLC-MS/MS analysis. Finally, prior to comparative analysis, protein data were globally normalized by a scaling factor for each sample. The scaling factor is the average spectral count of all samples divided by total spectral counts for each sample. Hence, the sum of the normalized spectral count values is constant across all samples.

Clinical Relevance

To mimic the environment during colorectal cancer (CRC) surgery with disruption of the epithelium barrier and translocation of bacterial products, six CRC cell lines were treated with the bacterial endotoxin lipopolysaccharide (LPS). Mass spectrometry-based proteomics was used to study the effect on cancer cell secretion. We report the largest CRC secretome dataset to date consisting of 5601 proteins, including soluble proteins and extracellular vesicle (EV) proteins, in response to bacterial endotoxin. Of note, LPS-impact on the global CRC secretome or EV proteome was minor with most effects being cell line-specific. A subset of proteins released in response to LPS was previously associated with CRC hepatic metastases. Endotoxin-induced factors may, therefore, potentially be involved in distant metastatic niche preparation. Other identified proteins were not previously linked to cancer, providing new candidates involved in this intercellular communication process.

A total of 5601 non-redundant proteins were identified, with an average of 3396 proteins per EV or SS sample (Table S1). To obtain a global overview of the EV and SS proteome datasets, unsupervised hierarchical clustering was carried out using the normalized spectral count data of all identified proteins (Figure 1B). The dataset is divided in two main clusters according to sample type: EV and SS fractions. LPS-treated samples and controls clustered per cell line, implicating that the effect of LPS is minor relative to the molecular differences between different CRC cell lines. Finally, biological replicates clustered together indicating good reproducibility of the analysis.

Venn analysis showed that 632 proteins were uniquely identified in the EV fractions while 526 proteins were only identified in the SS fractions (Figure S2A). Exosome markers, such as tetraspanin CD63, a constituent of the late endosome and typically found in secreted EV following the fusion of endosomes with the plasma membrane, the tetraspanins, CD9 and CD81 [32], TSG101 and PCDC6IP, were enriched in the EV fractions as compared to the SS fractions (Figure S3) [33]. For PDCD6IP this difference was significant in all cell lines; for CD9 in CaCo-2, LoVo and SW480, and CD81, in LoVo. These results indicate that our EVs isolated by ultracentrifugation were indeed enriched for vesicles. Comparison to published CRC cancer cell line data compiled in Vesiclepedia [30], using the bioinformatics

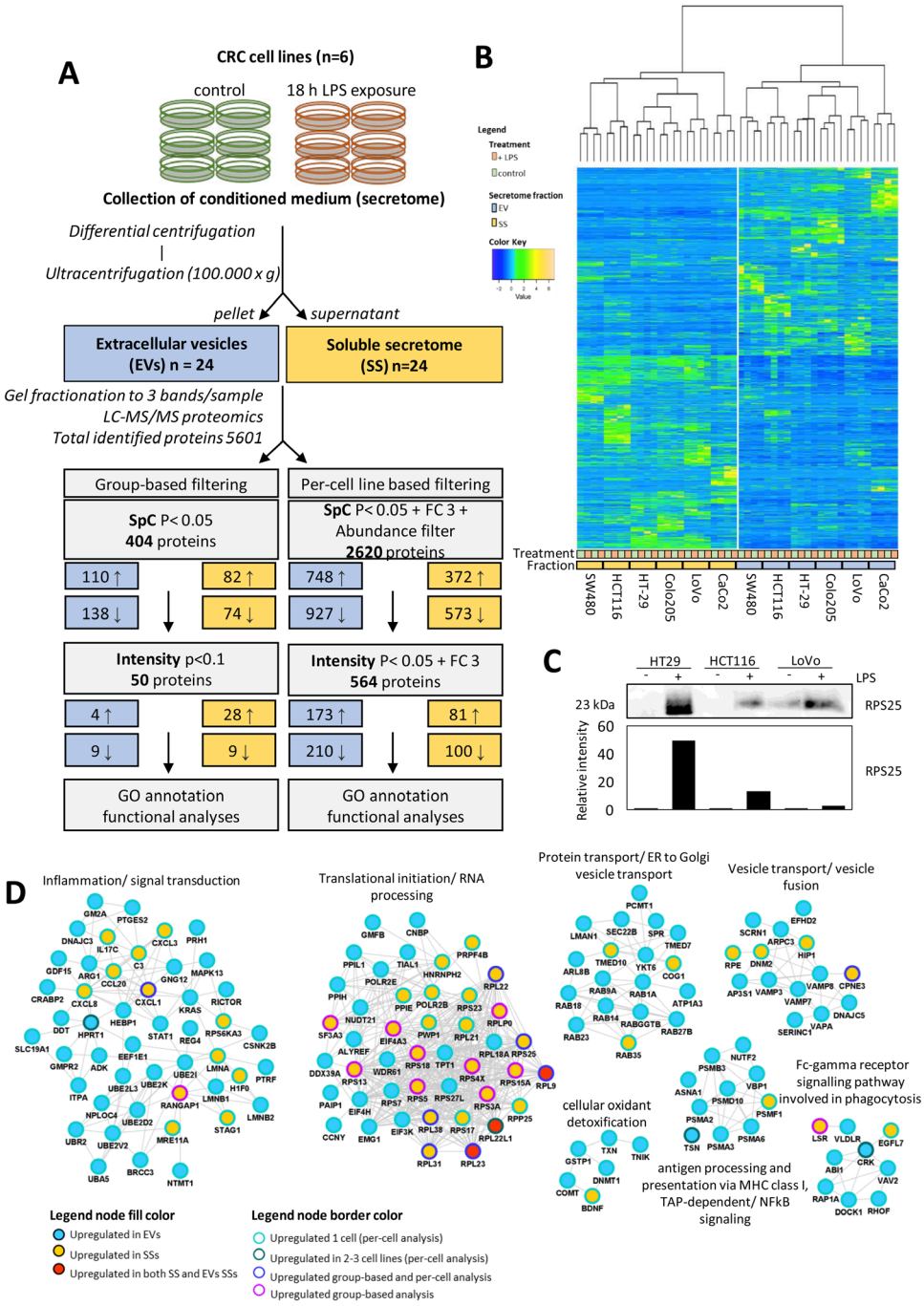

FIGURE 1 Workflow. (A) Cells were grown to confluency, lipopolysaccharide (LPS) was added and supernatant was collected after 18 h. EVs were isolated by ultracentrifugation, and all proteins were identified by LC-MS/MS and two independent statistical tests and different filters were applied to pinpoint regulated proteins. A GO analysis was performed with regulated proteins. (B) Unsupervised hierarchical clustering using the Euclidean distance measure to obtain the proximity between samples. EVs and SS fractions cluster in two branches, underscoring the differential abundance of most proteins in these fractions, despite high overlap of identified proteins. Within each secreted fraction, samples from the same cell line cluster together. Moreover, biological replicates cluster together, showing the reproducibility of the workflow. (C) Validation by western blot of RPS25 regulation. (D) Gene Ontology functional analysis on LPS-regulated proteins

tool FunRich [31], revealed that 267 EV proteins were not previously described in CRC EVs (Figure S2B and Table S2).

To pinpoint proteins with differential abundance after LPS treatment of CRC cell lines, two analyses were performed (Figure 1): 1. across all samples in a group-based analysis of LPS-treated versus control samples (Table S3); and 2. per cell line (Table S4). Moreover, these differential proteins were assessed by two independent quantitative analyses: spectral counting coupled to the paired beta-binominal test [34,35] and ion intensity-based quantification using LFQ values from MaxQuant coupled to differential analysis using limma [36].

TABLE 1 Overview of the number of regulated proteins per fraction in the group-based analysis and per cell line analysis

		Soluble secretome				Extracellular vesicles			
		Up		Down		Up		Down	
Group-based analysis		Count	Int	Count	Int	Count	Int	Count	Int
		Regulated 82	170	74	162	110	49	138	53
		Overlap 28		9		4		9	
		Soluble secretome				Extracellular vesicles			
		Up		Down		Up		Down	
Cell line	CCS	Count	Int	Count	Int	Count	Int	Count	Int
CaCo-2	No CCS	Regulated 146	45	37	13	159	54	76	24
		Overlap 24		3		24		13	
COLO205	CCS1	Regulated 18	26	111	43	83	15	245	113
		Overlap 13		18		10		57	
HCT116	CCS2	Regulated 74	23	64	30	318	138	80	17
		Overlap 7		13		93		8	
HT-29	CCS3	Regulated 38	105	53	18	55	20	250	113
		Overlap 21		6		9		55	
LoVo	CCS2	Regulated 48	27	159	49	32	107	79	40
		Overlap 9		27		18		19	
SW480	CCS3	Regulated 48	20	149	74	101	48	197	110
		Overlap 7		33		19		58	

UP represents proteins increased in LPS-treated samples, while DOWN refers to proteins with decreased abundance in LPS-treated samples. Overlap refers to candidates confirmed by both independent analyses.
CCS, colorectal cancer subtype described in 2014 by De Sousa et al.; count, Spectral count analysis; int, Intensity-based analysis.

In the group-based analysis of protein spectral counts, 63 proteins were regulated in the SS fraction and 93 in the EVs ($p < 0.05$). Of these, 37 SS proteins and 13 EV proteins were confirmed by the intensity-based analysis ($p < 0.1$, Table S4) and one candidate was validated by western blot (Figure 1C). Clustering shows that these proteins are involved in two major biological processes, one involved in translation, and the second in inflammation/signal transduction (Figure 1D).

Overall, LPS yielded a heterogeneous response across the six CRC cell lines; therefore, we also analyzed the LPS effect per cell line. In this analysis, the following stringent filtering was applied to select for discriminatory proteins based on spectral counting (Table 1): fold-change >3, a minimum of three spectral counts, no missing values, and consistent regulation, ensuring that the maximum value of the lower expressed condition had to be less than minimum of the higher expressed condition. See Table S4 for a summary of the regulated proteins per fraction per cell line. This filtering gave the following numbers of overlapping differentially secreted proteins in SS/EV fractions: CaCo-2: 27/37; COLO-205: 31/67; HCT116: 20/101; HT-29: 17/64; LoVo: 36/37 and SW480: 40/77. Altogether this analysis shows that most of the regulated proteins were found in the EVs (68%).

To find common biological functions in the LPS-regulated secretome proteins of all six CRC cell lines, Gene Ontology (GO) mining was performed using DAVID bioinformatics tool [37]. For the group-based analysis, the most commonly LPS-regulated proteins were ribosomal and other nucleolar proteins, especially in SS, such as RPS3A, RPL9, RPL38 or RPS25. As for the terms retrieved for proteins with differential abundance in the per cell line analyses (Table 2), LPS-treated SS fraction proteins were associated with cytokines and other extracellular proteins, whereas EVs were associated, among others, with proteins involved in cytoskeleton, non-membrane bounded organelles, vesicle trafficking and integrins. Ribosomal and RNA processing proteins were upregulated in all LPS treated cell lines. GO terms retrieved for proteins enriched in EVs upon LPS treatment included: cytoskeleton, intercellular junctions and integrins (Table 2).

To rule out that the observed intracellular proteins are related to differential cell death or differential apoptosis of LPS-treated and control cell cultures, we inspected the spectral counts of markers for the ER (Calnexin), mitochondria (Cytochrome-C), and Golgi (GM130) as well as Caspases 3, 8 and 9 (Figure S5). This analysis did not reveal any difference in the levels of these proteins, underscoring that our results cannot merely be attributed to differential cell death or apoptosis induction.

To validate our LPS-regulated candidates, we performed western blot analysis of 3 candidates from the group-based analysis with available antibodies: RPS25, RPL38, and HMGB1, in two independent experiments. One candidate could not be evaluated due to an antibody that did not show protein bands at the expected molecular weight (RPL38, Figure S4A). One candidate showed inconclusive results with validation of the downregulation in EVs in 2/3 cell lines tested in experiment 1 but not in experiment 2 (HMGB1, Figure S4B). Finally,

TABLE 2 Retrieved terms using the DAVID bioinformatics tool with the list of regulated proteins per cell line as an input. The tables list significant retrieved terms in (A) SS fraction enriched proteins and SS fraction for decreased proteins in (B) EV fraction enriched proteins; EV fraction for decreased proteins

A	CaCo-2	COLO-205	HCT116	HT-29	LoVo	SW480
Terms for LPS-upregulated proteins in SS fraction						
Ribosome	■	■	■	■	■	■
Nuclear and organelle lumen proteins	■	■	■	■	■	■
RNA processing	■	■		■	■	
Basement membrane		■				
Enzyme inhibitor	■	■				■
DNA metabolic process	■	■		■		■
Cytokines and other extracellular proteins		■		■		■
Terms for LPS-downregulated proteins in SS fraction						
Nuclear and lumen proteins		■	■		■	
Pigments granule		■			■	■
Proteasome		■			■	
RNA transport and processing		■	■		■	■
B	**CaCo-2**	**COLO-205**	**HCT116**	**HT-29**	**LoVo**	**SW480**
Terms for LPS-upregulated proteins in EVs fraction						
Nucleotide binding	■	■	■	■	■	■
RNA processing	■	■	■	■	■	■
Cytoskeleton and non-membrane bounded organelles	■	■	■	■	■	■
Molecular assembly	■	■	■	■	■	■
Ribosome	■	■	■	■	■	■
RNA processing	■	■	■		■	■
Pigment granule, vesicles	■	■	■	■	■	■
Organelle lumen	■	■	■	■	■	■
Nucleotide binding	■	■	■	■	■	■
Redox activity			■			■
Differentiation and development			■	■		■
Protein location	■	■	■	■	■	■
Vesicle trafficking	■	■	■	■	■	
Nucleotide synthesis		■	■	■	■	■

(Continues)

TABLE 2 (Continued)

B	CaCo-2	COLO-205	HCT116	HT-29	LoVo	SW480
Ubiquitination						
Annexins and enzime inhibitors						
Ribosome biogenesis						
Integrins						
Intercellular junctions						
Apoptosis and cell death						
Chaperone binding						
Calcium binding						
Terms for LPS-downregulated proteins in EVs fraction						
Vesicles and pigment granules						
RNA processing						
Nucleotide binding						
Molecular assembly						
Organelle lumen						
Homeostasis						
Negative regulation of cell death						
Intracellular transport						
No synthesis						

EV, extracellular vesicle; SS, soluble secretome.

the upregulation of RPS25 in the soluble secretome fraction of LPS stimulated CRC cell lines was clearly validated in both western blot experiments (Figure S4C).

All increased and reduced proteins were investigated in Pubmed database to evaluate the link of LPS-regulated proteins to cancer and to CRC-hepatic metastasis. To this end, each protein was included in one of the four following categories: 1) CRC hepatic metastasis, 2) CRC-, 3) cancer other than CRC and 4) no cancer-previously reported. In the group-based analysis, 13% of proteins had already been studied in the context of CRC metastasis, either in clinical or experimental studies, or up to 44% in CRC. Furthermore, from the per cell line analysis, up to 50% of all EV LPS-regulated proteins were previously reported for their association with CRC, and 10% were previously linked to CRC hepatic metastasis (Table S5). For SS LPS-regulated proteins, 14% of proteins higher abundance in LPS-treated samples and 8% of proteins with lower abundance had a link with CRC metastasis (Table S5).

Interestingly, some of the LPS regulated EV proteins, such as RPS27L, uniquely expressed in LPS-treated HCT116 EVs [38] GDF15 [39], RAB27B, uniquely found in LPS-treated LoVo EVs [40], KRAS (upregulated in HCT116 EVs) and HMGB1 (downregulated in CaCo-2 EVs) [41,42], were previously detected in human tissue or stool samples and/or in CRC cell lines, and a correlation with CRC prognosis or metastasis has been proposed. The upregulation of inflammatory cytokines (CXCL1, CXCL3 and CCL20) and RAS proteins, by CRC cells upon LPS-treatment may provide a means to prepare the future metastatic site in the liver [43–46], as previously reported for macrophages educated by CRC-derived exosomes [47]. Here, we expand on previous observations of the prometastatic potential of LPS [48]. Further underscoring the above, the LPS-triggered EV proteins PSMD10, REG4 and LGALS8 were previously reported to be linked to hepatic metastasis. Taken together, bacterial endotoxins stimulate the release of proteins implicated in formation of hepatic metastasis, a finding that warrants further exploration.

RPS25 regulation, seen in both the group-based and per cell line analysis, was confirmed by western blot in three CRC cell lines. RPS25, which extra-ribosomal functions are not fully known, has been found to interact in the MDM2-p53 axis [49]. Released during ribosomal stress, RPS25 and other ribosomal proteins stabilize MDM2 and trigger p53 activities. In this context, extracellular RPS25 could represent a marker of cellular stress, such as the damage triggered by pro-inflammatory and even pro-metastatic stimuli. Further studies are needed to accurately determine the underlying molecular mechanisms, although increasing evidence points at a role of ribosomal proteins in CRC carcinogenesis [50].

Finally, LPS is recognized by Toll-like Receptor 4 (TLR4) and triggers an inflammatory response though NF-κB translocation to the nucleus and inflammation-related genes transcription [51]. To evaluate whether NF-κB activation upon LPS treatment occurs in CRC cells, we carried out an immunofluorescence and image analysis to assay the translocation of the p65 subunit to the nucleus. The changes in nuclear fluorescence intensity/total intensity, calculated as percentage referred to the untreated sample, is depicted in Figure S6. CaCo-2 cell line showed a significant increase in NF-κB nuclear signal after 5, 10, and 55 min of treatment with LPS. For HCT116, differences were seen at 5, 10 and 45 min. These latter two cell lines showed a cyclic NF-κB nuclear translocation (Figure S6). This pattern was previously described [52,53], due to the alternative pathways activated upon LPS recognition, that leads to both early and delayed NF-κB nuclear translocation through MyD88 and TRIF pathway, respectively [54]. Interestingly, NF-kB activation has been described to strengthen Wnt signalling, inducing dedifferentiation of non-stem cells, acquiring a tumor initiating phenotype [55]. Thereby, supporting the high number of LPS-associated proteins linked to CRC metastasis found in the secretomes of CaCo-2 and HCT116. These included inflammation-related molecules, such as ABI1 [56], studied in colon inflammation; HMGB1 [57], Ras-proteins (KRAS) [53]; LCN2 [58]; Galectin-8 [59] and PSMD10 [60,61]. Taken together, an initial inflammatory response to bacterial endotoxin was observed in the tested CRC cell lines.

To summarize, in this study, we provide an in-depth inventory of the cancer secretome, including soluble and EV proteins, released from six CRC cell lines in response to LPS treatment. We show that bacterial endotoxin has minor impact on CRC cell line secretome of the cell panel taken as a group, yet it changed the secreted protein repertoire in cell line-specific manner. This modulation might play a role in orchestrating the development of a permissive environment for CRC liver metastases, especially through EV-communication.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data were deposited to the ProteomeXchange Consort via the PRIDE partner repository with the dataset identifier PXD11611.

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SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/prca.201900119 in the Supporting Information section at the end of the article.

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