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Cu isotope ratios are meaningful in ovarian cancer diagnosis

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ABSTRACT

Background: Ovarian cancer diagnosis is currently based on imaging and circulating CA-125 concentrations with well-known limits to sensitivity and specificity. New biomarkers are required to complement CA-125 testing to increase effectiveness. Increases in sensitivity of isotopic separation via multi collector inductively coupled plasma-mass spectrometry have recently allowed highly accurate measurement of copper (Cu) isotopic variations. Studies in breast cancer patients have revealed changes of serum copper isotopic composition demonstrating the potential for development as a cancer biomarker. Evaluating $^{65}Cu/^{63}Cu$ ratios ($\delta^{65}Cu$) in serum samples from cancer patients has revealed a strong correlation with cancer development. In this study blood samples from forty-four ovarian cancer patients, and 13 ovarian biopsies were investigated.

Results: Here we demonstrate that changes in Cu isotopes also occurs in ovarian cancer patients. Copper composition determined by multiple collector inductively coupled plasma mass spectrometry revealed that the copper isotopic ratio $\delta^{65}Cu$ in the plasma of 44 ovarian cancer patient cohort was significantly lower than in a group of 48 healthy donors, and indicated that serum was enriched for ^{63}Cu. Further analysis revealed that the isotopic composition of tumour biopsies was enriched for ^{65}Cu compared with adjacent healthy ovarian tissues.

Conclusions: We propose that these changes are due to increase lactate and Cu transporter activities in the tumour. These observations demonstrate that, combined with existing strategies, $\delta^{65}Cu$ could be developed for use in ovarian cancer early detection.

1. Introduction

Copper (Cu) is a vital nutrient absorbed by intestinal cells and transported to the liver where it is stored thus controlling its concentration in blood [1,2]. Cu circulates mainly complexed to ceruloplasmin (60–95%) and albumin (10 %) [3,4]. In cells, Cu is complexed to metallochaperones including COX17 and ATOX1 [5] which deliver Cu to cytochrome c and ATPase 7B respectively. Modifications to Cu concentration and relative abundance of Cu isotopes (fractionation) have been linked to modified metabolic processes (oxidative phosphorylation, hypoxia, angiogenesis), and thus to health and disease [6].

Ovarian cancer diagnosis is currently based on circulating cancer antigen 125 (CA-125) concentrations where it is elevated in 50 % of early stage ovarian cancer cases [7], but is also increased in pregnancy, endometriosis [8] and other benign clinical conditions, which reduces its specificity. However it remains an effective approach for following the response to chemotherapy on patients and detecting relapse [9].

To complement clinically available diagnostic methods, new biomarkers including circulating tumour DNA, tumour serum proteins, circulating cancer cells or serum levels of metals such as Cu and zinc [10,11] are being developed due to increased sensitivity of multi collector inductively coupled plasma-mass spectrometry (MC-ICP-MS).

Copper is present in the form of two ions Cu(I) and Cu(II) in cells and in blood. Transport and uptake of these two Cu ions is the cause of the selective distribution (fractionation) of the copper isotopes ^{63}Cu(I/II) and ^{65}Cu(I/II) between the cells and the blood [6,12]. Recent studies have measured a strong decrease of the ratio ($\delta^{65}Cu$, see Eq. 1) of Cu isotopes in serum of breast or colorectal cancer patient [13].

The aims of the present study were to compare $\delta^{65}Cu$ ratios i) from whole blood in group of ovarian cancer patients compared to a healthy control group and ii) evaluate a small series of ovarian cancer biopsies

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and healthy ovary tissue.

2. Material and methods

2.1. Clinical samples

Ethical approval for this study was obtained from NHS HRA Wales6 REC (15/WA/0065) to collect tissue and serum samples from ovarian cancer patients and non-cancer controls. Formal written consent was obtained from all patients at the time of recruitment into the study. Patients attending general gynaecology clinics or gynaecology oncology clinics in Swansea Bay and Cwm Taf Morgannwg NHS University Health Boards (SBUHB and CTMUHB) were recruited into this study. Forty-four women with histologically confirmed ovarian cancer were recruited in this project. These were post-menopausal patients that presented to primary care or emergency services with symptoms suggestive of ovarian pathology (pain, abdominal bloating, weight loss and change in bowel habit). An ovarian mass was subsequently identified on imaging investigations (ultrasound, CT, MRI) and tumour marker measurements [CA-125] performed. The histological evaluation of the ovarian biopsies and the cancer diagnosis was confirmed by the Pathology Department as part of the patient's routine clinical care. However, only patients with a confirmed ovarian tumour diagnosis were included in the study. Patients with infection, chronic inflammation, autoimmune disease and other cancers were excluded from this study. Amongst the patients, thirteen gave serum and biopsies and 31 gave only serum. Analysis of the CA-125 marker is reported in Table 1. Women were between 43 and 83 years old with a mean of 64.4 years old. Control serum (from the Etablissement Français du Sang, patient agreement obtained through MTA13 − 1728) was obtained from women between 19 and 65 years old with a mean of 33 years old were analysed. Data are included in supplementary 1 (Télouk et al., submitted to Journal of Hepatology). Blood was sampled into dry test tubes, centrifuged immediately, and stored at −80 °C. Two hundred microliters of serum were mineralized on a hot plate in a mixture of 2 mL of nitric acid and 0.5 mL of hydrogen peroxide and processed on a macroporous anion-exchange resin AGMP1 100–200 mesh (Biorad, UK) to separate Cu [14]. Biopsies (Table 2) placed into culture medium at the time of surgery were cut in pieces and one piece retained for isotopic measurements. These pieces were weighed and mineralized on a hot plate in a mixture of 2 mL nitric acid and 0.5 mL hydrogen peroxide and processed on a macroporous anion-exchange resin to separate Cu.

2.2. Spectrometry

Element concentrations were determined as previously described [14,15] by ICP-MS using an iCAP Q (Thermofisher Scientific, Bremen, Germany). $^{65}Cu/^{63}Cu$ ratios of the blood and biopsy samples were determined using a Nu instrument multiple-collector inductively coupled plasma mass spectrometer (MC-ICP-MS) Nu Plasma HR 500 (Wrexham, UK). As mass spectrometry isotopic measurement by MC-ICP-MS suffers from a stable bias between samples, a correction was systematically applied. For this, a constant and known standard made of zinc was systematically added in the purified samples. The Zinc acts as a mass bias corrector and standard in sample bracketing as it is measured at the same time as copper and is used as reference. This allow to reduce the MC-ICP-MS mass bias inducing wrong measurement compared to the real isotopic composition in copper.

A conventional delta value $\delta^{65}Cu$ is used throughout to report the Cu isotope abundances. $\delta^{65}Cu$ is a dimensionless parameter defined as:

$$\partial^{65}Cu = \left[\frac{(^{65}Cu/^{63}Cu)\text{sample-}(^{65}Cu/^{63}Cu)\text{ref}}{(^{65}Cu/^{63}Cu)\text{ref}}\right] \times 10^3 \sin^{-1}\theta \quad (1)$$

This represents the relative deviation of the $^{65}Cu/^{63}Cu$ ratio in the measured sample from its value in the reference material NIST SRM 976 in parts per 1000 (‰). Typical reproducibility on $\delta^{65}Cu$ at the 95 percent confidence level as determined from three replicates of serum samples is 0.05‰. Natural variations of $\delta^{65}Cu$ in inorganic and organic material do not exceed +/- 3‰ [16]. These δCu variations are due to metabolic processes and biological variability.

3. Statistical analysis

Normality of the data was analysed using a Kolmogorov Smirnov test.

Statistically significant differences on δCu values between healthy serum vs OC serum samples were assessed using a 2-samples *t*-test as distributions of the datasets were normal. Statistically significant differences on δCu values between tumour vs non tumour ovarian biopsies were assessed using a Mann Whitney test as distributions of the datasets were not normal.

In order to evaluate the correlation between age and δCu of ovarian cancer patients, as the datasets were normally distributed we undertook a Pearson's Correlation Test. In order to evaluate the differences between number of chemotherapy and $\delta^{65}Cu$ values, we performed a Mann-Whitney test between the different groups we described in the results. Finally, the differences between cancer stage and $\delta^{65}Cu$ values was assessed using a Kruskal Wallis test between the different groups described in the results.

4. Results

4.1. Clinical diagnosis and treatment

Forty-four ovarian cancer patients were included in this study and results compared to data from 48 healthy patients (See Supplementary 1. Télouk et al. unpublished). The mean age of the women with ovarian cancer in the study was 64.4 years, and women were post-menopause (Table 1). In total 22 women were diagnosed with FIGO (Federation Internationale de Gynécologie et d'Obstétrique) stage 3 ovarian cancer, seven with FIGO stage 4 cancer and 8 FIGO stage 1 or 2 and seven benign masses.

We obtained ovarian biopsy samples from 13 patients from one or from both ovaries, 11 were tumoral and 10 were non tumoral (Table 2). The 13 patient cohort included those diagnosed with high grade serous ovarian cancer (HGSOC) (n = 6), borderline ovarian tumour (n = 1), endometrioid ovarian adenocarcinoma (n = 2) and benign tumour masses (n = 4). The levels of CA-125 were measured at diagnosis. Patients presenting before Pt chemotherapy had high levels of the marker (mean of the group = 1301 ± 1370). The effect of Pt treatment was followed by monitoring CA-125 levels. Samples were taken when the marker had significantly decreased (mean of the group = 205 ± 250).

4.2. Association between $\delta^{65}Cu$ and cancer

Healthy patient serum $\delta^{65}Cu$ values were compared to that of ovarian cancer patients (Fig. 1). Ovarian cancer patient serums had a copper isotope ratio ranging from -1.59 to -0.14‰ (median -0.80‰, mean -0.78‰ ± 0.05), a distribution that was significantly lower ($p < 0.001$) than the control group (between -0.71 and 0.05‰, median -0.23‰, mean -0.23‰ ± 0.02). Comparison of the copper isotope ratio in eleven ovarian tumour biopsies and ten non-tumour ovarian biopsies showed that in tumour biopsies, $\delta^{65}Cu$ values were between -0.19 and 0.59‰ (n = 11, median -0.09‰, mean 0.09‰ ± 0.08). $\delta^{65}Cu$ values were significantly lower ($p < 0.05$) in healthy ovaries with values ranging from -0.63 to 0.08‰ (n = 10, median of -0.16‰, mean of -0.16‰ ± 0.06 $p < 0.05$). Finally, no difference was found between $\delta^{65}Cu$ of the 13 patients we obtained serum and biopsies from and the 31 patients with only serum samples ($p > 0.05$).

Table 1
Ovarian cancer: diagnostic, stage, number of cycles of chemotherapy and analytical data on serum samples (CS = Cancer Serum, * = pre-menopausal women, N/A = not applicable, NC = non communicated).

Sample Number	Age	Cancer Diagnosis	Stage	Nb of chemotherapy	Initial CA-125 at diagnosis	CA-125 at recruitment in the study	δCu(‰)
CS1	43	HGSOC	4A	3	1146	32	−0.25
CS2	57	HGSOC	4B	4	363	19	−0.98
CS3	73	Metastatic colorectal adenocarcinoma to the ovaries	4B	NC	202	202	−0.84
CS4	68	HGSOC	4A	3	> 25,000	517	−0.57
CS5	71	HGSOC	4B	4	1857	21	−0.59
CS6	71	HGSOC	4B	6	4248	500	−0.47
CS7	76	HGSOC	4A	4	1656	35	−0.81
CS8	74	HGSOC	3C	3	198	53	−0.79
CS9	62	HGSOC	3C	3	3682	1154	−0.6
CS10	60	HGSOC	3C	6	3150	30	−0.87
CS11	50	HGSOC	3C	6	721	22	−1.36
CS12	82	HGSOC	3C	6	2085	57	−0.53
CS13	60	HGS Primary Peritoneal disease	3C	4	227	< 6	−0.9
CS14	69	HGSOC	3C	6	796	20	−0.97
CS15	59	HGSOC	3C	6	11,450	59	−0.8
CS16	71	HGSOC	3C	5	423	14	−0.65
CS17	78	HGSOC	3C	9	2429	2227	−0.89
CS18	67	HGSOC	3C	6	81	19	−0.39
CS19	78	Ovarian Carcinosarcoma (Tubo)	3C	None	160	160	−0.97
CS20	44	HGSOC	3C	None	785	785	−0.67
CS21	55	Ovarian Carcinosarcoma	3C	3	831	122	−0.61
CS22	82	HGSOC	3C	4	836	155	−0.69
CS23	71	HGSOC	3C	None	1161	1613	−1.22
CS24	83	HGSOC	3C	3	2538	206	−1.01
CS25	58	HGSOC	3C	4	634	231	−0.53
CS26	80	HGSOC	3C	4	58	35	−1.13
CS27	66	HGSOC	3C	6	340	35	−0.19
CS28	73	HGSOC	3C	4	204	37	−1.56
CS29	82	HGSOC	3C	4	634	231	−1.6
CS30	56	HGSOC	2C	4	302	24	−1.1
CS31	47	Endometrioid adenocarcinoma ovary	2A	None	492	492	−0.14
CS32	62	Ovarian Clear Cell	1C2	None	241	431	−0.82
CS33	67	Ovarian Granulosa cell tumour	1C2	None	38	38	−0.98
CS34	68	Ovarian Borderline Serous Cystadenoma	1C2	None	37	18	−0.68
CS35	54	Endometrioid adenocarcinoma ovary	1A	None	74	74	−0.53
CS36	57	Borderline Serous Carcinoma	1A	None	67	67	−0.91
CS37	60	Borderline Ovarian Serous tumour	1A	None	64	52	−0.84
CS38	60	Ovarian Fibroma	Benign	N/A	55	68	−0.49
CS39	55	Mucinous Cystadenoma	Benign	N/A	54	54	−1.05
CS40	51	Ovarian Fibroma	Benign	N/A	73	64	−0.76
CS41	64	Ovarian Fibroma	Benign	N/A	13	13	−1.17
CS42	51	Mucinous Cystadenoma	Benign	N/A	52	52	−0.16
CS43	46	HGSOC	N/A	None	12	12	−0.93
CS44	74	Ovarian Fibroma	Benign	N/A	37	37	−0.38

4.3. Association between δ^{65}Cu and cancer status

Serum δ^{65}Cu values were compared based on stage of the cancer disease. Using a Kruskal Wallis test, no association was found between cancer stage and δ^{65}Cu in blood, with a median of -0.58‰ (mean -0.61‰ ± 0.10) for stage 4, -0.83‰ (mean -0.86‰ ± 0.07) for stage 3, -0.87‰ (mean -0.79‰ ± 0.08) for stage 2 and -0.80‰ (mean -0.72‰ ± 0.12) for stage 1 patients ($p = 0.95$). Moreover, no association was found between stage in the disease and δ^{65}Cu in tumour mass. The low number of tumour mass samples from stage 4 patients may explain the lack of correlation.

The number of cycles of chemotherapy appeared to influence the δ^{65}Cu value in the biopsies. Patients receiving at least four cycles of chemotherapy had a significantly ($p = 0.05$) higher δ^{65}Cu ($n = 6$, median = -0.01, mean = 0.12‰ ± 0.08) than patients receiving no chemotherapy ($n = 7$, median = -0.10, mean = -0.15‰ ± 0.06).

Finally we decided to evaluate if the differences on δ^{65}Cu levels between patients were dependent on the patients age at the time of sample collection using a Pearson's Correlation Test. No significant association was observed between the patient's age and δ^{65}Cu (p-value = 0,115, r = -0272).

5. Discussion

In the present study the degree of copper fractionation was measured in blood and tissue samples obtained from ovarian cancer patients, and δ^{65}Cu was used to determine the ratio of the two stable copper isotopes. A positive δ^{65}Cu indicated depletion of circulating ^{63}Cu isotope, possibly due to an uptake by tumour cells, and conversely a negative value indicated an enrichment of the lighter ^{63}Cu isotope in serum. We observed that δ^{65}Cu was lower in the serum of ovarian cancer patient compared to that from healthy donors. Thus, as in breast and colorectal cancer [13], we observed that patients with ovarian tumours have higher levels of ^{65}Cu in the tumour mass than in blood. No correlation was made between the stage of cancer and serum δ^{65}Cu. Thus, whilst δ^{65}Cu correlates with ovarian cancer diagnosis it displays no specificity for cancer type or stage [13,17].

The mechanism behind the decrease of δ^{65}Cu in the serum of cancer patients has been suggested to be a consequence of the hypoxic tumour environment that increases the relative level of lactate compared to normal tissue and thus of an increased carboxyl group concentrations available for complexation with Cu [13]. Since the heavy ^{65}Cu copper Cu(I) isotope forms more stable bonds with carboxyl groups of lactate

Table 2
Ovarian cancer: diagnostic, stage, number of cycle of chemotherapy and analytical data on biopsy samples (CB = Cancer biopsy, * = pre-menopausal women, N/A = not applicable).

Sample Number		Age	Cancer Diagnosis	Stage	Nb of chemotherapy	Tumour δCu (‰)	Non Tumour δCu(‰)	Comments
CB6	Right	71	HGSOC	4B	6	−0.09		HGSOC
	Left		HGSOC			0.01		HGSOC
CB17	Right	78	HGSOC	3C	9	0.33		HGSOC
	Left							Not sampled
CB18	Right	67	HGSC of L fallopian tube	3C	6	−0.06		Benign
	Left					0.26		Benign
CB25	Right	58	HGSOC	3C	4	0.57		HGSOC
	Left					0.59		HGSOC
CB27	Right	66	HGSC of L fallopian tube	3C	6	−0.13		HGSOC
	Left					−0.11		HGSOC
CB28	Right	73	HGSC of L fallopian tube	3	4		−0.28	Benign
	Left					0.29		HGSOC
CB31	Right	47	Grade 3 endometrioid type	2A	None		−0.09	Normal
	Left							Endometrioid adenocarcinoma
CB34	Right	68	Borderline R Ovarian serous cystadenoma	1C2	None	−0.09		Borderline R Ovarian serous cystadenoma
	Left						0.08	Several serous inclusion cysts
CB35	Right	54	L Ovarian endometrioid adenocarcinoma	1A	None		−0.02	Endometriosis foci
	Left							Endometrioid adenocarcinoma with big endometriosis and cystadenoma
CB39	Right	55	Benign L mucinous cystadenoma	Not cancer	N/A		−0.22	Normal
	Left						−0.26	Benign L mucinous cystadenoma
CB41	Right	64	Benign R fibroma	Not cancer	N/A			Benign R fibroma
	Left						0.06	Normal
CB42	Right	51	Benign R mucinous cystadenoma	Not cancer	N/A		−0.22	Benign mucinous cystadenoma
	Left							Normal
CB43	Right	46	Leiomyosarcoma favoured to be of broad ligament origin	N/A	None		−0.63	Cellular spindle cell tumour with fascicular growth pattern w/ moderate to severe atypia
	Left						−0.11	Normal

than lighter Cu isotopes it would be preferentially chelated and thus retained in cancer cells, resulting in an apparent decrease of serum δ^{65}Cu [17]. In cells, copper is present predominantly as Cu(I) and is always complexed with transport proteins to overcome its reactivity, therefore avoiding the production of reactive oxygen species that can cause cellular damage. Ab initio calculations on the ability of organic molecules to coordinate with copper have been undertaken [18,19]. O donor (glutamine, threonine), N donor (histidine) and S donor (cysteine, methionine) amino acids19] were found to be preferentially bind ^{63}Cu light copper isotopes of Cu(II), and can also be assumed to fractionate as Cu(I) [20].

In ovarian cancer, patients often relapse following an initial positive response to platinum chemotherapy, developing resistance and thus reducing subsequent therapeutic options [9]. The most common mechanisms for resistance are the overexpression of ATP-dependent efflux pumps such as MDR1 and ATPases 7A and B [21,22]. The ATP-dependent copper transporter ATPases 7A and 7B supplies the Golgi with copper needed for enzyme synthesis. While ATPases 7A and 7B allows fine control of copper concentration in cells, its activity also allows tumour cells to detoxify platinum-based drugs which increase the resistance of the cells to treatment [23–25]. It seems likely therefore that copper fractionation in ovarian cancer following chemotherapy could occur due to the overexpression of ATP7ase A drug efflux pump [26,27] to detoxify Pt treated patients (Fig. 2). This is supported by our mass spectrometric analysis that revealed a decrease in the δ^{65}Cu value in ovarian cancer patients corresponding to a decrease in circulating ^{65}Cu in these patients (δ^{65}Cu = -0.80‰), and an increase δ^{65}Cu value in tumours (δ^{65}Cu = -0.01‰) compared to healthy tissue.

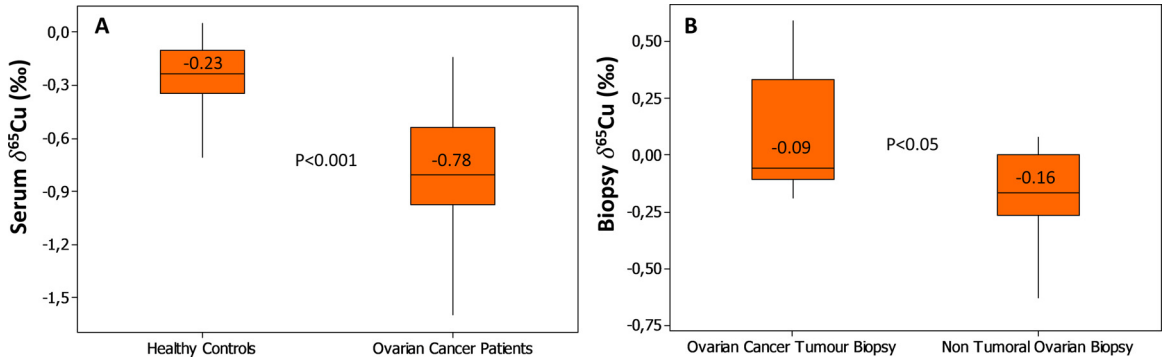

Fig. 1. Comparison of δ^{65}Cu between healthy blood donor and ovarian cancer blood and between ovarian cancer mass and non-tumour ovarian biopsy.
A) Whisker plot of serum δ^{65}Cu values for healthy women (48) compared to ovarian cancer patients (44). Boxes represent the 75 percent middle quantile and the whiskers 95 percent quantiles. Horizontal line: median. Separation between ovarian cancer patients and healthy women is significant ($p < 0.001$).
B) Whisker plot of 11 ovarian cancer mass and 10 contra-lateral ovaries that are either healthy either withdraw to be checked for cancer development. Significant difference has been measured between the two conditions ($p < 0.05$).

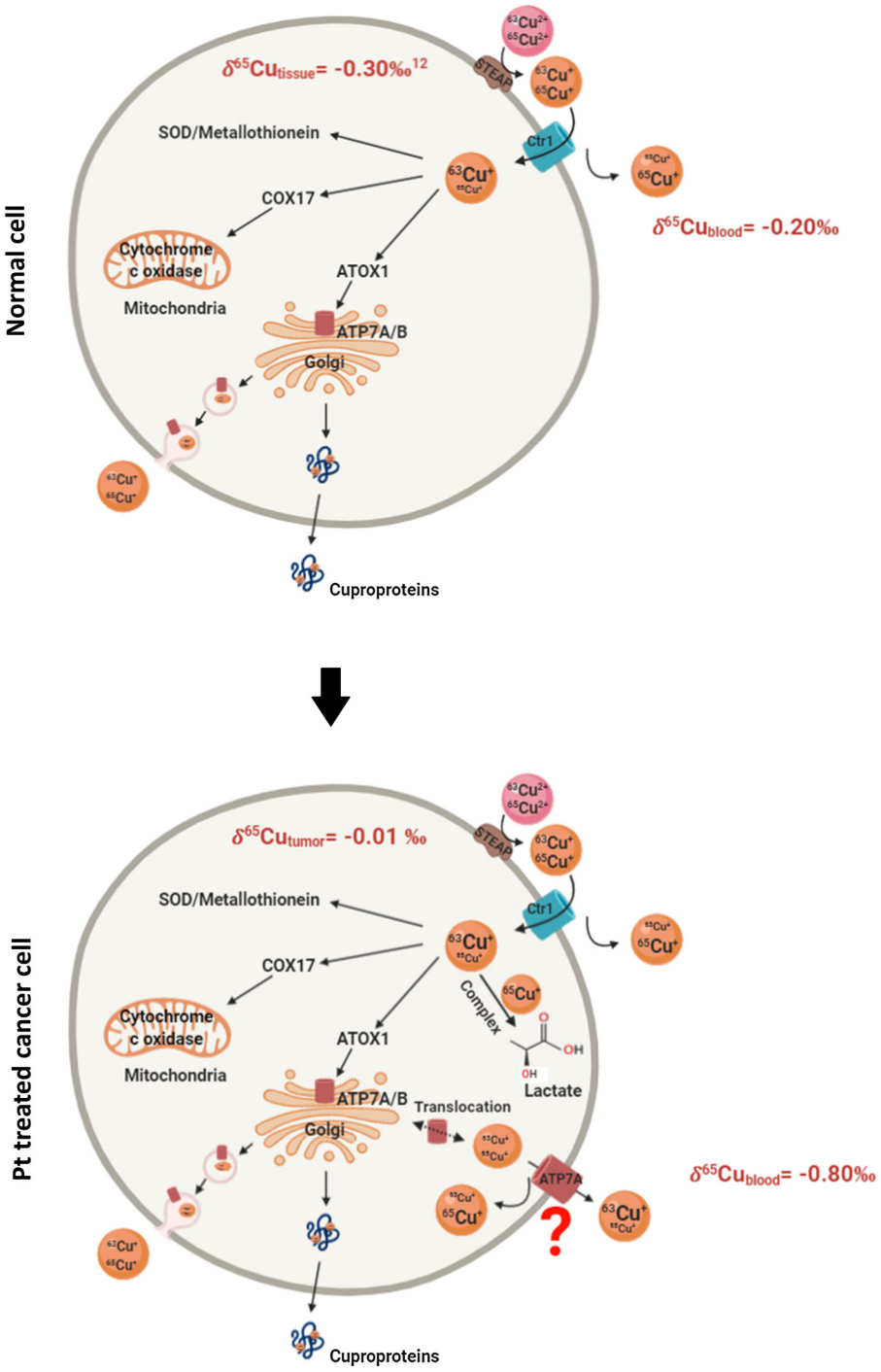

Fig. 2. Proposed mechanisms of copper fractionation between normal cells and platinum treated cancer cells.
Changes in the circulating ^{65}Cu levels from patients who have received chemotherapy may be multifactorial. The tumour environment is hypoxic and results in an increase in tumour cellular lactate metabolism leading to the preferentially chelation of heavy copper by lactate thus retaining this isoform of Cu in the tumour cells. In addition, selectivity of copper transporter toward light copper isotopes ^{63}Cu occurs due to transporter amino acid sequence composition [20]. In platinum treated cancer cells the copper transporter ATP7A would selectively export ^{63}Cu. This would result in ^{65}Cu being selectively retained in the tumour cells by lactate and increased expression of efflux copper transporter increasing the relative amount of ^{63}Cu in blood.

6. Conclusion

Here we have demonstrated that Cu serum $\delta^{65}Cu$ levels are lower in ovarian cancer patients than in healthy donors. Conversely, ovarian cancer biopsies display a higher $\delta^{65}Cu$ than serum from healthy volunteers. Our study highlights the potential of Cu as a functional biomarker for the detection of ovarian cancer. Current development of new automatized tools to perform the chemistry on biological samples and increased performance of measurement of MC-ICP-MS will allow the future implementation of $\delta^{65}Cu$ to aid ovarian cancer detection when CA-125 and other imaging techniques are not conclusive. A larger clinical study is now required to define $\delta^{65}Cu$ thresholds that would be indicative of the presence of the disease.

Ethics approval and consent to participate

This research was approved by NHS HRA Wales6REC (15/WA/0065) for collection of tissues and serum samples from ovarian cancer patients and non-cancer controls. Formal written consent was obtained from all patients at the time of recruitment into the study. Patients attending general gynaecology clinics or gynaecology oncology clinics in Swansea Bay and Cwm Taf Morgannwg NHS University Health Boards (SBUHB and CTMUHB) were recruited into this study. For control samples, blood samples were obtained by the Etablissement Français du Sang after patient agreement obtained through MTA13-1728.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

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Authors' contributions

Data collection, data analysis and manuscript drafting was performed by Benoit Toubhans. Alexandra T Gourlan, Laurent Charlet and Philippe Télouk contributed to the study conception and design. Clinical samples and data collection/analysis was performed by Lavinia Margarit and Kerryn Lutchman-Singh. R Steven Conlan, Deyarina Gonzalez and Lewis W Francis contributed to study design, critical interpretation of data and drafting of the manuscript. The authors read, amended and approved the final manuscript.

Declaration of Competing Interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2020.126611.

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