Experimental Cancer Medicine Centres (ECMC) Network and

National Cancer Research Institute (NCRI) Biomarkers & Imaging Clinical Studies Group

Cell-free DNA Consensus Meeting

Meeting report

Charles Wilson Building, University of Leicester
24th November 2014

In collaboration with

The Experimental Cancer Medicine Centre Initiative is jointly funded by Cancer Research UK, the National Institute for Health Research in England and the Health Departments for Scotland, Wales and Northern Ireland

Meeting Summary

34 invited attendees (listed in Appendix 1) representing expertise in cfDNA in 14 of the 18 ECMCs and 4 industry partners held at the University of Leicester.

Chaired by Prof. Robert Brown (Imperial ECMC) and hosted by Prof. Jacqui Shaw (Leicester ECMC). The meeting aimed to gain consensus from the ECMC and NCRI communities on:

- cfDNA techniques and analysis
- key research questions and clinical strategy

Recommendations

- To focus efforts and accelerate cfDNA in areas of greatest need, we recommend that research collaborations are developed in the top 5 priority disease areas identified on the day as having high unmet clinical need and as being tractable targets for cfDNA research (non-small cell lung, breast, ovarian prostate, colorectal cancers and in relapsed disease,– see page 5).
- To ensure consistent standards across the UK, we recommend that a generic Standard Operating Procedure (SOP) be developed for the use of different preservative tubes; EDTA, which is widely used, and alternatives such as the CellSave Preservative Tubes (Janssen Diagnostice) & Cell-Free DNA BCT (Streck Inc).
- To identify existing best practice in the clinic and the lab, in cancer and beyond, we recommend that a systematic review should be commissioned. This review should seek to identify factors known to affect quality and yield of cfDNA, and should identify existing efforts to tackle these issues.
- Developing studies looking at the unknown factors affecting variation in cfDNA quality and yield listed in this report and identified in a systematic review will allow the community to make evidence-based recommendations on handling, processing and analysis in cfDNA allowing for more comparable results between centres.

Key words:

cfDNA - circulating cell free DNA
ctDNA - circulating tumour DNA
CTC - circulating tumour cell,
MRD - minimal residual disease

Introduction

On 24th November 2014 the NCRI Biomarkers & Imaging Clinical Studies Group and the ECMC network held a consensus meeting comprising of research teams in the ECMC centres investigating cell-free DNA (cfDNA). The main aim of the day was to build on the NCRI Workshop held in January[1] and gain consensus on key areas identified in that meeting.

Summary overview and aims of the day

Prof. Bob Brown opened the meeting giving background to how the consensus meeting was developed. Following the National Cancer Research Institute (NCRI) Biomarkers and Imaging Clinical Studies Group workshop on cfDNA held in Manchester, the NCRI and Experimental Cancer Medicine Centres (ECMC) network jointly sought to bring together expertise from around the ECMC network on this topic.

Prof. Jacqui Shaw summarised the aims of the day to gain consensus on cfDNA techniques, analysis, research questions and clinical strategy and to generate a report/ position document/ publication to share with the wider community using cfDNA and industry via the ECMC and NCRI networks. The agenda and outline document was developed with an organising committee (listed in Appendix 2) with discussion of each of the items on the agenda to be led by a member of the committee.

Clinical application of cfDNA

Dr James Brenton outlined the aims of this session to turn the discussion in the January workshop into concrete recommendations and actions.

What is happening nationally was discussed to set context:

- *Genomics England Limited (GEL)* - 100, 000 genomes in NHS patients with 40% in cancer and 60% infectious diseases. Timescale is short aiming to collect 10,000 samples across England between January and April. There are now plans to bolt on ctDNA to this and other circulating nucleic acids (CNAs) e.g. miRNAs in a subset of the patients. The aim is to look at germline and somatic mutations (this represents a substantial investment).

[1] http://csg.ncri.org.uk/wp-content/uploads/2014/07/cfDNA-report_v2.pdf

- *TRACERx trial* – provides opportunities for looking at intra-tumoural heterogeneity (ITH), disease progression and emergence of .
- *Matrix Trial* – Prof. Gary Middleton outlined the opportunity for cfDNA research in this large CRUK trial with pharma partners, including 8 drugs and 20 molecular targets. Each arm will have a cohort of 30-40 well characterised patients with ctDNA for pre-, post- and during treatment.
- *Haematology* has led the field of liquid biopsy especially in acute leukaemia, which defined the concept of MRD; now able to address questions in solid tumours with CTCs and cfDNA.

Attendees discussed the clinical context into which cfDNA falls. These were:

- *Minimal residual disease (assessment of)*
- *Response markers* – to assess toxic treatments with unclear benefits
- *Liquid biopsy* – for prognostic markers or substituting for diagnostic test where there is a highly specific mutation, can a blood test replace biopsy in patients where a targeted agent could make a difference?
- *Broad understanding of ITH*– What is the relative dominance of a driver mutation in a treatment pathway, ITH and impact on patient care, understanding mechanism of resistance especially in the relapse setting and metastatic disease. *Poor performance status* – rare tumours and monogenetic tumours where driver mutations can be easily identified.

Attendees discussed criteria for selecting tumours of interest for cfDNA research. These were:

- *Mutation profile* – copy number aberrations/variation (CNA/V) and/or non-synonymous mutations (NS)
- *Availability in blood* – yield, is the tumour a secretor?
- *Sensitivity* – this was discussed in greater detail later in the meeting
- *Opportunity/ utility* – where a prospective trial has opportunity for serial liquid biopsy (e.g. Matrix) exists

Dr Tim Forshew raised whether the group should be looking beyond plasma. The possibility of expanding to urine and CSF was discussed but the scale for validating cfDNA in these samples would be a large jump and plasma analysis was considered more feasible for the near future.

Whether ct/cfDNA is representative of ITH and the benefits of mapping this for clinical utility was discussed. Dr Mariam Jamal-Hanjani suggested this could be viable by focussing on the driver mutations in ITH and use this as a predictor for relapse. Dr Brenton added that constant validation of new hotspots and drivers may not be

necessary and that looking at ITH is not useful for long-tail mutations. Dr Shirley Henderson queried whether clinicians would treat based on actionable mutations picked up in cfDNA and not tissue e.g. KRas? Prof. Gary Middleton suggested this could happen in EGFR-mutant lung cancer where biopsy may not be possible. Dr Liz Harrington added that AstraZeneca are working with Qiagen to make an assay commercially available to test for EGFR mutation on ctDNA (December 2014 gained FDA approval in USA).

Dr Chris Baker queried whether the assays would be looking for there/not there (+/-) mutations or more specific information on mutation frequency and if technology may be a limiting factor for the latter. Dr Harrington suggested that the group could develop a grid for new cfDNA markers looking at yield, there/not there assays, mutation spectra, actionable mutations available and how important a liquid biopsy is to the cohort to help investigators prioritise disease areas.

Dr Henderson commented that establishing a positive/negative cut-off level for mutation detection in cfDNA might be difficult. This difficulty had occurred in some areas of Haematology e.g.JAK2. Dr Henderson noted that she has a 50-gene panel on ion torrent, the signature mutation profile in some patients differs between biopsy and plasma.

Priority disease areas

By vote members prioritised the following disease areas based on the criteria and areas discussed above (additional comments made have been included):

1. *Non-small cell lung cancer*

Prof. Gary Middleton – proper targeted therapies, concentration high, good yield and highly mutagenic tumour

2. *Breast cancer*
3. *Relapsed disease*
4. *Prostate cancer*
5. *Colorectal cancer*

Dr Brenton – MRD in Duke's B opportunity.

6. *Ovarian cancer*
7. *Melanoma*
8. *Pancreas cancer*

Prof. Middleton – huge unmet need with pancreas because of the difficulty in getting tumour biopsy. Field is used to having small biopsies and doing a lot with the sample. Could do a study of pre-op ctDNA and then compare to resected tumour.

9. *Cancer of unknown primary (CUP)*
10. *Lymphoma*
11. *Oesophageal cancer*

Dr Brenton – opportunity for sequential biopsy to match with liquid biopsy.

Significant challenges

The group discussed disease areas which may represent significant challenges for cfDNA. These were:

- *Mesothelioma*

Prof. Shaw – not much evidence of any cfDNA studies here as yet and has genomic losses and gains and few common point mutations.

- *Pancreas*

Dr Brenton – strong correlation with disease burden but is KRas mutation representative of those who have failed treatment?

- *Brain*

Bettegowda paper[2] showing different cfDNA yield in different tumours especially brain due to blood-brain barrier.

- *Copy number variations*

CNV/A challenge large indels as cfDNA Is predominantly low molecular weight DNA.

Recommendations for collection of samples

Prof. Shaw summarised her group's findings in looking at different reported processes for cfDNA sample collection and analysis across centres. 2 spins (blood to isolate plasma, then re-spin plasma to pellet any residual cellular material) appears to be standard for many protocols however the rest is variable. Between 800-2000g for 1st and 2000-16000g for the 2nd spin appear to be tolerable variables. Prof. Shaw's lab runs with a lower first spin and higher second to avoid stressing cells and releasing cell DNA. The critical factor between spins appears to be pipetting technique used by the person. There is also significant variation between patients and there could be variation on how useable the sample is depending on factors like their meal before sample. Prof. Shaw demonstrated the difference in turbidity between samples from patients with and without a high-fat meal prior to blood draw.

[2] Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies. Science Translational Medicine. 2014;6:224ra24–4

Practical issues to consider limiting quality and yield variation

- *Extraction kit*

Some better for larger volumes e.g. CNA kit is better for plasma volumes > 1ml

- *Concentration in plasma*

Prof Shaw noted that different sized amplicons will give different data. When percentage recovery is the same, higher molecular weight amplicons will give a lower estimate cfDNA concentration.

- *Ability of centre to do double spin*

Dr Brenton noted that some centres don't have microfuges (for second high plasma spin) and have therefore been unable to run studies like ICON8. Dr Harrington suggested this could be taken into consideration and planned for when visiting sites and implementing a protocol.

- *Time to processing*

Long delay allows lysis of white blood cells (WBC) and causes contamination. Up to 2 hours appears to be similar to immediate processing but between 2-20 hours has variation due to contamination. This contamination with WBC DNA could be enough to mask a low frequency mutation. It was suggested that the Streck or CellSave tubes could reduce variation in processing particularly where delays are anticipated to help guarantee a higher mutant fraction.

- *Freeze and thaw effect*

Dr Frank McCaughan noted a KCL study has shown there is a difference in immediate processing and freezing and thawing before processing (even if not repeated). Dr Karen Page commented that Leicester have managed to process 12-year-old samples and recover cfDNA from plasma. Dr Guttery noted that quantification for these may be different using digital PCR to pick up single copies of genes. Prof. Shaw added that a 3rd spin may be possible after thawing before using the Qiagen kit to pellet any nuclei and reduce recovery of high molecular weight DNA.

- *CellSave/ Streck vs EDTA tubes*

Dr Dominic Rothwell summarised a number of studies in Manchester comparing CellSave with EDTA tubes with varied processing times, posted batches and looking at the effect of preservative in CellSave tubes on low-res NGS for both healthy normal volunteers and cancer patients. The results will be published in due course. He added that for blood processing the Manchester 'Gold standard' is to have sample for CTCs and cfDNA in the same tube so that info from these can be combined with PMBC for germline mutations and CTC derived xenograft (CDX)

tumours. There was good correlation of low-res genome-wide sequencing amongst all samples.

Dr Dana Tsui commented that Cambridge had looked at EDTA vs Streck tubes and found no significant difference introduced by the preservative in Streck tubes. It was noted that there is a significant cost difference between EDTA and CellSave (the latter £8/7.5ml tubes) and that EDTA tubes are widely available in NCRN phlebotomy labs.

- *Sample size*

Samples appear to vary by tumour type and downstream application. 5ml plasma is the maximum for the Qiagen CNA kit. It was noted that practicalities of taking larger blood volumes may be difficult but it is dependent on the patient's health and alternative biopsy options.

- *Picking up other mutations in germ-line DNA*

Ms Elliann Fairbairn commented that Wellcome Trust Sanger Centre are currently running a public survey[3] to establish attitudes to genetic findings, which could be used as a reference for ethics. Dr Brenton added that in a recent ovarian study 95% of participants wished to be informed of unexpected but clinically relevant findings and of genetic information as a concept.

Not critical factors appear to be:

- Spin speed (within the ranges mentioned previously)
- Temperature (at spin), room temp and 4^{o}C widely used
- Time to spin with EDTA tubes – minimum time preferred but can leave up to 6 hours on bench
- Temperature to store:-80^{o}C is standard if not immediately processed (but repeated freezing and thawing has a significant impact)

Key unknown factors affecting variation

- *Intra- and inter-site variation*

Prof. Middleton suggested a direct comparison on the same sample in one site and then between sites as a simple study to look at variation. Prof Shaw mentioned that this is being done by several groups.

- *Fasting*

Prof. Shaw noted the effect of high-fat meal prior to blood draw on analysis is unknown. Dr Harrington suggested that in proteomics 1/3 of samples are lycopoenic and that bringing up the spin or fasting would reduce the effect. Dr Brenton

[3] http://www.sanger.ac.uk/about/press/2013/130416-genomethics.html

cautioned that compliance is an issue with fasting especially for sequential samples but restriction of certain foods could be a viable alternative.

- *Time after therapy*

Prof. Brown queried whether there could be a rebound element in cfDNA as there is for PET where the metabolism kicks back in after therapy. Dr Rothwell noted this rise and fall can be seen in BRAF in melanoma. Dr Forshew added that a spike was seen in Luis Diaz's mouse model study of colorectal cancer[4]. Prof. Brown suggested looking at cytokeratin –18 where an apoptosis peak can be seen 48-72 hours after treatment. A study could be done in matched samples tying in with an existing biomarker to look for the peak after chemotherapy, radiotherapy and surgery.

- *Effect of undiagnosed cancer, microemboli and blood transfusions*

It was noted that white blood cells should be dead, in the case of transfusions, by the time of blood draw for cfDNA. Dr Brenton added it is critical to record microemboli as these may have an effect.

- *Mechanism of cfDNA release/ clearance and half-life in the body*

Dr Forshew commented that half-life of cfDNA in pre-natal is 1 hour but this could be different in ctDNA depending on mechanism. Dr Brenton added that cfDNA in urine appears to have passed through the liver so the process of filtration could be biochemical clearance/degradation rather than size.

- *Yield variation between patients*

Higher cfDNA yield is generally seen in cancer patients compared to health normal volunteers but yield also varies between cancers e.g. higher in lung (also see previous section Bettegowda paper) and within patients with the same cancer due to multiple factors including size, stage and individual variation.

Dr Henderson commented that variation between ethnicities is seen in foetal DNA e.g. lower yield in Afro-Caribbean women [5]and Prof Shaw added that similar variation is seen between individuals in forensic sciences with respect to cell/DNAshedding. Dr Forshew also noted that patient size could also be a factor e.g. detecting trisomy in larger patients is difficult because of larger blood volume.

- *Relationship between CTC and cfDNA yield*

[4] Rago C, et al. Serial assessment of human tumor burdens in mice by the analysis of circulating DNA. Cancer Res. 2007 Oct 1;67(19):9364-70. PubMed PMID: 17909045.

[5] Cell-free DNA levels in pregnancies at risk of sickle-cell disease and significant ethnic variation. Gerovassili A1, Nicolaides KH, Thein SL, Rees DC, Br J Haematol. 2006 Dec;135(5):738-41

Some tumours correlate (e.g. prostate) and some show good yield in one but not the other (e.g. Ovarian good cfDNA and poor CTCs by CellSearch).

State of the art for cfDNA technologies including industry perspective

Dr Alison Hargreaves, CRUK Account Lead, introduced her colleagues at Qiagen: Dr Marco Polidori and Dr Manuel Frietsch who gave an overview of current products available at those in development.

Dr Polidori summarised the products to assess liquid biopsy for free circulating nucleic acids, CTCs and exosomes and the challenges with these. Qiagen currently look at which areas of workflow can be automated and optimised for higher sensitivity and volume.

Dr Frietsch summarised the capabilities of the current kits: QIAamp DSP circulating NA kit and the QIAsymphony custom protocol & kit compared to the QIAsymphony DSP circulating DNA kit in development. The new kit will have optimised chemistry to support EDTA and Streck tubes as well as higher throughput than the existing customized kit.. He referred to the Spindler[6] paper which uses QIAsymphony customised kit to demonstrate analysis of KRas in plasma as an alternative to tissue analysis.

Dr Frietsch outlined a study[7] performed by QIAGEN in collaboration with LifeCodexx AG looking at blood sample processing in EDTA vs Streck showing the QIAsymphony kit in development has equivalent performance to the QIAamp DSP circulating NA kit.

Validation of different volumes was discussed and, although 2-4ml volumes have been validated, it was felt that having wider volume variation may not be an advantage.

Prof. Brown noted that the data from DNA exosomes with Qiagen's new kit could give good insight into epigenetics.

Dr Harrington, Director of Translational Science, introduced her colleague at AstraZeneca, Dr Simon Dearden from the personalised healthcare and biomarkers

[6] Spindler, KLG et al. Quantitative Cell-Free DNA, KRAS, and BRAF Mutations in Plasma from Patients with Metastatic Colorectal Cancer during Treatment with Cetuximab and Irinotecan, Clin Cancer Res February 15, 2012 18:1177-1185;

[7] "Purification of Circulating Cell-Free DNA from plasma using the automated Large-Volume Extraction on the QIAsymphony SP Instrument", poster presentation, AMP 2014

group and outlined industry interest in liquid biopsies for identifying patients, monitoring relapse and guiding combinations strategies.

Dr Dearden informed the group that at the end of September the MHRA had approved a label extension for Iressa to allow identification of patients with cfDNA where tissue is not available to evaluate. A recent study of 150 patients showed a single false positive and similar efficacy between cfDNA and tumour positives. In response to a question Dr Dearden noted that the label extension is going through the marketing companies, a CE marked kit will be available at the end of 2014 but discussions regarding funding are currently ongoing.

Dr Henderson noted that numerous companion diagnostics would not be viable for future targeted treatments and added that these test could be done with NGS. Dr Harrington responded that a CE marked companion diagnostic is required to ensure access.

Dr Harrington outlined where there is potential to go beyond hotspot assays with NGS using targeted assays compared to BEAMing.

Dr Brenton commented that in ovarian the mechanism of resistance to PARP inhibitors appears to be rearrangements in BRCA 1/2; cfDNA may face challenges picking up complicated or larger rearrangements but could look at MRD burden or driver mutations. Prof Shaw noted that CNA also has potential but sensitivity is a potential issue as 10% ctDNA would be needed for a reliable signal.

Prof. Middleton stressed the need to understand the clinical pertinence of markers in response and relapse e.g. T790 in lung. Dr Brenton added this understanding with liquid biopsy could provide an opportunity for refinement or drug holidays as seen in haematology. Prof. Middleton commented that in lung holidays could help provide protection for sensitive clones to help understand resistant clones however he noted that flare-ups during drug holidays can be fatal with prognosis as poor as 8 days.

Dr Henderson commented that allele frequency is currently collected but not reported to clinicians as the significance is not understood. Dr Brenton explained that in high-grade cirrhotic ovarian genes are expressed as an allele fraction of p53 to show heterogenetity.

Prof. Shaw pointed out the difficulty of using cfDNA as a one-off test, given variables discussed earlier, and that reference to a reliable base line is needed.

The suggested cut-off point for a reliable signal was discussed with 4% agreed as important (e.g. this is the range where a difference is seen in PFS for T790).

Sampling at the time of biopsy was also considered a potential variable as biopsy could increase cfDNA.

Parameters for validation of cfDNA biomarkers

Dr Jonathan Tugwood summarised the requirements for quantitative and qualitative biomarkers. Qualitative scoring samples and there/not there measures could be done with cfDNA. Dr Tugwood outlined various parameters and to what extent these need to apply to validation of cfDNA biomarkers including considerations for sample selection and size to validate. Dr Tugwood emphasised the need to assess what is feasible in cost and availability, reporting confidence intervals, where meaningful, and good implementation of QA and QC.

Prof. Brown suggested it would be useful in the detailed report to include how many current cfDNA tests fall into the parameter matrix included in Dr Tugwood's presentation.

Members discussed the issue of standardisation and sharing samples and procedures between ECMCs. It was acknowledged that although pre-clinical consortia exist to share procedures that sharing samples across the network remains an issue. Prof. Brown agreed to raise this issue with the ECMC Leads.

Conclusion and next steps

Prof. Brown thanked members for their input into the meeting and summarised the actions:

- A meeting report to be circulated to ECMC and NCRI communities by the ECMC office
- A link to the online video on centrifuge and blood handling to be shared by Prof. Shaw
- A more detailed report of guidelines and recommendations to be developed by Prof. Shaw and the team at Leicester ECMC

Recommendations

- To focus efforts and accelerate cfDNA in areas of greatest need, we recommend that research collaborations are developed in the priority disease areas identified on the day as having high unmet clinical need and as

being tractable targets for cfDNA research (non-small cell lung, breast, ovarian prostate and colorectal and in relapsed disease, – see page 5).

- To ensure consistent standards across the UK, we recommend that a generic Standard Operating Procedure (SOP) be developed for the use of different preservative tubes; EDTA, which is widely used, and alternatives such as CellSave & Streck.
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Appendix 1: Attendees ECMC cfDNA Consensus meeting 24th November 2014, Leicester ECMC

Name	Surname	Centre/ Organisation
Javier	Armisen-Garrido	Horizon Discovery Group plc
Chris	Baker	CRT / CRUK Translational Research Partnerships
James	Brenton	Cambridge ECMC
Robert	Brown	Imperial ECMC / NCRI BI CSG
Suzanne	Carreira	ICR ECMC
Simon	Dearden	Astrazeneca
Carole	Evans	Birmingham ECMC
Elliann	Fairbairn	ECMC
Tim	Forshew	UCL ECMC
Manuel	Frietsch	Qiagen
Anthony	Fullam	Wellcome Trust Sanger Institute
Davina	Gale	Inivata/ Cambridge ECMC
David	Guttery	Leicester ECMC
Alison	Hargreaves	Qiagen
Liz	Harrington	Astrazeneca
Shirley	Henderson	Oxford (NHS/ Diagnostics)
Lynn	Howells	Leicester ECMC
Mariam	Jamal-Hanjani	UCL ECMC
Frank	McCaughan	KCL ECMC
Gary	Middleton	Birmingham ECMC
Daniel	Nelmes	Cardiff ECMC
Brendan	O'Sullivan	Birmingham ECMC
Karen	Page	Leicester ECMC
Marco	Polidori	Qiagen
Craig	Robson	Newcastle ECMC / NCRI BI CSG
Dominic	Rothwell	Manchester ECMC
Jacqui	Shaw	Leicester ECMC
Gillian	Smith	Dundee ECMC
Fiona	Taylor	Sheffield ECMC
Fiona	Thomson	Glasgow ECMC
Dana	Tsui	Cambridge ECMC
Jonathan	Tugwood	Manchester ECMC
Steven	Walker	Belfast ECMC
Shen	Wei	Horizon Discovery Group plc

Appendix 2: Organising committee for the ECMC cfDNA consensus meeting

Name	**Surname**	**Centre/ Organisation**
Seema	Alexander	NCRI
Ged	Brady	Manchester ECMC
James	Brenton	Cambridge ECMC
Robert	Brown	Imperial ECMC / NCRI BI CSG
Elliann	Fairbairn	ECMC
Jacqui	Shaw	Leicester ECMC
Jonathan	Tugwood	Manchester ECMC