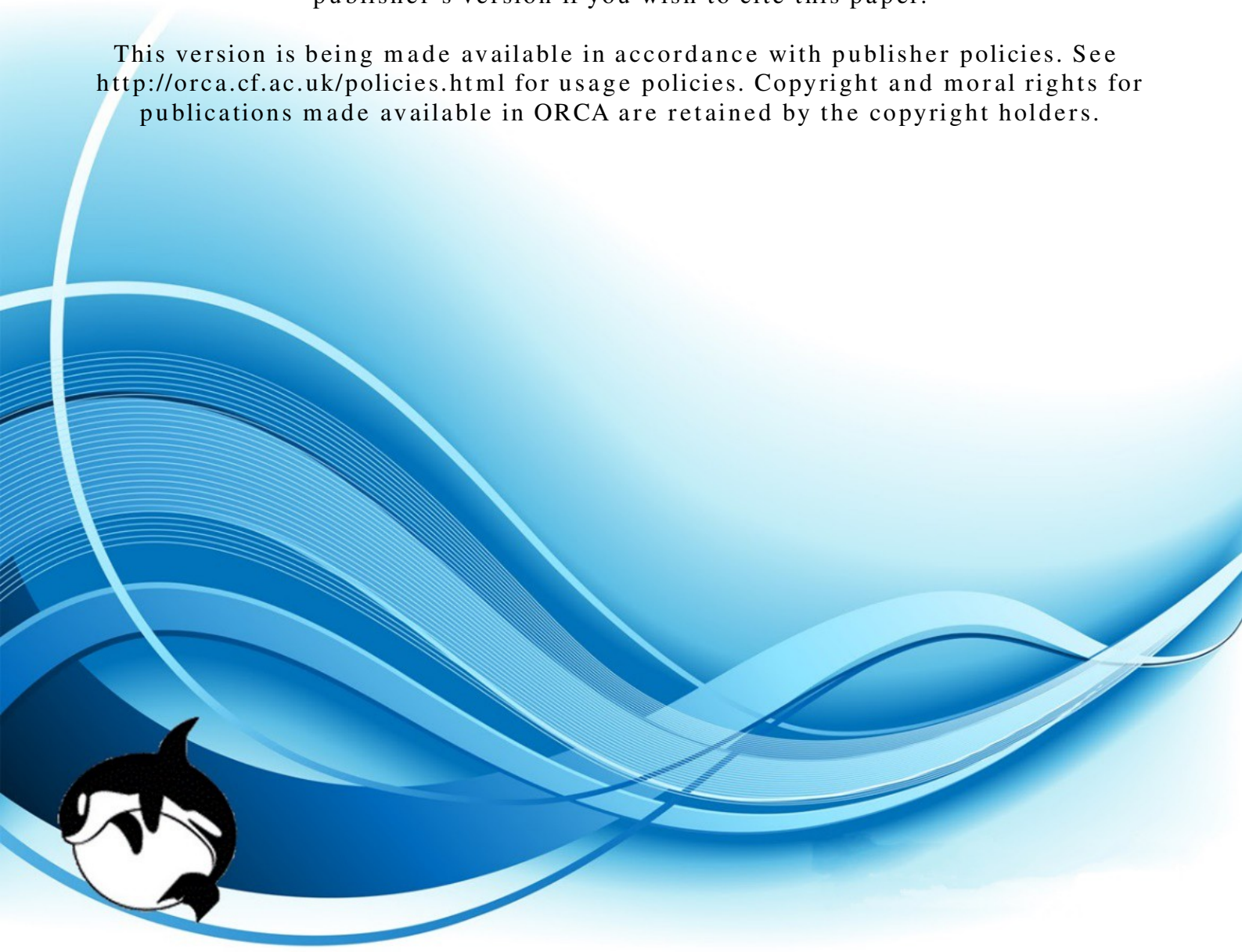

Gene panel testing for breast cancer should not be used to confirm associations with syndromic genes in particular for *NF1* pathogenic variants

Running Head: Panel testing for syndromic gene cancer associations

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In recent years there has been an explosion in the use of multi-gene panels to test for cancer predisposition often utilising large panels across many tumour types. More recently the results of these tests have been used as a form of case control study to assess genes for cancer associations, especially with breast cancer. Case control analysis is arguably the most informative method to identify gene-cancer associations as it also provides confirmation of the level of any increased risk. Ideally the 'cases' should be derived from a truly unselected series of individuals with the relevant cancer. Similarly the controls should come from the same population, and can be either true 'population' controls from representative, unselected and ideally age matched individuals, or 'super' controls (older/age matched individuals known not to have the malignancy being investigated). Particularly for rare diseases (present in less than 1 in 2000 individuals), very large case control series are required to confirm moderate risk elevations of only 2-3 fold. Whilst traditional methods such as positional cloning from family linkage were used for identifying the *BRCA1* and *BRCA2* genes in 1994 and 1995, most other breast cancer predisposition genes were identified as causal from candidate gene approaches using case control series often enhanced by using familial samples. However, even these have identified potentially spurious associations, because analyses of breast/ovarian cancer families has identified a real association with 'ovarian' cancer, but a potentially false association with breast cancer[1,2]. In particular, initial breast cancer associations with *RAD51C, RAD51D* and *BRIP1* were later called into question with breast cancer specific analysis [1,2]. Hence, these three genes are not on the UK's National Health Service breast cancer panel,[3] but they do appear on most commercial 'breast cancer' specific panels. Other DNA repair gene associations, such as for *ATM, CHEK2* and *PALB2*, have been well validated in multiple cohort studies and added to breast cancer specific gene panels.[3] In contrast to the aforementioned genes which have no recognisable syndromic phenotype for an individual, the phenotypes from germline pathogenic variants in *NF1, PTEN* and *STK11* (table 1) are usually easily recognisable in single individuals and there are well validated diagnostic criteria which allow a clinical diagnosis without the need for molecular confirmation.[4,5]

Four recent articles based on multi-gene panel testing, published in high impact oncology journals, have nonetheless concluded that there are no associations between pathogenic variants in these three syndromic genes (*NF1, PTEN* and *STK11*) and breast cancer risk.[6-9] Whilst these analyses have identified potential new gene associations, the negative results concerning syndromic associations should be tempered. In the first of their three articles Ambry's 21 panel gene test[6] was evaluated in 41,611 consecutively tested white women with breast cancer. In the second[7] 9,639 patients with breast cancer were assessed, whilst the third assessed the risk of triple negative breast cancer in 8,753 women. Whilst two studies used control data from the ExAc database[6,8] the second used a combination of controls tested for non-cancer indications.[7] The larger initial study[6] identified *NF1* gene variants in 0.1% compared to 0.11% in ExAc controls. No control frequency was provided for the second study although a frequency of around 0.15% was said to be non-significant[8]. The third study also found a frequency of 0.15% in triple negative breast cancer which was also non-significant.[8] The first two studies effectively excluded *BRCA1* and *BRCA2* as they confirmed that there had been a high degree of pre-testing for these genes. The first also excluded what they 'termed' 'syndromic' genes including *PTEN, CDH1* and *TP53.* However, it is unclear why neurofibromatosis 1, caused by pathogenic variants in the *NF1* gene, was not also excluded as being

syndromic, as it is far more recognisable from patient characteristics than even PTEN hamartoma syndrome.[4]

There are two main flaws in the conclusions that *NF1* is not associated with breast cancer. The first is that panel testing for breast cancer is selective based on family history, age and patient/clinician choice. A clinician who already has an 'explanation' for a breast cancer in a patient with NF1 is unlikely to send off for a gene panel. Indeed the syndromic learning problems associated with NF1 may also preclude gainful employment and thus reimbursement for panel testing. The link to breast cancer based on cohort studies is now irrefutable with six studies reporting Odds Ratios of 4-11 fold for NF1 women aged <50 years of age[10,11]. Furthermore, driver *NF1* pathogenic variants have been identified in the Cancer Genome Atlas and from NF1 patients are associated with higher tumour grade and Human Epidermal Growth Factor Receptor 2 (HER2) overexpression,[10,12,13] further evidence against a chance association. The link with breast cancer and NF1 has been established since at least 2007[11]. All of the reported panel tests were performed since March 2012[6-8], after four of the cohort studies had reported showing a probable causal association with breast cancer and NF1[11]. The authors of the Ambry reports clearly admit that there was already preselection for BRCA testing[6-8]. If a clinician wished to test for *NF1* they would use a substantially more sensitive RNA based approach than a panel[14]. A similar concern should also be expressed for the apparent lack of association with *PTEN* in the second and third Ambry studies[7,8] as these were not powered to assess the lower frequency of *PTEN* pathogenic variants and suffer from the same issue of lack of requirement for a panel test when an explanation for breast cancer was already present. The same criticism can be put forward for the absence of *STK11* variants being identified in 2000 familial breast cancer samples tested in the LIFEPOOL study.[9] Peutz-Jeghers disease caused by *STK11* variants is easily identified by peri-oral pigmentation and usually presents symptomatically in early life with multiple intestinal polyps (Table 1).

The second potential flaw is the use of ExAc controls. The very high frequency of apparent pathogenic variants of 1/900 is simply not consistent with the estimates of birth incidence from highly ascertained populations of between 1 in 2,000-2,600[15,16]. Due to early deaths in NF1 patients prevalence in an adult population is nearer 1 in 3,000-4,500.[15,16] It is therefore unclear whether this high estimate in controls is due to over assessment of pathogenic variants, selection for children to have exomes with NF1 features or due to some variants being silent clinically.

Whilst panel tests appear to be a useful agnostic test for cancer associations with non-syndromic genes, they are not when considering easily recognisable syndromes, as these create biases in selection against any association and may also contaminate controls. The only way to robustly assess for links with syndromes in a case control study is for ALL patients with breast cancer to be tested on a population basis with appropriate controls tested of a similar age, also without selection.

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Table 1: Syndromic genes identifiable from clinical features in a single woman that increase breast cancer risk from cohort studies

Gene	Syndrome	Birth incidence	Clinical features	Breast cancer risk to 50 yrs/lifetime	Other malignancy risk
NF1	Neurofibromatosis 1	1 in 2,000-2,500	*Café au lait* Cutaneous neurofibromas Iris Lisch nodules	10%/20%	Malignant peripheral nerve sheath tumor, glioma
PTEN	PTEN hamartoma syndrome (Cowden)[3]	1 in 100,000-200,000	Macrocephaly, mucocutaneous lesions (eg Trichilemmomas)	50%/85%	Thyroid, endometrial
STK11	Peutz-Jeghers[4]	1:25,000 to 1:280,000	Peri-oral pigmentation, hamartomatous bowel polyps	Nk/37-55%	Colorectal, stomach, small bowel, ovary, cervix, pancreas, testes