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# Clinical disease course and survival outcomes following disease recurrence in adenoid cystic carcinoma with and without NOTCH signaling pathway activation

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ABSTRACT

*Background:* Adenoid cystic carcinoma (ACC) is a rare salivary cancer. The highest rates of disease recurrence are in patients with NOTCH pathway activation, reported in up to 20%. Novel drugs targeting NOTCH signaling are under investigation in the recurrent/metastatic (R/M) setting. To understand their clinical utility, there is an urgent need to better characterize the disease course and outcomes following current standard of care treatment.
*Methods:* 120 patients with R/M ACC underwent clinical review at a single UK Cancer Centre. Patients were retrospectively assessed for tumor NOTCH pathway activation using next generation sequencing (NGS) targeting NOTCH1/2/3 genes and/or NOTCH1 intra-cellular domain (NICD1) immunohistochemistry. Demographic and treatment data were extracted from the clinical notes. Kaplan-Meier survival analysis was performed using log rank test.
*Results:* NOTCH pathway activation was identified in 13/120 patients (11 %). In 12/101 patients analyzed by NGS, NOTCH1/3 activating somatic mutations were identified, and a further patient was identified with NICD1 diffuse nuclear staining in whom NGS testing was not possible. Patients with NOTCH pathway activation had shorter median RFS (1.1 vs 3.4 years, $p = 0.2032$) and significantly reduced median OS from diagnosis (4.0 vs 16.3 years, $p < 0.0001$). There was significantly reduced median OS from time of disease recurrence/metastasis (1.9 vs 9.6 years, $p < 0.0001$).
*Conclusion:* This study clearly demonstrates a reduction in OS from time of first confirmed disease recurrence/metastasis for patients with NOTCH pathway activated ACC. This provides support for developing new drugs for this sub-group of patients, for whom clinical outcomes are significantly worse and effective treatments are lacking.

## Background

Adenoid cystic carcinoma (ACC) is a rare cancer which is resistant to systemic therapies in most cases [1]. The majority originate within salivary glands with a smaller number occurring in other secretory glands within the trachea, lung and breast [1]. Most cases present with localized disease, however, ACC is characterized by high rates of recurrence and distant metastasis [2].

Whole exome sequencing of ACC tumour samples has identified NOTCH pathway activating alterations in up to 20 % of patients [3,4]. The NOTCH family consists of four transmembrane protein receptors (NOTCH-1, −2, −3, −4). There are five ligands involved in the activation of NOTCH receptors: Delta-like ligand (DLL) −1, −3 and −4 and Jagged (JAG) −1 and −2 [5]. Ligand activation results in pathway activation via release of the NOTCH intracellular domain (NICD) and transcriptional activator complex formation. NOTCH signaling is involved in a number of oncogenic processes including cell proliferation, survival, migration, angiogenesis, stem cell renewal and metastasis.

In addition to next generation sequencing (NGS) to identify NOTCH mutations, immunohistochemistry (IHC) staining for NICD1 is another

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established approach to identifying NOTCH pathway activation. NOTCH pathway activation, as indicated by increased NICD protein expression in the nucleus has been observed in patients with gain-of-function NOTCH1 and NOTCH3 mutations compared to those with wild-type NOTCH [6,7]. These authors reported that NOTCH1/3 mutations were significantly associated with decreased relapse-free survival and overall survival following primary diagnosis compared to NOTCH1/3 wild-type patients.

Therapies are under development to target NOTCH including receptor/ligand antibodies, gamma secretase inhibitors and transcription complex inhibitors [8,9,10]. The majority of ACC patients have resection of localized disease followed by a disease-free interval of many years prior to recurrence [7] and drug therapies are being evaluated in the recurrent or metastatic setting. To better understand the clinical utility of candidate drug therapies targeting NOTCH pathway activated ACC there is a need to better characterize the disease course and survival outcomes following disease recurrence, as the point from which systemic therapies are being evaluated.

## Patients and Methods

### *Patient consent*

One hundred and twenty patients with ACC provided informed consent to an ethically approved study permitting analysis of their clinical and genomic data. The study was granted research ethics approval under the MCRC Biobank Research Tissue Bank Ethics (NHS NW Research Ethics Committee 18/NW/0092) and was performed in accordance with the Declaration of Helsinki.

### *NOTCH-1, −2 and −3 analysis by next generation sequencing*

DNA was extracted from FFPE tumour blocks from 101 ACC patients in the Manchester Centre for Genomic Medicine and assessed for alterations in NOTCH1, NOTCH2 and NOTCH3 genes by next generation sequencing (Illumina TST-170, using a minimum DNA input of minimum 40 ng per sample) covering the entire exonic regions of NOTCH1/2/3 genes.

Genomic somatic alterations were detected from raw reads using Illumina's somatic variant caller Pisces (with min-variant-frequency filter 0.02 and min-depth-filter 100). We assessed only mutations falling inside two specific areas of the NOTCH1/2/3 gene, one area spanning the heterodimerization-negative regulatory region (NRR) and the other area spanning the proline, glutamic acid, serine, threonine-rich (PEST) domain. Alterations were classified as gain of function if the mutations disrupted the NRR or PEST domains. A further patient was found to have a NOTCH activating mutation from an existing FoundationOne® report and was therefore included in NGS analysis.

### *NICD1 analysis by immunohistochemistry*

FFPE sections from 88 ACC patients were stained for NOTCH1 intracellular domain (NICD1) immunohistochemistry (IHC) using cleaved NOTCH1 (Val1744 (D3B8) rabbit mAb (Cell Signaling mAb #4147) on Leica Bond Max instrument [11]. MDA-MD-157 cell line and normal tonsil tissue were used as positive controls. Normal tonsil tissue stained without the primary antibody were used as negative controls. Cases were classed as NICD1 positive if on pathology review there was diffusely positive nuclear staining in tumor cells.

### *Clinical data collection*

Patients who were referred to assess their suitability for systemic therapies within clinical trials underwent clinical review at a single UK Cancer Centre from 2017 to 2020. Treatment data including surgery, radiotherapy and systemic therapies were extracted and patients followed up for survival outcomes.

### *cBioportal analysis of NOTCH pathway activation*

One thousand and two hundred patients were reviewed via cBioportal. One hundred and fifty with complete survival data were identified. Twelve were identified as having NOTCH1/3 pathway activation mutation with criteria applied as in section 2.2.

### *Statistical analysis*

To evaluate for any difference in the clinical characteristics between patients with and without NOTCH pathway activation, univariable analysis was performed between the groups using the Fisher's exact test for categorical data and an unpaired *t* test for continuous variables.

Recurrence free survival (RFS), survival from recurrence and overall survival (OS) rates were estimated using the Kaplan-Meier method and the log-rank test. Data was censored on December 2020. RFS was calculated from diagnosis to the date of first confirmed disease recurrence or metastasis. Survival from recurrence was calculated from the date of first confirmed disease recurrence or metastasis to the date of death of any cause or last known follow up. OS was calculated from diagnosis to the date of death of any cause or last known follow up. Statistical analyses were conducted using GraphPad Prism (Version 9.0, Graph- Pad Software, San Diego, CA, USA) and SPSS (version 27.0; IBM Corporation, Somer, NY, USA).

## Results

### *Patient and disease characteristics*

For all patients in this study, median age at diagnosis was 48 years (range 17 to 75 years), with 50 (42 %) male and 70 (58 %) female patients. The primary site was major salivary gland in 58 (48 %) patients, with 62 (52 %) from a minor salivary gland site. Ninety-six percent (115/120) of patients had confirmed recurrent or metastatic disease, with lung being the most common site of recurrence (n = 80, 79 %). Other sites of recurrence include localized (n = 38, 33 %), liver (n = 16, 13 %) and bone (n = 13, 11 %).

### *Classification of NOTCH pathway activation by NGS*

As the predominant mechanism for NOTCH pathway activation in ACC is a gain of function mutation in the NOTCH1/2/3 genes [6], NGS was performed to determine NOTCH1/2/3 status in patients for whom sufficient DNA was available for analysis. NOTCH1/3 activating somatic mutations which were predicted to disrupt the function of the NRR or PEST domains were identified in 12 % (12/101) of ACC patients by NGS (Fig. 1Ai and ii and Table 1). The majority of the alterations were in NOTCH1 (9/12) with 3 patients having gain of function mutation in NOTCH3. These NOTCH1/3 alterations are consistent with those described in 15 % (n = 22/144) of ACC patients in cBioportal (Fig. 1Bi-ii). The majority of alterations affecting the NRR were missense single amino acid substitutions (5/6), with one insertion. Of the alterations disrupting the PEST domain, almost all (8/9) resulted in truncating frameshifts, with one missense substitution.

Of the 12 patients who had a NOTCH*1/3* activating mutation identified using NGS, 58 % (7/12) were analyzed on DNA extracted from the primary tumour site and 42 % (5/12) were from sites of metastatic disease (Table 1). In comparison, of the 89 patients with wild-type NOTCH*1/3* identified using NGS, 47 % (42/89) were analyzed on DNA from the primary tumour site and 44 % (39/89) were from metastatic disease. The site of biopsy was unknown in 9 % (8/89) of patients in the NOTCH wild-type group.

Lollipop plots of NOTCH1/3 mutated genes are shown in Fig. 1. 265 distinct NOTCH alterations (missense, frameshift or inframe-indels)

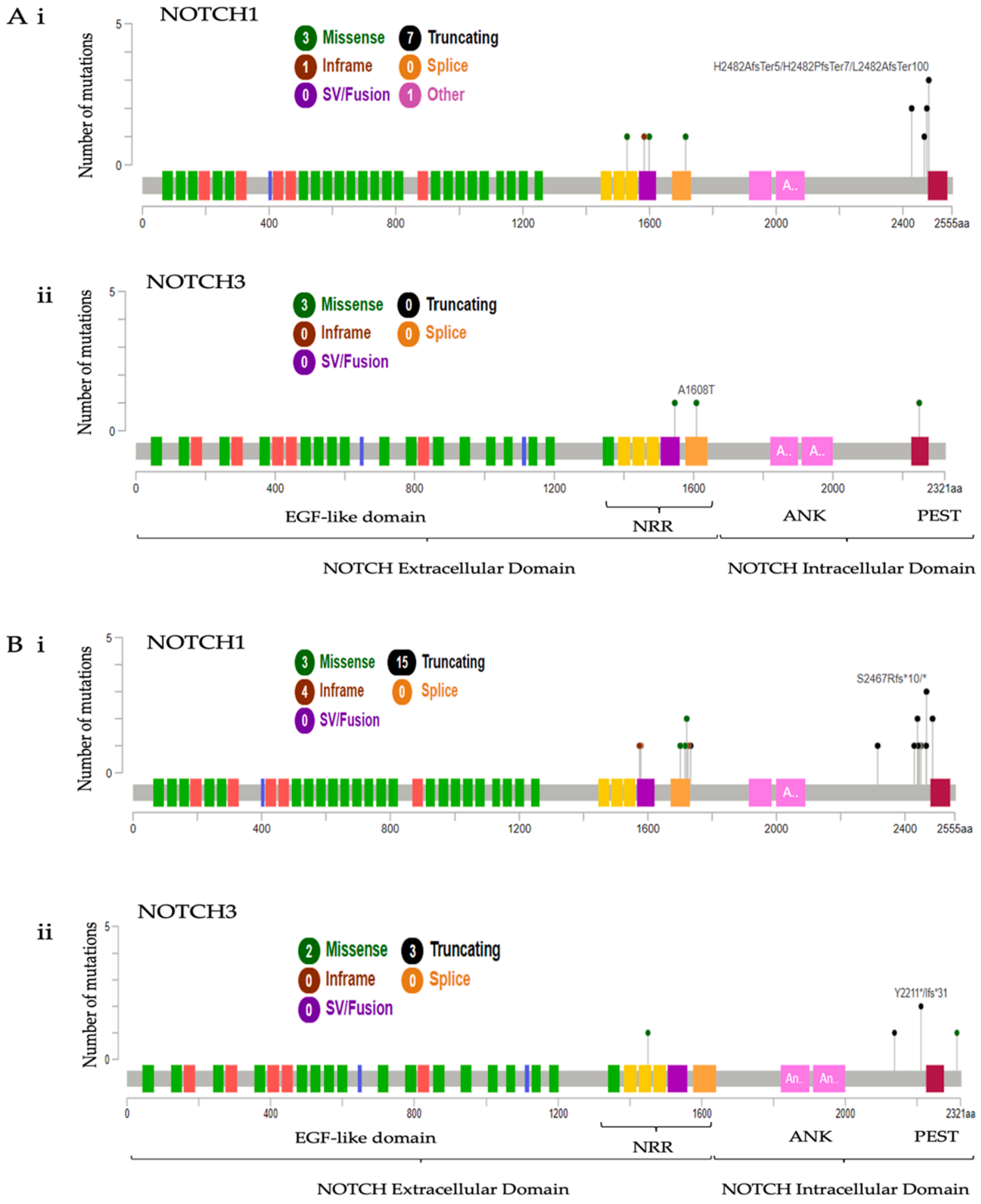


**Fig. 1.** NOTCH1/3 gain of function mutations in ACC in the current study (Ai-ii) and in the cBioportal analysis (Bi-ii). NRR = negative regulatory region; ANK = ankyrin; PEST = Pro-Glu-Ser-Thr-rich domain.

were observed, with 14 considered activating mutations found in hotspot regions (NRR and PEST domains).

Identification of additional patients with NOTCH pathway activation using NICD immunohistochemistry.

As it is possible for NOTCH pathway activation to occur independently of NOTCH gene gain of function mutation [5], where FFPE tumor blocks were available (88/120 patients), patients were analyzed for NICD1 diffuse nuclear staining by IHC (Fig. 2A). This approach identified one further patient with NOTCH pathway activation in whom there was insufficient tumour for DNA based NGS. In total, combining patients with NOTCH gain of function mutation by NGS and diffuse NICD1 nuclear staining by IHC, 13/120 (11 %) patients had evidence of NOTCH pathway activation. Of the 5 patients with NICD-positive IHC staining, all 5 were analyzed on tissue samples obtained from the primary tumour site (Table 1).

Comparison of the clinical characteristics for ACC with and without NOTCH pathway activation.

The clinical characteristics of patients with and without NOTCH pathway activation are summarized in Table 2. In NOTCH pathway activated ACC, primary site was major salivary gland in 7/13 (54 %).

**Table 1**
Summary of cases with NOTCH pathway activation.

| NOTCH mutation (1) | Amino acid change (1) | NOTCH mutation (2) | Amino acid change (2) | NOTCH Domain | NICD1 IHC | Primary Site | Histology | NGS biopsy site | NICD1 IHC biopsy site |
|---|---|---|---|---|---|---|---|---|---|
| **NGS analysis alone identifying NOTCH 1/3 gain of function mutation** | | | | | | | | | |
| NOTCH1 | His2428AlafsTer5 | NA | NA | PEST | NA | Minor (sphenoid sinus) | NA | Primary tumour | NA |
| NOTCH1 | Val1599Gly | NOTCH1 | Val2476CysfsTer9 | NRR/PEST | NA | Major | NA | Metastatic | NA |
| NOTCH3 | Arg1546Cys | NA | NA | NRR | NA | Minor (maxillary sinus) | NA | Primary tumour | NA |
| NOTCH3 | Ala1608Thr | NOTCH3 | Ser2248Phe | NRR/PEST | NA | Major | NA | Primary tumour | NA |
| **NGS and NICD1 analyses identifying both NOTCH1/3 gain of function mutation and diffuse NICD1 nuclear staining** | | | | | | | | | |
| NOTCH1 | Leu2482AlafsTer100 | NA | NA | PEST | Positive | Major | Solid & Tub/ Crib | Metastatic | Primary tumour |
| NOTCH1 | Pro1582_Glu1583insSerValProValLeuMetProPro | NA | NA | NRR | Positive | Major | Solid & Tub/ Crib | Metastatic | Primary tumour |
| NOTCH1 | His2428ProfsTer7 | NA | NA | PEST | Positive | Minor (palatal) | Solid & Tub/ Crib | Primary tumour | Primary tumour |
| NOTCH1 | His2428ProfsTer7 | NA | NA | PEST | Positive | Minor (carinal) | Solid | Primary tumour | Primary tumour |
| **NGS and NICD1 analyses identifying NOTCH1/3 gain of function mutation without diffuse NICD1 nuclear staining** | | | | | | | | | |
| NOTCH1 | Ile1714Leu | NA | NA | NRR | Negative (H-score 100) | Major | Tub/Crib | Primary tumour | Primary tumour |
| NOTCH1 | Leu2468CysfsTer9 | NA | NA | PEST | Negative (H-score 10) | Major | Scant NOS | Primary tumour | Primary tumour |
| NOTCH1 | His2428ProfsTer7 | NOTCH1 | Val2476ArgfsTer6 | PEST | Negative (H-score 60) | Minor (tracheal) | Solid & Tub/ Crib | Metastatic | Metastatic |
| NOTCH3 | Ala1529Pro | NA | NA | NRR | Negative (H-score 30) | Minor (floor of mouth) | Solid & Tub/ Crib | Metastatic | Metastatic |
| **NICD1 analysis alone identifying diffuse NICD1 nuclear staining** | | | | | | | | | |
| NA | NA | NA | NA | NA | Positive | Major | Solid | NA | Primary tumour |

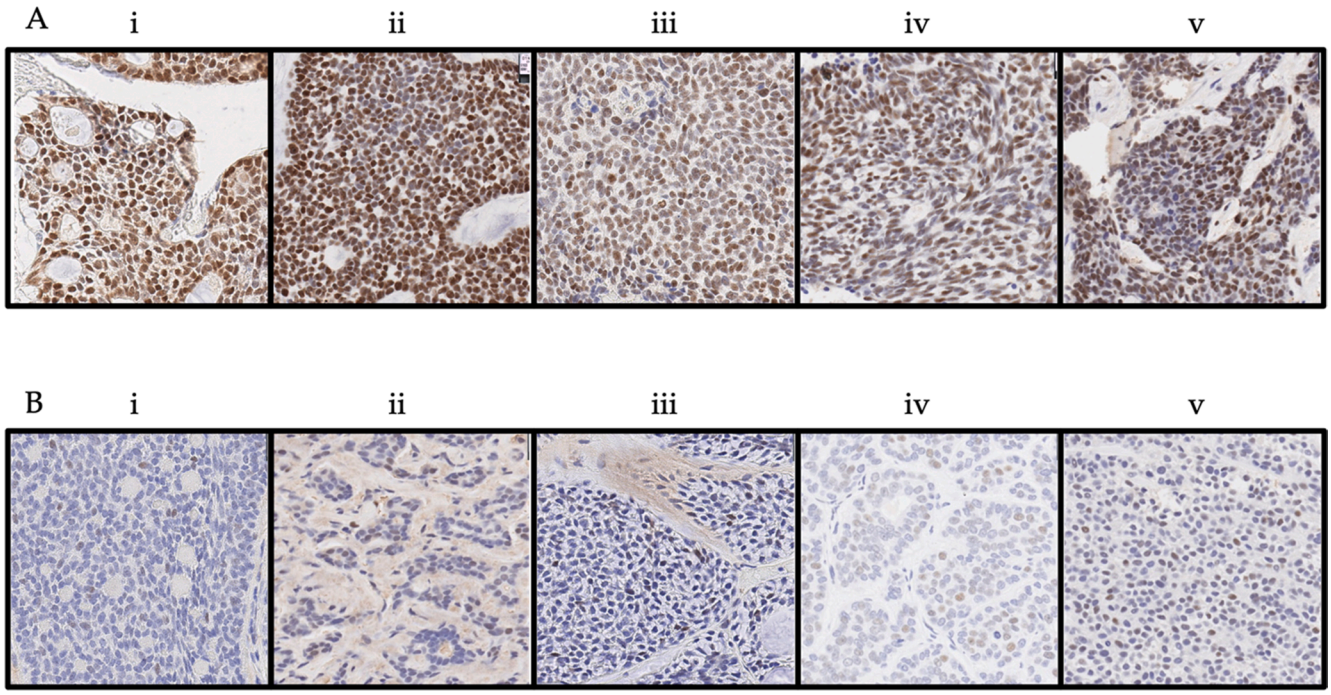


**Fig. 2.** NOTCH intracellular domain IHC in ACC in A) NICD1 positive cases, B) NICD1 negative cases.

**Table 2**
Baseline characteristics.

| Characteristic | Total population (n = 120) | NOTCH activation (n = 13) | No NOTCH activation (n = 107) | *p* value |
|---|---|---|---|---|
| **Age at diagnosis, median (range)** | 48 (17 to 75) | 46 (22 to 74) | 48 (17 to 75) | 0.614 |
| **Sex (%)** | | | | 1.000 |
| **Male** | 50 (42) | 5 (38) | 45 (42) | |
| **Female** | 70 (58) | 8 (62) | 62 (58) | |
| **Primary site** | | | | 0.773 |
| **Major** | 58 (48) | 7 (54) | 51 (48) | |
| **Minor** | 62 (52) | 6 (46) | 56 (52) | |
| **Site of metastasis** | | | | |
| **Local** | 38 (32) | 5 (39) | 33 (31) | 0.547 |
| **Lung** | 80 (67) | 7 (54) | 73 (68) | 0.355 |
| **Liver** | 16 (13) | 3 (23) | 13 (12) | 0.379 |
| **Bone** | 13 (11) | 2 (15) | 11 (10) | 0.632 |
| **Lymph node** | 5 (4) | 1 (8) | 4 (4) | 0.442 |
| **Skin** | 5 (4) | 1 (8) | 4 (4) | 0.442 |
| **Treatment** | | | | |
| **Surgery** | 107 (89) | 9/13 (69) | 98/107 (92) | 0.035* |
| **Adjuvant RT/ CRT** | 98 (82) | 6/13 (46) | 92/107 (86) | 0.002* |
| **Palliative SACT** | 33 (28) | 6/13 (46) | 27/107 (25) | 0.184 |
| **Lines of palliative SACT** | **n = 33/120** | **n = 6/13** | **n = 27/107** | 0.379 |
| **1** | 21/33 (64) | 5/6 (83) | 16/27 (59) | |
| **2+** | 12/33 (36) | 1/6 (17) | 11/27 (41) | |
| **Chemotherapy** | 14/33 (42) | 3/6 (50) | 12/27 (44) | 1.000 |
| **TKI** | 12/33 (36) | 0 | 12/27 (44) | 1.000 |
| **Experimental therapy/ clinical trial** | 14/33 (42) | 4/6 (67) | 12/27 (44) | 0.229 |

Abbreviations: RT - radiotherapy; CRT - chemo-radiotherapy; SACT - Systemic anti-Cancer Therapy; TKI - Tyrosine Kinase Inhibitor. * denotes a statistically significant *p* value.

These patients had a higher proportion of metastases to the liver (23 % vs 12 %; p = 0.379) and bone (15 % vs 10 %; p = 0.632) compared to NOTCH wild-type patients. All patients had a specialist multi-disciplinary review to determine their suitability for treatment with curative intent at their first clinical presentation. Significantly fewer patients with NOTCH activated ACC presented with operable disease and underwent surgery (69 % vs 92 %; p = 0.035) or received adjuvant radiation/chemoradiation (46 % vs 86 %; p = 0.002) due to either the presence of inoperable locally advanced disease or of distant metastasis. A higher percentage of patients with NOTCH activated ACC received palliative systemic therapy (46 % vs 25 %; p = 0.184) compared to NOTCH wildtype patients. This clinical decision making followed national guidelines and took place prior to any knowledge of the individual patients NOTCH status.

Comparison of recurrence free survival and overall survival from primary diagnosis.

Consistent with previous reports [6], patients with NOTCH pathway activation (n = 13) had shorter RFS (median RFS 1.1 vs 3.4 years, $p = 0.2032$) and significantly reduced OS from diagnosis (median OS 4.0 vs 16.3 years, $p < 0.0001$) (Fig. 3A). Median follow up from diagnosis was 7 years (range 0 to 38 years).

Comparison of recurrence free survival and overall survival from first confirmed recurrence.

As drug therapies are being evaluated in ACC in the recurrent or metastatic setting, our primary aim was to characterize the clinical outcomes following disease recurrence in patients with and without NOTCH pathway activation. There was significantly reduced OS from time of first confirmed disease recurrence or metastasis (1.9 vs 9.6 years, $p < 0.0001$) (Fig. 3B). This reduction in OS for NOTCH activation following recurrence was seen consistently whether patients were classified using NGS (2.2 vs 9.6 years, $p < 0.0001$) or NICD1 IHC (0.8 vs 9.6 years, $p < 0.0001$) (Fig. 3Bii and iii).

Fig. 4 highlights the significant reduction in RFS, OS from diagnosis and OS from first recurrence in patients with NOTCH pathway activation compared to those without. At time of censoring, only 2/13 (15 %) patients with NOTCH pathway activation were alive compared to 76/107 (71 %) of patients without NOTCH pathway activation.

Previous studies have reported an association with poor overall survival in ACC patients with TP53 loss of function mutations [11]. TP53 status was available for 102/120 patients in the current study; 12/13 patients with NOTCH pathway activation and 90/107 patients without. One patient (1/12, 8 %) in the NOTCH pathway activation group was

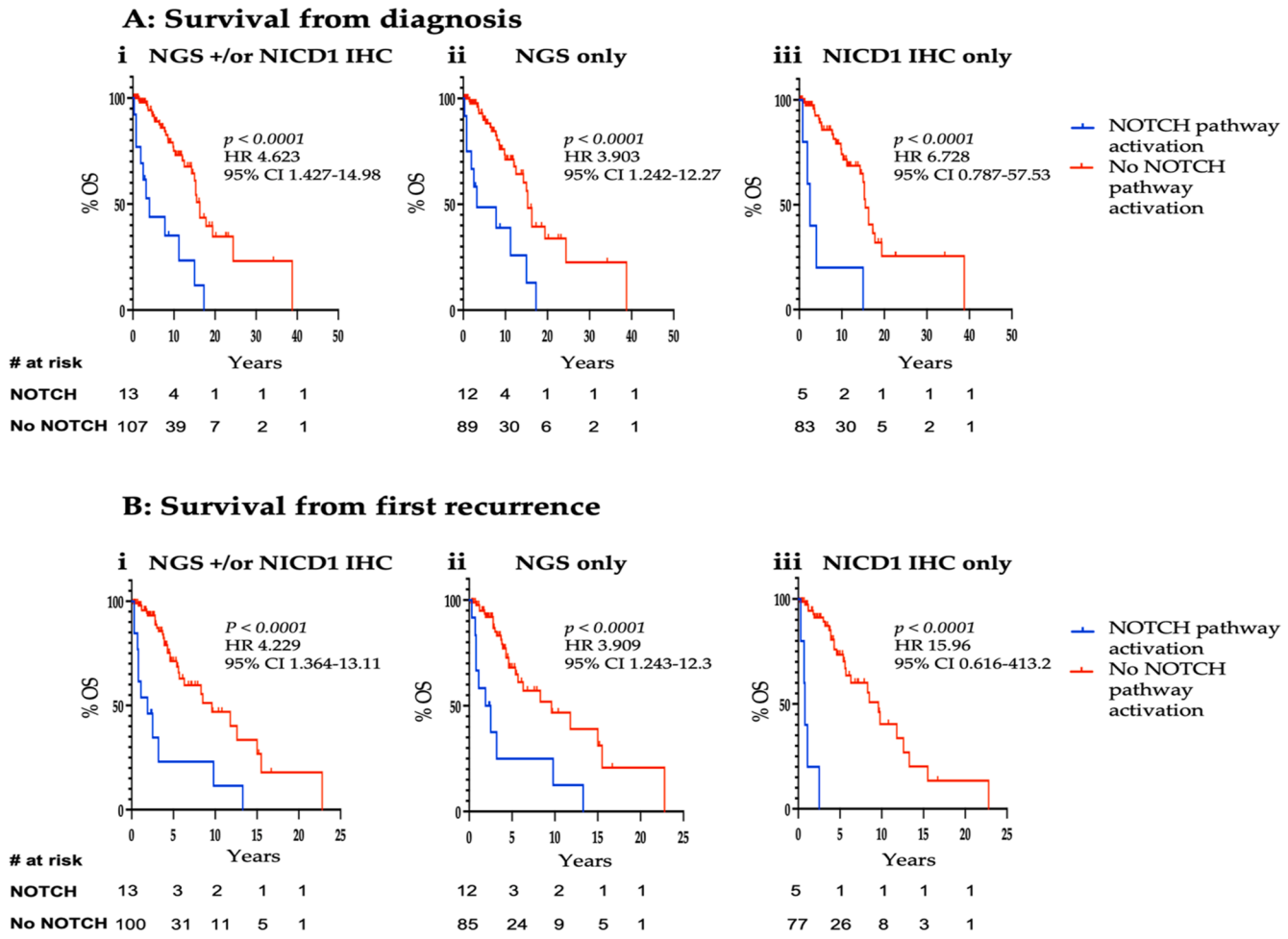


**Fig. 3.** Kaplan-Meier estimate for: (Ai-iii) Overall survival from diagnosis, defined as time from diagnosis to death from any cause; (Bi-iii) Survival from first recurrence, defined as time from first recurrence to date of death from any cause, dashes indicate censored events.

identified as having a TP53 loss of function mutation (Fig. 4). A similar percentage (8/90, 9 %) of patients were identified as having a TP53 loss of function mutation in the group without NOTCH pathway activation (Fig. 4).

## Discussion

The NOTCH signaling pathway plays a critical role in regulating development and homeostasis [11]. However, NOTCH signaling is also integral to tumorigenesis and has been shown to facilitate both tumour suppression and oncogenesis across numerous tumour types [12]. Overall, combining the NOTCH NGS and NICD1 IHC approaches, the incidence of NOTCH pathway activation in this cohort was 11 % (13/120). Of these 13, 54 % (7/13) were assessed for both NOTCH1/3 activating mutation and NICD IHC staining. Of these 7, 71 % (5/7) were analyzed using the same biopsy site for both NGS and NICD1 IHC (Table 1). Forty-three percent (3/7) showed concordance in NOTCH activation (NOTCH activating mutation identified with positive NICD1 IHC staining), whilst 57 % (4/7) were discordant (NOTCH activating mutation identified without NICD1 IHC positive staining).

There is no established clinical approach to which testing should be applied to identify patients with NOTCH pathway activation. This is the first study that has shown that when using the combined approach of DNA based NGS and NICD1 IHC to a large cohort of patients seeking clinical trial therapies that both assays have the potential to provide additional utility in patient classification as NOTCH pathway activated.

As mentioned previously, activation of the NOTCH signaling pathway has been reported as a key component in ACC pathogenesis with an associated aggressive phenotype and poor prognosis [6]. As systemic therapies are being developed in patients with R/M ACC, it is important to understand the clinical impact of NOTCH pathway activation on patient outcomes following disease recurrence. Earlier studies have reported the outcomes for patients with NOTCH mutated ACC from the point of first diagnosis [3,4,6,7]. However this has the potential to over-estimate the longevity of these patients as there is frequently a significant disease-free interval from primary diagnosis which may be many years. In the current study, despite a lower frequency of detection of NOTCH activating mutations, a similar association with poor overall survival from diagnosis was observed and, notably, from the time of first confirmed disease recurrence or metastasis. To our knowledge this is the first study to describe clinical outcomes and patient survival times from the point of disease recurrence, and the median overall survival of less than two years from the point of recurrence demonstrated emphasizes the clinical need for the development of new treatments in this group. Over the past decade, different classes of drugs therapeutically targeting NOTCH have been clinically tested. Novel NOTCH inhibitors have been investigated in phase II clinical trials (NCT03691207; NCT03422679).

To define genomic sub-groups of ACC patients using DNA based NGS, only a few studies have variably distinguished between any NOTCH mutations and NOTCH activating mutations, and these activating mutations have been limited to NOTCH1 mutations [4,6,7]. In the clinical characterisation described in this study we have applied the approach of secondary functional classification of NOTCH gene mutations as activating only if they were predicted to disrupt the function of the NRR or

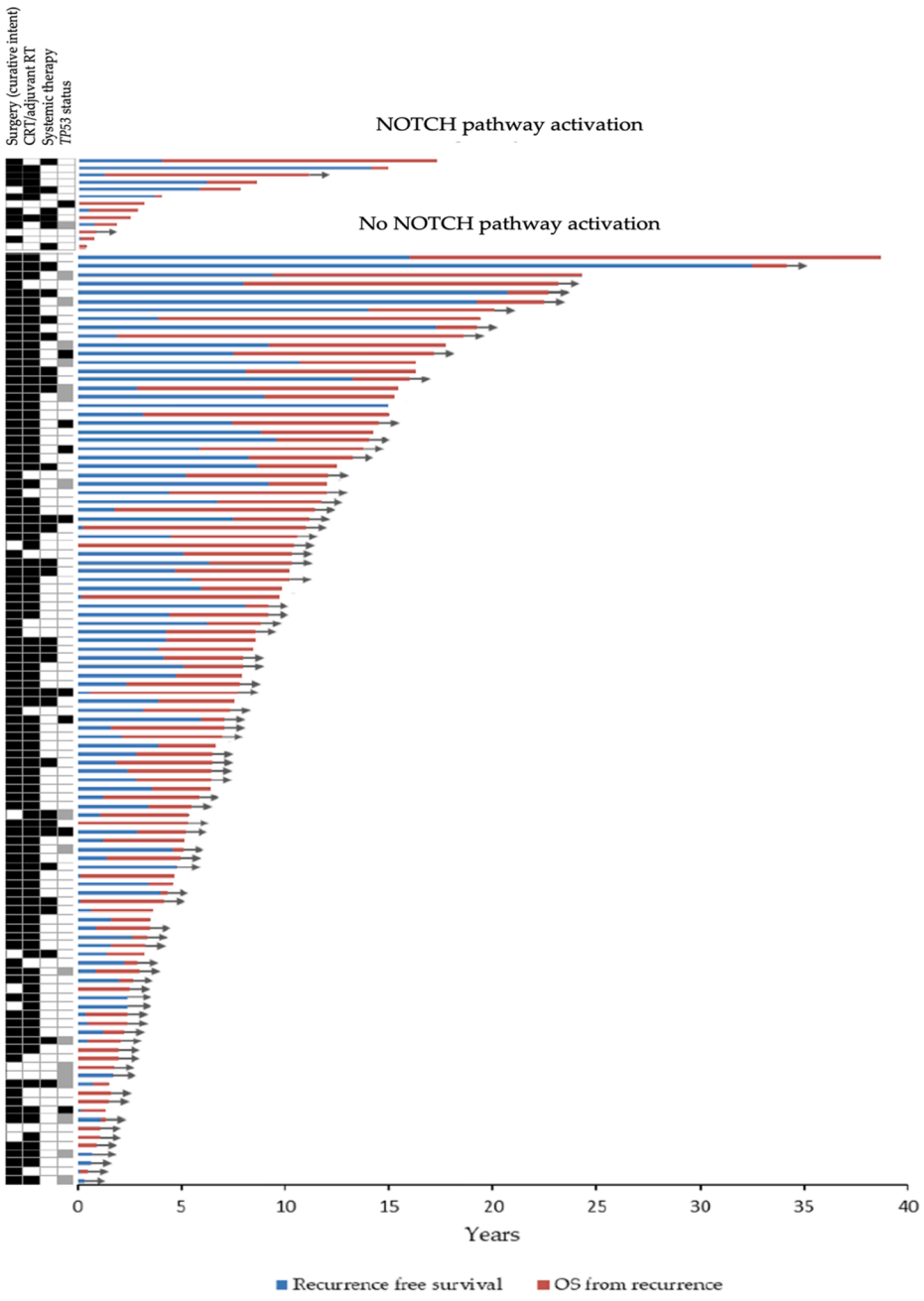


**Fig. 4.** Swimmer's plot of time from diagnosis to first recurrence (blue bar) and survival from first recurrence to death or last known follow-up (red bar) for patients with NOTCH pathway activation and No NOTCH pathway activation. Each bar represents an individual patient, with the length of the bar corresponding to the time of overall survival. Arrow indicates patient is alive. Columns along the y axis indicate whether the patient was (black box) or was not (white box) treated with surgery (curative intent), chemoradiotherapy (CRT)/adjuvant radiotherapy or palliative systemic therapy. TP53 status indicated as mutant (black box), wild-type (white box) or unknown (grey box). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PEST domains as NOTCH gene mutation causing NOTCH signaling pathway activation was considered to be a biologically relevant population. This approach is however more restrictive and the current study has described a lower described frequency of patients in the NOTCH pathway activated sub-group (11 %) compared with those with NOTCH mutated ACC reported previously (up to 20 %) [3,4,6].

Previous studies have reported a higher prevalence of NOTCH pathway activation in R/M ACC compared to primary ACC [4]. This distinction was not clearly evident in our cohort with a similar proportion of patients with NGS analysis carried out on tissue obtained from metastasis in the NOTCH pathway activation (42 %, 5/12) and no pathway activation (44 %, 42/89) groups. Furthermore, all patients (5/5) with NICD-positive IHC staining were analyzed on tissue samples obtained from the primary tumour site. Comprehensive data on the site of biopsy for the majority of patients who underwent NICD1 IHC was not available.

Intra-patient heterogeneity and tumour evolution are very well described across multiple cancer types [13]. NOTCH pathway genes have been identified as early genetic events in ACC [14]. Previous reports including primary and metastatic samples to assess NOTCH gene mutations, without any additional functional classification or IHC classification such as carried out in the current study, describe a 23 % frequency in primary tumours compared to a 27 % frequency from the metastasis [4]. However, there is no secondary functional analysis of NOTCH gene mutations to describe the frequency of NOTCH activating mutations in this earlier analysis and this remains an area of ongoing investigation specifically with respect to NOTCH signaling pathway activation in ACC.

Patients with NOTCH pathway activation mutation have been reported to exhibited a higher likelihood of developing liver and/or bone metastasis [6]. Several studies have investigated the interplay between epithelial-to-mesenchymal transition (EMT) and NOTCH signaling in the context of vascular intravasation facilitating distant metastasis [15–17]. However, the precise role of NOTCH signaling in this context

remains uncertain.

Although TP53 is mutated in many cancers, it is not as frequently mutated in ACC, as observed in this study with 9/102 (9 %) ACC patients reported as having a TP53 mutation [18]. In mouse models, concomitant NOTCH activation and TP53 loss of function have been shown to promote epithelial-to-mesenchymal transition (EMT) and enhance invasive or aggressive phenotypes [19]. Within this study cohort, there did not appear to be an association between NOTCH pathway activation and TP53 loss of function mutations.

A limitation of the current study is the lack of availability of TNM staging data at first diagnosis for all the patients studied. This was the case as patients were referred from multiple centres in the UK, and formal TNM staging at first presentation was performed through external centres and sometimes a number of years prior to recurrence. There would however be potential utility of comparison between genomic classification and TNM classification to provide additional insight into this clinical sub-group and further analysis in a surgically annotated cohort with TNM data would be of value to investigate this further.

In the cohort of patients included in the study, recurrent or metastatic disease was present in 96 % at the time of analysis. In part, this high frequency of recurrent or metastatic disease is due to the long duration of follow up for patients included in this analysis, with a median follow up of 7 years and a range of up to 38 years. However, in addition, patients included in the current study were undergoing clinical review to consider systemic therapies within clinical trials. As the role of systemic therapies within clinical trials is predominantly restricted to the recurrent or metastatic setting, this results in the high frequency of recurrent/metastatic disease described. Although the greatest need for new therapies is in the setting of disease recurrence, the results of the current study should be interpreted in this context and further studies in a cohort of patients having disease resection with curative intent are warranted. The authors acknowledge that for this case series patients were selected based on recurrence/metastatic disease and by definition the cohort is therefore biased towards those with worse outcomes. Furthermore, survival from diagnosis may be underestimated as the population does not include patients who underwent surgical resection with no recurrence. However, the findings of this study are in keeping with published data and adds to our understanding of the more rapidly progressive disease course experienced by ACC patients with NOTCH activating mutations.

## Conclusion

This is the first study to report OS from time of first confirmed disease recurrence or metastasis for patients with NOTCH pathway activated ACC. Although R/M ACC is frequently considered an indolent disease, this provides support for developing new drugs for the sub-group of ACC with NOTCH pathway activation, for whom effective therapies remain devastatingly short.

## Funding

This research was funded by Ayala Pharmaceuticals, Syncona Foundation, The Infrastructure Industry Foundation and The Christie Charity.

## Institutional Review Board Statement

The study was granted research ethics approval under the MCRC Biobank Research Tissue Bank Ethics (NHS NW Research Ethics Committee 18/NW/0092) and was performed in accordance with the Declaration of Helsinki.

## Informed Consent Statement

All subjects provided informed consent to collection of demographic, clinical and genomic data included in this study.

## CRediT authorship contribution statement

**Laura Feeney:** Data curation, Writing – original draft, Visualization. **Brindley Hapuarachi:** Formal analysis, Data curation, Writing – original draft, Visualization. **Helen Adderley:** Data curation. **Sam Rack:** Formal analysis, Data curation, Visualization. **David Morgan:** Formal analysis. **Russell Walker:** Conceptualization, Resources, Data curation. **Rami Rauch:** Resources. **Elad Herz:** Resources. **Joel Kaye:** Resources. **Kevin Harrington:** . **Robert Metcalf:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [KH conflict of interest includes Arch Oncology, AstraZeneca, Bristol-Myers Squibb, Boehringer Ingelheim, Merck Sernon, MSD, Oncolys BioPharma, Replimune, Inzen Therapeutics, Pfizer, Codiak Biosciences and Merck Sharp & Dohme. RM conflict of interest includes Bristol-Myers Squibb, Merck Sharp & Dohme, Roche, Bayer, Achillies Therapeutics, Aptus Clinical, PCI Biotech, Ayala Pharmaceuticals and OxSonics. All other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.].

## Data Availability Statement:

Data will be made available to clinical and academic partners upon request within the terms of the relevant ethical agreements provided.

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