Progress report to AHS project no. 140 "Investigation of IL13-RA2, EGFR and EphA2 as tumor associated antigens and targets for immunotherapy in canine brain tumors"

1. Summary of the project (with keywords), from the grant proposal

Malignant brain tumors cause severe neurological deficits and are associated with poor prognosis due to aggressive growth and high recurrence rates. No specific therapies leading to complete tumor remission are currently available for **canine malignant brain tumors**. It is expected that increasing accuracy in diagnosis and prognosis of canine brain tumors together with the pet's changing role in societal perception will raise the demand for **new tumor-specific treatment modalities** in dogs. Our research teams are aiming at investigating microbial (vaccine) vectors for **targeted therapy of canine brain tumors** using canine distemper virus and *Listeria monocytogenes*. To prove the efficiency of our targeted microbial vector therapy strategy in canine brain tumors we are in need of eligible **brain tumor associated antigens (TAA)**. However, systematic and unbiased "discovery" studies for TAA in canine brain tumors are lacking. Therefore, we propose here to screen three potential brain TAA candidates (**EGFR, IL-13Rα2 and EphA2**) for their suitability as canine brain TAA. The identification of suitable brain TAA will be a significant step forward in the diagnosis and targeted therapy of canine brain tumors as it will allow the **development of tools** for the specific ***in vivo* diagnosis** and **therapeutic targeting** of brain tumors.

2. Progress of the project

2.1. Tissue sampling

Tissue sampling of normal and fresh canine tissues for tumor antigen (TAA) detection is finalized. The fresh/frozen tissue sampling of canine brain tumors is still ongoing due to the limited tumor availability.

2.2. Canine brain tumor microarray

The canine brain tumor microarray has been constructed at the DBMR, University of Bern (Prof. Inti Zlobec). The array contains annotated regions of 39 oligodendrogliomas, 32 astrocytomas and 29 meningiomas in triplicates and additionally 47 control tissues. All canine primary brain tumors that are included in the tissue microarray have been characterized and graded.

2.3. Immunohistochemistry

2.3.1. EGFR (Master thesis Luigina Lanci, PhD thesis William Pownall)

Epithelial growth factor receptor (EGFR) expression was assessed by immunohistochemistry. The receptor is not expressed in the normal brain and most other organs (Figure 1 and Figure 2A). However, EGFR is expressed at low to high levels in epithelial organs such as skin, oesophagus, liver, kidney and bladder (Master's thesis Luigina Lanci, Figure 2A). William Pownall analysed EGFR expression in the brain tumour microarray (section 2.2.) designed specifically for this study. EGFR was expressed in different types of brain tumours. The prevalence and intensity of EGFR expression was highest in astrocytomas (Figure 2B), and expression was clearly associated with tumour grade: while low-grade astrocytomas (grade I and II) did not express EGFR, a high proportion of grade III and IV astrocytomas strongly overexpressed EGFR. Expression was particularly consistent and strong in gliomatosis cerebri, a subtype of grade III astrocytoma. EGFR expression was inconsistent and relatively low in meningiomas, oligodendrogliomas and oligoastrocytomas (Figure 2B).

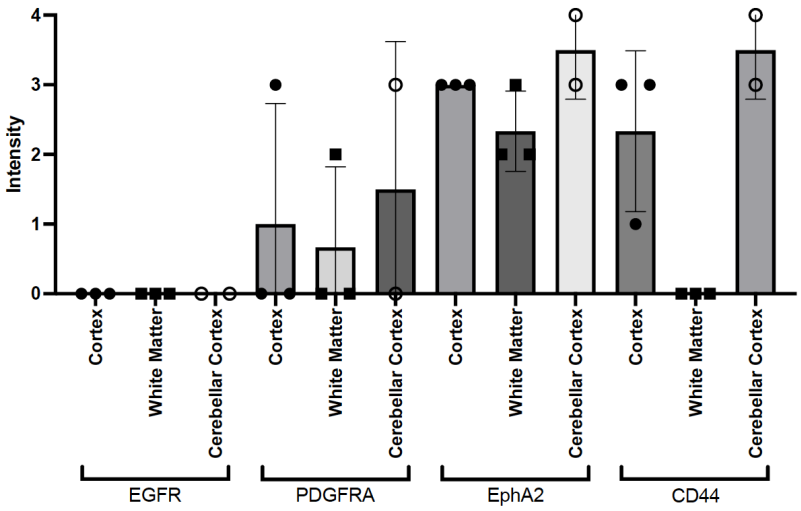


Figure 1: Expression of investigated TAA candidates in normal brain. While EGFR is not expressed, PDGFRA, EphA2 and CD44 are expressed at varying levels.

Our results suggest that EGFR could be a potential TAA candidate for immunotherapy of astrocytomas/gliomatosis cerebri. However, although EGFR is not expressed in the normal brain, the strong expression of this growth factor in skin, urinary tract and oesophagus suggests that these organs may be targets of autoimmune reactions and that therapeutic intervention may result in off-target reactions.
Therefore, in a follow-up project, the EGFR of astrocytomas will be sequenced to identify mutated EGFR that could be used as a tumour-specific antigen and targeted by immunotherapy to avoid or reduce off-target reactions.

2.3.2. EphA2

Ephrin type A receptor 2 (EphA2) was the tumour antigen with the strongest and most consistent expression in both brain tumours and normal tissues (Figure 1, 3A and B). These results suggest that EphA2 is widely expressed in the body and is not be suitable as a TAA for immunotherapy because of the high risk of widespread autoimmune response following targeting.

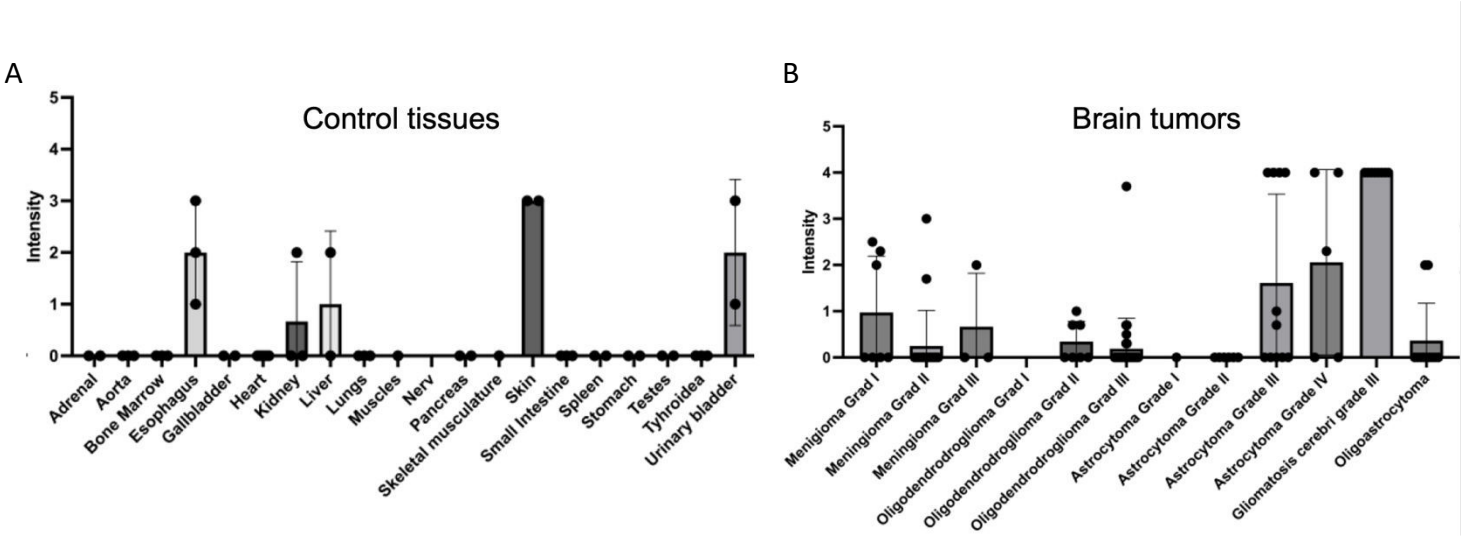


Figure 2: Expression of EGFR in healthy control tissues and in brain tumors. A) EGFR is not expressed in most normal tissues, but strongly expressed in the skin, moderately expressed in the esophagus and at low levels in the urinary bladder. B) EGFR is strongly expressed in high grade astrocytomas including gliomatosis cerebri. Other brain tumors express EGFR more irregularly and at lower levels.

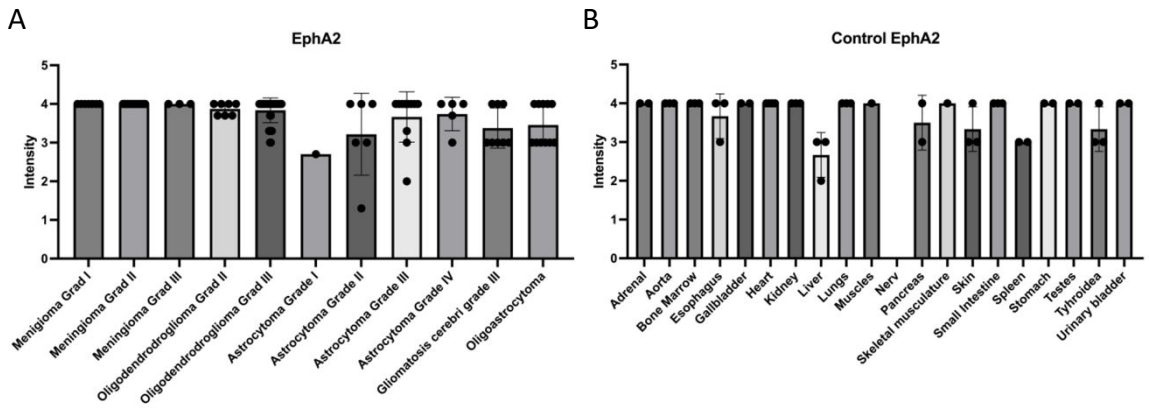


Figure 3: Expression of EphA2 in healthy control tissues and in brain tumors. EphA2 is strongly expressed in brain tumor types (A), but also in all control tissues (B). The peripheral nerve was not analyzed.

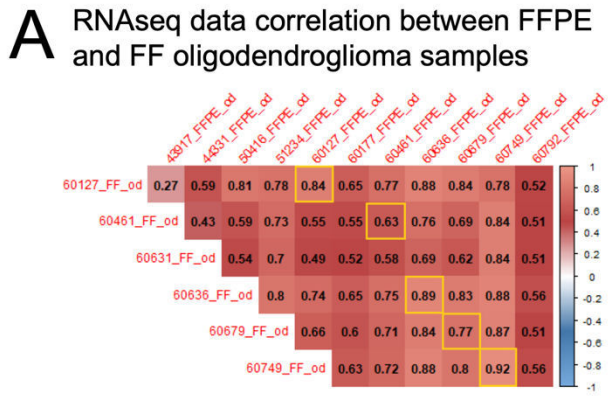
2.3.3. IL13-Rα2

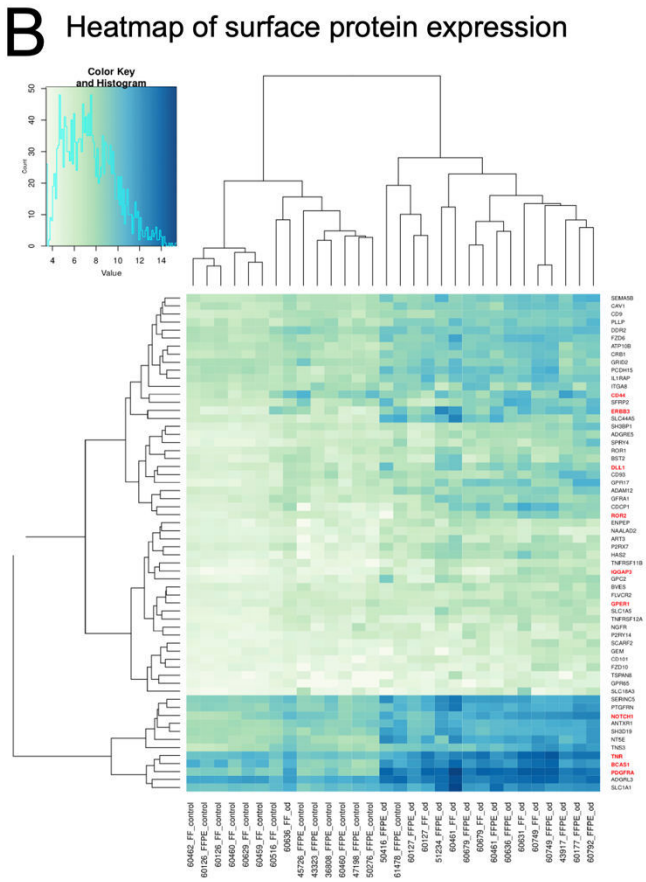
We did not yet manage to find an IL13-Rα2 antibody that cross-reacts with canine L13-Rα2 in FFPE tumor tissue. The search is ongoing.

2.4. Further TAA candidates

The above mentioned TAA candidates may have limitations as autoimmune reactions have to be considered due to their expression in healthy tissues. Therefore, we plan to screen brain tumours for TAA and TSA candidates using multi-faceted and unbiased -omic approaches. We started with RNA sequencing to investigate patterns of overexpression in brain tumours. To test the feasibility of transcriptome analysis in FFPE tumour tissue and to gain first unbiased insights into potential TAA candidates, we performed a pilot transcriptome study in 12 canine oligodendrogliomas (six French Bulldogs, four Boxers, one Pomeranian, one Border Terrier) and 13 control dogs that died of other diseases not related to the CNS (five French Bulldogs, four Boxers, one Border Terrier), Pomeranian and Border Terrier) and 13 control dogs that had died of other diseases not related to the CNS and did not have brain tumours (five French Bulldogs, four Boxers, one Bolonka Zwetna, one Hovawart, one mixed breed and one Bernese Mountain Dog). RNA was extracted in parallel from snap frozen and FFPE material using the Qiagen RNeasy mini kit, Lexogen CORALL Total RNA-Seq libraries were generated and 50 base paired-end sequencing was performed on an Illumina NovaSeq6000 system. The results showed good quality of libraries generated from FFPE material and correlation between sequencing data from matched snap frozen and FFPE oligodendroglioma and control samples (Figure 2A). 82% (49 out of 60, Figure 2B) of overexpressed genes encoding surface proteins were identified in the FFPE tumour samples, and in particular, basically all highly overexpressed genes were detected (Figure 2C), indicating that RNA sequencing of FFPE tumours is a suitable approach to increase the tumour sample size. While IL13-Rα2 overexpression was not identified by RNA sequencing, we identified 10 potential candidates for TAA: PDGFRA, NOTCH1, DLL1, GPER1, TNR, IQGAP3, CD44, ERBB3, ROR2, BCAS1. It is planned to further analyse the strong hits for somatic gene mutations in a follow-up project and to establish WGS sequencing on FFPE tissue to screen for somatic mutations with an unbiased approach.

Two of the 10 potential TAA candidates were further analyzed for tumor expression by immunohistochemistry: PDGFR+, CD44. Antibodies against two further TAA candidates (CD44 and Notch 1) did not cross-react with canine tissue. In brain tumors, PDGFRA overexpression was most consistent and strongest in meningiomas and oligodendrogliomas, while astrocytomas and oligoastrocytomas showed





C Top 10 surface protein encoding genes overexpressed in FF and/or FFPE oligodendroglioma

	Fresh-frozen oligodendroglioma versus fresh-frozen control		FFPE oligodendroglioma versus FFPE control	
	baseMean	logFC	baseMean	logFC
PDGFRA	9502.16	4.67	6800.68	3.05
NOTCH1	1315.45	2.60	1976.001	2.37
DLL1	224.03	3.26	281.26	2.66
GPER1	60.09	2.35	86.15	1.06
TNR	5358.33	2.99	4358.11	2.83
IQGAP3	49.89	4.37	46.85	3.4
CD44	349.34	2.04	648.63 (no FDR significance)	0.56 (no FDR significance)
ERBB3	592.53	2.62	764.95	2.42
BCAS1	4754.64	2.48	3004.59	2.83
ROR2	190.5	3.42	143.979	2.37

Figure 4: Pilot bulk RNA sequencing study comparing fresh-frozen (FF) and formalin fixed paraffin embedded oligodendroglioma and control brain. A) Correlation between FF and FFPE oligodendroglioma samples. Yellow encased values indicate high correlation between FF and FFPE from the same tumor. B) Heatmap of expression of surface protein encoding genes that are upregulated in oligodendroglioma. Separation between oligodendroglioma and control transcriptomes occurs across FF and FFPE samples. C) The top 10 overexpressed genes encoding for surface proteins show similar logFC values between FF and FFPE oligodendrogliomas.

lower expression (Figure 5A). However, in many control organs, the expression of PDGFRA was high (Figure 5B) and comparable or stronger than in brain tumors. These data suggest that auto-immune reactions need to be considered if targeting PDGFRA in immunotherapy.

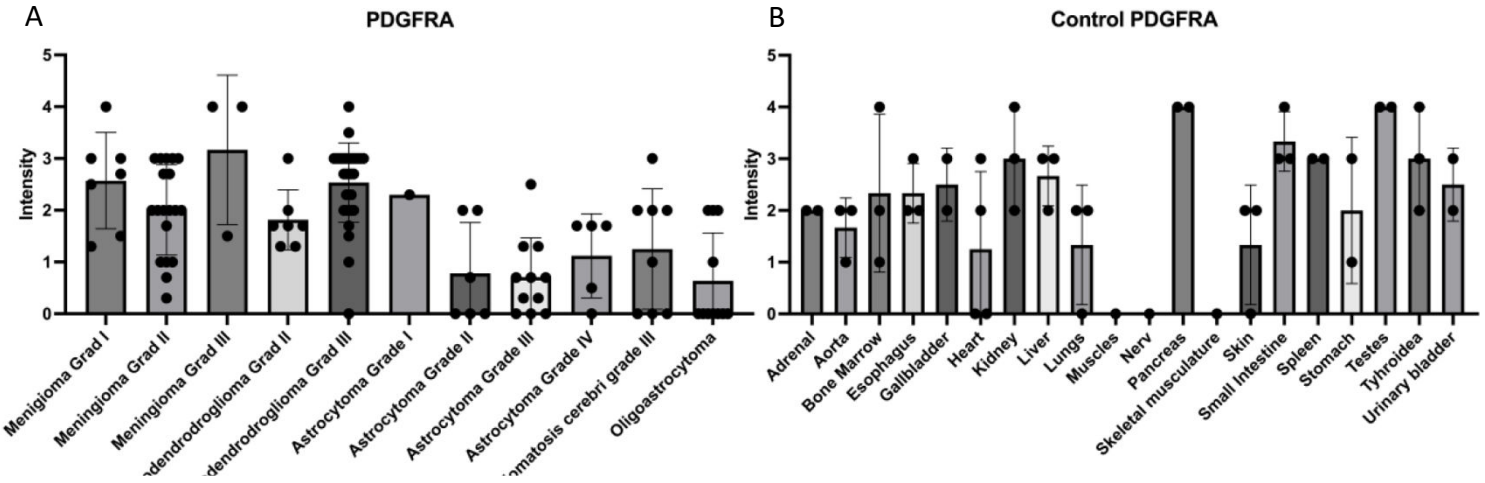


Figure 5: PDGFR expression in brain tumors (A) and control tissues (B).

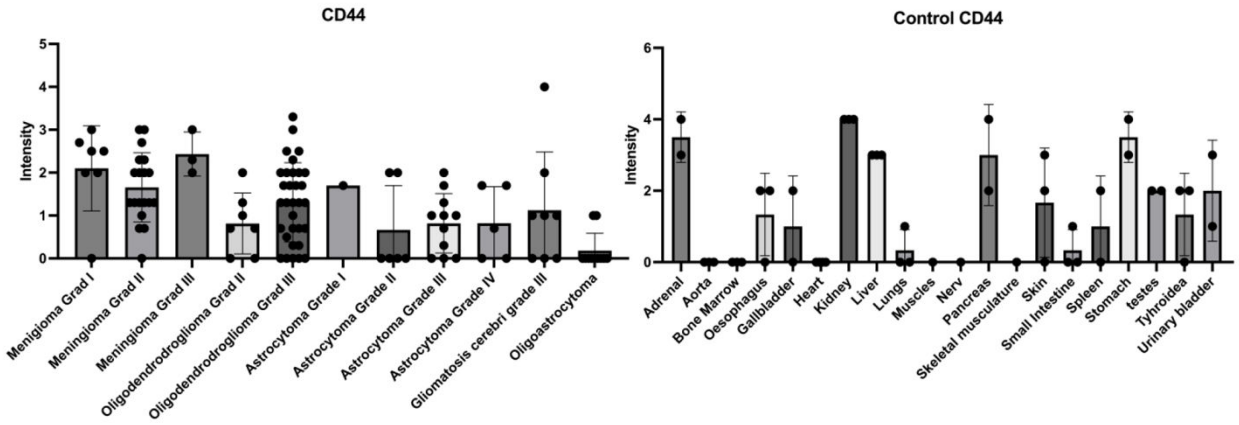


Figure 5: PDGFR expression in brain tumors (A) and control tissues (B).

CD44 was focally to diffusely expressed in most brain tumors, but at a relatively low level (Figure 6A). Expression in control tissues such as adrenal gland, kidney, liver, pancreas and stomach was much stronger. The data suggest that CD44 is not a good TAA candidate and that off-target reactions need to be expexted.

3. Timeplan/outlook

The manuscript drafts are still in preparation for publication and are expected to be ready in 2023 as William Pownall defends his PhD project on the 16th of March 2023. We are currently establishing and optimizing the bioinformatical pipelines for TAA/TSA identification based on genome and RNA sequencing of tumors and matched control tissues.