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## **Plasma galectins and metabolites in advanced head and neck carcinomas: evidence of distinct immune characteristics linked to hypopharyngeal tumors**

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1 **Plasma galectins and metabolites in advanced head and neck carcinomas:**  
2 **evidence of distinct immune characteristics linked to hypopharyngeal tumors**

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38  
39 **Running title:** Plasma galectin-9 and kynurenine in hypopharyngeal carcinomas

1 **Abstract**

2 Extra-cellular galectins 1, 3 and 9 (gal-1, -3 and -9) are known to act as soluble  
3 immunosuppressive agents in various malignancies. Previous publications suggest that their  
4 expression is dependent on the metabolic status of producing cells and reciprocally that they  
5 can influence metabolic pathways in their target cells. Very few is known about the status of  
6 gal-1, -3 and -9 in patients bearing head and neck squamous cell carcinomas (HNSCC) and  
7 about their relationships with the systemic metabolic condition. This study was conducted in  
8 plasma samples from a prospective cohort of 83 HNSCC patients with advanced disease.  
9 These samples were used to explore the distribution of gal-1, -3 and -9, along with two  
10 immuno-inflammatory markers (C-reactive protein and CXCL9) and simultaneously to profile  
11 a series of 87 metabolites assessed by mass spectrometry. We identified galectin and  
12 metabolic patterns within five disease categories defined according to the primary site and  
13 human papillomavirus (HPV) status (HPV-positive and -negative oropharyngeal carcinomas,  
14 carcinomas of the oral cavity, hypopharynx and larynx carcinomas). Remarkably, samples  
15 related to hypopharyngeal carcinomas displayed the highest average concentration of gal-9  
16 ( $p = 0.017$ ) and a trend towards higher concentrations of kynurenine, a potential factor of  
17 tumor growth and immune suppression. In contrast, there was a tendency towards higher  
18 concentrations of fatty acids in samples related to oral cavity. These observations emphasize  
19 the diversity of HPV-negative HNSCCs. Depending on their primary site, they evolve in  
20 distinct types of immune and metabolic landscapes which seem to be congruent with  
21 specific oncogenic mechanisms.

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# 1 Introduction

2 Most malignant tumors of the upper aero-digestive tract or of the head and neck  
3 area are squamous cell carcinomas (HNSCCs). They represent the 6th leading cause of cancer  
4 worldwide with an overall incidence of 650 000 new cases per year (Leemans, Braakhuis et  
5 al. 2011, Bhat, Hyole et al. 2021). In most cases, the main etiological factors are alcohol and  
6 tobacco abuse. However, a fraction of them is related to infection by oncogenic viruses,  
7 notably human papillomavirus (HPV), mainly for two subtypes of oropharyngeal carcinomas:  
8 tonsil and basal tongue carcinomas (Näsman, Du et al. 2020). The risk of distant metastases  
9 or locoregional recurrences is lower in HPV-positive carcinoma patients. Nevertheless,  
10 surgery and/or concomitant chemo-radiotherapy are still the standard of care for the  
11 treatment of non-metastatic primary HNSCCs regardless of their viral or non-viral etiology.  
12 Distant metastases and locoregional recurrences of HNSCCs remain a major therapeutic  
13 challenge requiring better treatments than conventional chemotherapy. For HNSCCs, like for  
14 other human malignancies, immunotherapy, especially immune checkpoint inhibitors (ICIs)  
15 targeting the PD1/PD-L1 axis, have improved patients outcomes. Immunotherapy can  
16 achieve durable responses for patients previously regarded as beyond therapeutic  
17 resources. However, only 20% of the patients with recurrent and/or metastatic disease  
18 benefit from immunotherapy, and it remains challenging to predict which patients will  
19 benefit from ICIs (Burtness, Harrington et al. 2019, Borel, Jung et al. 2020, Clarke, Eriksen et  
20 al. 2021).

21 The prevalence of alternative immune inhibitors is one mechanism suspected to  
22 explain primary or secondary resistance to ICIs in HNSCCs as well as in other human  
23 malignancies. Some of these alternative inhibitors are released in the extra-cellular medium  
24 and diffuse in the peripheral blood with potential systemic effects. This is the case for  
25 example for soluble Lag3 or HLA-G (Botticelli, Zizzari et al. 2021, Kluckova, Durmanova et al.  
26 2021). One category of alternative immune inhibitors often detected in the context of  
27 human malignancies are galectins, especially galectin-1, -3 and -9 (gal-1, -3 and -9)(Koyama,  
28 Akbay et al. 2016, Limagne, Richard et al. 2019, Navarro, Martínez-Bosch et al. 2020). In  
29 brief, galectins are lectins with selective affinity for  $\beta$ -galactoside disaccharides that are  
30 contained in the carbohydrate moiety of glycoproteins and glycolipids. They are present in  
31 various intra-cellular compartments and involved in multiple cellular functions from RNA  
32 splicing to plasma membrane organization and surface receptor recycling (Johannes, Jacob  
33 et al. 2018). Although they have no signal sequence, several galectins can be secreted in the  
34 extra-cellular space through non-conventional pathways like translocation through the  
35 plasma membrane or association with extra-cellular vesicles (Popa, Stewart et al. 2018).  
36 Galectins released by malignant or stromal cells can behave like cytokines having mainly  
37 immunosuppressive functions (Gordon-Alonso, Hirsch et al. 2017, Chou, Chen et al. 2018,  
38 Navarro, Martínez-Bosch et al. 2020). Regarding HNSCCs, there are clues suggesting that  
39 several extra-cellular galectins, especially gal-1, gal-3 and gal-9 play a role in tumor immune

1 escape (Saussez, Lorfevre et al. 2008, Klibi, Niki et al. 2009, Nambiar, Aguilera et al. 2019).  
2 There are also data suggesting reciprocal interactions between galectin production and  
3 metabolic alterations occurring in the context of malignant diseases. For example, hypoxia  
4 has been shown to enhance gal-3 expression in malignant cells and/or tumor-associated  
5 macrophages (TAMs) in various types of malignancies (de Oliveira, Ribeiro et al. 2015, Wang,  
6 Li et al. 2020, Gu, Meng et al. 2021). Reciprocally, gal-3 facilitates the uptake of glucose  
7 especially in endothelial cells at least in part by up-regulation of the membrane transporter  
8 Glut-4 (Darrow and Shoheit 2015). Also, high production of lactate enhances gal-9 expression  
9 in HNSCCs (Chang, Xu et al. 2021). Therefore, this work intended to provide more  
10 information about the status of gal-1, -3 and -9 in HNSCCs in connection with the systemic  
11 metabolic status. We chose to profile gal-1, -3 and -9 and simultaneously a panel of  
12 metabolites in the peripheral blood of HNSCC patients more precisely in plasma samples.  
13 The first reason for this approach is that galectins and some cancer-related metabolites are  
14 known to promote immune suppression not only in the tumor microenvironment but also at  
15 the systemic level, including in the peripheral blood. A second reason is that regarding  
16 biomolecules released by malignant cells or stromal cells, investigations on peripheral blood  
17 is a way to overcome the difficulties related to spatial tumor heterogeneity. The presence  
18 and concentration of these biomolecules in plasma samples are expected to reflect their  
19 status in various portions of the primary tumor or in multiple metastatic sites. Finally, in view  
20 of future longitudinal investigations, it is useful to rely on biological samples requiring only  
21 minimally invasive procedures (Duchemann, Remon et al. 2020).

22 Practically, we assessed by ELISA the plasma concentrations of gal-1, -3 and -9, of C-  
23 reactive protein (CRP, a marker of inflammation) and CXCL9 chemokine (a marker of  
24 interferon- $\gamma$  impregnation) in a prospective series of 83 patients with relapsed and/or  
25 metastatic HNSCCs. Simultaneously, we assessed by GC-MS the concentration of 87  
26 metabolites detectable by our platform of metabolomics across most of our plasma samples  
27 with satisfactory quality controls. The whole work was made as an ancillary study of the  
28 TopNivo trial (NCT03226756). We found that galectin and metabolite distribution was  
29 dependent on disease categories defined according to the primary tumor site and the HPV  
30 status. High concentrations of gal-9 and kynurenine (Kyn) were recorded in plasma samples  
31 related to hypopharyngeal carcinomas. In contrast, high concentrations of fatty acids were  
32 recorded in samples related to carcinomas of the oral cavity. This is a reminder that inside  
33 the vast group of HPV-negative HNSCCs, there are distinct types of tumor  
34 macroenvironments. High plasma levels of gal-9 appear to be characteristic of advanced  
35 hypopharyngeal carcinomas.

36

# 1 **Materials and Methods**

## 2 **Patients and healthy controls**

3 TopNivo is a safety multicentric study of Nivolumab in patients with recurrent and/or  
4 metastatic platinum-refractory HNSCCs. Nivolumab injections were given every 2 weeks, up  
5 to 12 cycles (1 cycle = 28 days). A total of 351 patients have been enrolled in TopNivo  
6 (NCT03226756). In the last stage of the clinical trial recruitment, EDTA blood samples were  
7 obtained from 83 consecutive patients, at baseline, prior to the onset of Nivolumab. Plasma  
8 samples from anonymous healthy donors were used as controls. They were purchased from  
9 Zenbio (NC, USA )(ages comprised between 19 and 65). The HPV status of oropharyngeal  
10 carcinomas was determined according to p16 expression detected by immunohistochemistry

## 11 **Preparation of plasma samples**

12 For each patient, 6 to 9 ml of blood were collected on EDTA tubes and plasma was separated  
13 from cells by centrifugation at 1700g for 15 min at 20°C in less than 3h from the time of the  
14 blood collection. About 4 ml of plasma were recovered and stored at -80°C in 3 aliquots.

## 15 **ELISA kits for galectin-1, -3 and -9**

16 Plasma galectin-1 (gal-1), galectin-3 (gal-3) and galectin-9 (gal-9) concentrations have been  
17 determined for all patients using the corresponding Quantikine ELISA kits from  
18 BioTechne/R&D system (reference DGal 10, 30 and 90) as recommended by the  
19 manufacturer. All samples were treated in duplicate. According to previous reports, normal  
20 median concentrations for these assays are close to 18, 6 and 7 ng/ml for gal-1, -3 and -9  
21 respectively (Bellutti Enders, van Wijk et al. 2014, Martinez-Bosch, Barranco et al. 2018,  
22 Yogasundaram, Nikhanj et al. 2018, Zhao, Mao et al. 2019).

## 23 **Plasma CXCL9 and CRP assay**

24 Plasma CXCL9 concentrations have been determined using the Human CXCL9/MIG  
25 Quantikine ELISA Kit from Biotechne/R&D system (REF DCX900) as recommended by the  
26 manufacturer. All samples were treated in duplicate. CRP assays were made using an  
27 immunoturbidimetric assay (Atelicca CH C-Reactive Protein 2 kit) according to the  
28 manufacturer instructions (Siemens Healthineers France).

## 29 **Plasma metabolite profiling by Gas Chromatography/Mass Spectrometry** 30 **using the MRM mode (Multiple Reaction Monitoring)**

31 **Pre-analytical procedure.** For each patient, 50 µl of crude plasma were mixed with 500 µl of  
32 ice-cold extraction mixture (methanol/water, 9/1 spiked with a cocktail of internal  
33 standards) and centrifuged after 5 min of incubation (10 min at 15 000 g, 4°C). One hundred  
34 and fifty µl aliquots of supernatant were transferred to glass vials and evaporated to be used

1 for Gas Chromatography combined with Mass Spectrometry (GC-MS). Then, 50  $\mu$ l of  
2 methoxyamine (20 mg/ml in pyridine) were added on dried extracts which were stored at  
3 room temperature in the dark, overnight. The day after, 80  $\mu$ L of N-Methyl-N-trimethylsilyl-  
4 trifluoroacetamide were added and final derivatization was made at 40°C during 30 minutes.  
5 Samples were then transferred in vials and directly injected into the GC-MS system.

6 **Sample analysis.** GC-MS/MS was performed with a 7890A gas chromatograph (Agilent  
7 Technologies, Waldbronn, Germany) coupled with a triple quadrupole 7000C (Agilent)  
8 equipped with a high sensitivity electronic impact source (EI) operating in positive mode.  
9 This method was inspired from developments reported in (Tsugawa, Tsujimoto et al. 2014).  
10 The scan mode was the MRM for biological samples. Peak detection and integration of the  
11 analytes were performed using the Agilent Mass Hunter quantitative software (B.07.01)  
12 (Viltard, Durand et al. 2019). All targeted treated data were merged and cleaned with a  
13 dedicated R package (version 4.0)(@Github/Kroemerlab/GRMeta).

14 **Quality control policy.** A daily qualification of the instrumentation was set up with  
15 automatic tune. These qualifications were completed with double injections of standards  
16 mixes, at the beginning and at the end of the run, in addition to blank samples to control the  
17 background impurities. Moreover, pools of tested samples were used to check the column  
18 before the analysis with the proper biological matrix. The same pool was re-injected during  
19 the batch to monitor and correct analytical bias occurring through the acquisition and post-  
20 acquisition treatment of the signal (m/z, retention time and sensitivity drifts). For each  
21 analyte, the corrected peak area was considered as proportional to its concentration.

22 **Data interpretation** was made using the MetaboAnalyst software  
23 (<https://www.metaboanalyst.ca>).

## 24 **Statistical analyses**

25 Most statistical tests were done using the Prism-Graphpad software except for  
26 interpretation of metabolomics profiles covered by the MetaboAnalyst software. The  
27 Kruskal-Wallis and Dunn's multiple comparisons tests were applied for comparisons of  
28 biomolecule concentrations in plasma samples related to distinct disease categories. The  
29 Spearman correlation test was used to assess correlations between distributions of various  
30 pairs of biomolecules.

## 31 **Ethical issues**

32 This study was conducted in accordance with the Declaration of Helsinki (1964) and subsequent  
33 amendments, ICH Good Clinical Practice (GCP) Guidelines (CPMP/ICH/135/95) and the following  
34 European Directive: 2001/20/CE. The Independent Ethics Committee "CPP Ile de France VIII"  
35 reviewed and approved the study documents, including the initial protocol, all subsequent  
36 amendments as well as all information and documents provided to each patient included in this

1 study. The initial protocol was given a favorable opinion on May 23<sup>th</sup>, 2017 (approval number: 2017-  
2 000424-10).

3

## 4 **Results**

### 5 **Elevated concentrations of gal-9, -1 and -3 in plasma samples from HNSCC** 6 **patients**

7 Our initial investigations were focused on plasma gal-1, -3 and -9. The main clinical  
8 characteristics of the patients are presented in details in **Supplementary Table 1** and  
9 summarized in **Table 1**. Their recurrent or metastatic diseases were classified in 5 broad  
10 categories on the basis of the primary tumor location and its HPV status. We recorded  
11 patients with lesions derived from 8 HPV-positive and 35 HPV-negative oropharyngeal  
12 carcinomas, 14 carcinomas of the oral cavity, 16 hypopharyngeal and 10 laryngeal  
13 carcinomas. This distribution of tumor categories is somehow surprising by comparison with  
14 other series of HNSCCs, especially from North America (Garneau, Bakst et al. 2018, Bhat,  
15 Hyole et al. 2021, Mito, Takahashi et al. 2021). The number of HPV-positive oropharyngeal  
16 carcinomas appears strikingly low (8/83) while the number of hypopharyngeal carcinomas is  
17 relatively high (16/83). This probably results at least in part from a selection bias. Indeed, our  
18 series gather patients with relapsed lesions and/or metastases, thus increasing the  
19 proportions of the most aggressive tumors which might result in a bias in favor of  
20 hypopharyngeal and against HPV-positive oropharyngeal carcinomas. Another reason of this  
21 peculiar distribution might be the lower frequency of HPV-positive carcinomas among  
22 HNSCCs in France compared to North America.

23 Median values for gal-1, -3 and -9 concentrations in samples from healthy donors were  
24 compatible with previous reports (**Supplementary Table 2**) (Bellutti Enders, van Wijk et al.  
25 2014, Martinez-Bosch, Barranco et al. 2018, Yogasundaram, Nikhanj et al. 2018, Zhao, Mao  
26 et al. 2019). As shown in **Figure 1A** and with more details in the **Supplementary Tables 2, 3**  
27 **and 4**, for a substantial number of HNSCC patients, plasma galectin concentrations were  
28 above the maximum values recorded in healthy donors. This was observed for gal-1 (58/83,  
29 70%), gal-3 (50/83, 60%) and to a lesser extent for gal-9 (19/83, 23%). Overall, the  
30 differences in median values between healthy donors and HNSCC patients were significant  
31 for each galectin (Mann Whittney:  $p < 0.0001$ , based on data presented in Supplementary  
32 Tables 2 and 3).

### 33 **Higher concentrations of plasma gal-9 linked to hypopharyngeal carcinomas**

34 Next, we investigated possible differences in plasma galectin concentrations  
35 according to the tumor category (based on the primary tumor site and its HPV status) and/or  
36 the number of metastatic sites. Regarding plasma gal-1 and -3, there was no evidence of



1 correlations between their concentrations and these clinical characteristics; except a trend  
2 towards higher concentrations of gal-3 for HPV-positive oropharyngeal carcinomas (**Figures**  
3 **1B and 1C**). In contrast, the average concentration of gal-9 was greater in patients with  
4 lesions derived from hypopharyngeal carcinomas ( $p = 0.018$ ) (**Figure 1D**). Consistently,  
5 plasma gal-9 was above the highest concentrations recorded in healthy controls for 9 of 16  
6 hypopharyngeal carcinomas cases versus 10 of 67 samples for other HNSCCs ( $p = 0.016$  –  
7 Fisher’s exact test) (**Supplementary Table 4**). Plasma gal-9 concentration was also greater in  
8 patients with 2 or more metastatic sites compared to patients free of metastatic lesions ( $p =$   
9  $0.028$ ) (**Figure 2**). Because hypopharyngeal carcinomas are known to be more aggressive  
10 than other HNSCCs, we wanted to determine whether the high levels of plasma gal-9  
11 recorded for this disease category were independent of their metastatic status (Garneau,  
12 Bakst et al. 2018). Therefore we resorted to multivariate analysis. Analysis by factorial anova  
13 showed a significant effect of the primary tumor category even among patients with  
14 multiple metastatic sites ( $p = 0.014$ ).

15

### 16 **Lack of correlations between galectin concentrations and levels of CRP and** 17 **CXCL9 in plasma samples from HNSCC patients**

18 It is well known that various cell types enhance their productions of gal-1, gal-3 or gal-9  
19 when they are in contexts of non-specific inflammation or high impregnation by  $\gamma$ -interferon  
20 (Anderson, Anderson et al. 2007, Henderson and Sethi 2009, Sundblad, Morosi et al. 2017).  
21 Such conditions are common in the microenvironment and macroenvironment of HNSCCs  
22 (Valdes, Villeda et al. 2020, Bhat, Hyole et al. 2021, Mito, Takahashi et al. 2021). Therefore,  
23 we used plasma CRP (C-reactive protein) as a marker of systemic inflammation and CXCL9 as  
24 a marker of impregnation by  $\gamma$ -interferon. Indeed, it is known that plasma levels of CXCL9  
25 often reflect the overall tissue production and activity of  $\gamma$ -interferon better than its own  
26 plasma concentration (De Benedetti, Prencipe et al. 2021). As shown in **Figure 3**, the  
27 correlations of galectin concentrations with CRP and CXCL9 concentrations were weak (low  $r$   
28 coefficient). This suggests that systemic inflammation or excessive production of  $\gamma$ -interferon  
29 are not the main determinants for the elevated concentrations of plasma galectins in  
30 HNSCCs and that additional mechanisms are at play.

### 31 **High levels of plasma kinurenine linked to high concentrations of plasma gal-9** 32 **and to hypopharyngeal carcinomas**

33 As mentioned earlier, several previous studies have highlighted reciprocal influences  
34 of metabolic regulations on the one hand and gal-1, -3 and -9 expression and activity on the  
35 other hand (Darrow and Shohet 2015, de Oliveira, Ribeiro et al. 2015, Wang, Li et al. 2020,  
36 Chang, Xu et al. 2021, Gu, Meng et al. 2021). Therefore, we investigated whether some  
37 plasmatic metabolites were preferentially associated with high concentrations of gal-1, -3

1 and -9 and/or with a specific tumor category. To do so we applied a method of targeted  
2 mass spectrometry called MRM (Multiple Reaction Monitoring) characterized by  
3 concomitant scanning of multiple mass windows. It was operated with an inclusion list of  
4 130 metabolites including metabolites of the tricarboxylic acid cycle, amino-acids and  
5 related molecules, fatty acids, eicosanoids and molecules related to plant-derived nutrients.  
6 The same plasma samples used for galectin assays were subjected to this procedure. After  
7 preliminary runs, 87 out of 130 metabolites were selected for subsequent analyses, based on  
8 their detection in the vast majority of plasma samples with satisfactory quality control (**list in**  
9 **Supplementary Table 5**). A correlation map including the 87 metabolites and the 5 proteins  
10 (gal-1, -3, -9, CRP and CXCL9) was built using the MetaboAnalyst software (**Figure 4**). Five  
11 main clusters were delimited on this map according to the boundaries of color contrasts and,  
12 to a lesser extent, the arborescence of the dendrogram. They were designated A, B, C, D, E  
13 (details on their content are provided in the legend of **Figure 4**). Briefly, the two biggest  
14 clusters, A and B, mainly consisted of free proteinogenic amino-acids. Clusters C and E  
15 contained various types of biomolecules. Several fatty acids were found in cluster D (oleic,  
16 linoleic, and palmitoleic acids) and the related 3-hydroxybutyric acid. Gal-1, gal-3, CRP and  
17 CXCL9 were not included in any of these 5 clusters. In contrast Gal-9 was included in cluster  
18 E. Additional information were provided by the supervised heat map showing relative  
19 plasma concentrations of the 87 metabolites and 5 proteins in connection with the tumor  
20 categories (**Figure 5**). According to the overall ANOVA statistical test, none of the  
21 metabolites showed significant differences in their distribution among distinct tumor  
22 categories. However, several metabolites close to gal-9 inside cluster E of the correlation  
23 map appeared to be more abundant in samples from hypopharyngeal and laryngeal  
24 carcinomas than from other tumor categories (**Figure 5**). This was the case for example for  
25 Kyn, arabinol, erythritol and threonic acids. In contrast three elements of cluster D, linoleic,  
26 palmitoleic and 3-hydroxybutyric acids were more abundant in samples from carcinomas of  
27 the oral cavity.

28 The distribution of plasma Kyn was analyzed with more details (**Figure 6**). As shown in  
29 **Figure 6A** there is a trend towards a higher concentration in samples related to  
30 hypopharyngeal and laryngeal carcinomas. Kyn is the main catabolite of tryptophane (Trp).  
31 As expected, an opposite trend was noted for the Trp/Kyn ratio which was smaller for  
32 hypopharyngeal and laryngeal carcinomas than for other HNSCCs (**Figure 6B**). Kyn was  
33 significantly more abundant in plasma samples from patients with two metastatic sites than  
34 in the absence of metastases (**Figure 6C**). Finally there was an overall correlation between  
35 gal-9 and Kyn concentrations among all HNSCCs ( $r$  close to 0.60) (**Figure 6D**). Interestingly  
36 this correlation was even greater among hypopharyngeal carcinomas ( $r$  close to 0.77) while  
37 it was absent among laryngeal carcinomas (**Figure 6E and 6F**).

38

39

# 1 Discussion

2 Our exploratory study on the distribution of galectins and metabolites in the plasma  
3 from patients bearing advanced HNSCCs has several original characteristics. First, it deals  
4 with the tumor macroenvironment, in other words with the systemic aspects of the disease  
5 in contrast with almost all previous studies focused on the microenvironment of tumor  
6 lesions. Next, we have investigated the status of three galectins simultaneously in the same  
7 series of patients in contrast with almost all previous studies focusing on one galectin in a  
8 given series. Lastly, we have combined the exploration of the galectin status with GC-MS  
9 profiling of 87 metabolites.

10 Our main results can be summarized as follows. First, we showed that gal-1, -3 and -9  
11 are detected in the plasma of a fraction of HNSCC patients at a higher concentration than in  
12 healthy donors. This applied to more than 50% of the patients for gal-1 and gal-3 and to  
13 about 25% of the patients for gal-9. Regarding gal-1 and gal-3, our data are consistent with  
14 previous publications reporting high concentrations of gal-1 and gal-3 in a fraction of HNSCC  
15 serum samples (Saussez, Lorfèvre et al. 2008). We found no significant differences in the  
16 distribution of gal-1 and gal-3 concentrations according to the tumor category, despite a  
17 trend towards higher concentrations in HPV-positive oropharyngeal carcinomas. This  
18 observation is consistent with a recent publication reporting a greater abundance of gal-3  
19 detected by immunohistochemistry in tumor sections from HPV-positive compared to HPV-  
20 negative HNSCCs (Coppock, Mills et al. 2021). Regarding gal-9, we found significant higher  
21 concentrations in plasma samples connected to the hypopharyngeal primary site, a  
22 correlation which was independent of the number of metastatic sites. We found no  
23 significant correlations between galectin concentrations and concentrations of the CRP and  
24 CXCL9. Regarding the metabolic profiles, our most exciting observations was the high  
25 concentrations of Kyn in samples from hypopharyngeal carcinomas; not only Kyn was  
26 abundant in this category but there was a strong correlation with gal-9 concentration, which  
27 was not the case for laryngeal carcinomas. Regarding carcinomas of the oral cavity, there  
28 was a trend towards higher concentrations of linoleic, palmitoleic and 3-hydroxybutyric  
29 acids.

30 Kyn is a metabolite related to amino-acids with important signaling capabilities. It  
31 results from the oxidation of tryptophan, one essential amino-acid which combines an indol  
32 heterocycle with a short lateral chain. Three dioxygenases can open the indol ring and  
33 produce Kyn: IDO1 (indoleamine 2, 3-dioxygenase 1), TDO (tryptophane 2,3-dioxygenase)  
34 and IDO2. These enzymes are expressed in various cell-types and tissues. IDO2 is present in  
35 the liver which is not the case for IDO1. Their expression is enhanced by interferon- $\gamma$  and  
36 lipopolysaccharide. TDO is present mainly in the liver, placenta and brain and it is up-  
37 regulated by glucocorticoids (Zhai, Ladomersky et al. 2018). All three enzymes are often  
38 expressed in malignant cells. Downstream of Kyn, there is the Kyn catabolic pathway which  
39 ultimately leads to the production of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) involving  
40 enzymes like kynureninase and kynurenine 3-monooxygenase (Castro-Portuguez and

1 Sutphin 2020). Kyn is involved in immunosuppression. Initially, several studies had pointed  
2 tryptophan starvation, rather than a direct effect of Kyn, as a major mechanism of T-cell  
3 inactivation. According to some ancient studies, the rise of Kyn production was mainly a  
4 side-effect concomitant of tryptophan catabolism. However more recent studies have  
5 challenged this interpretation especially since Kyn was identified as a ligand of the aryl  
6 hydrocarbon receptor (AhR), a ligand-activated transcription factor. Stimulation of AhR  
7 inside T-cells is known to favor the onset of immunosuppressive phenotypes especially their  
8 differentiation into T-regs (Mezrich, Fechner et al. 2010). In addition, it stimulates the  
9 cooperation of T-regs with tumor associated macrophages (TAMs) having M2 polarity  
10 (Campeato, Budhu et al. 2020). Moreover, Kyn has been reported to have direct oncogenic  
11 effects for example by activating  $\beta$ -catenin signaling in mouse colon cancer models  
12 (Bishnupuri, Alvarado et al. 2019). It can also induce a switch from programmed cell death to  
13 a program of tumor cell dormancy. This effect is dependent on AhR (Zhai, Lodomersky et al.  
14 2018). Therefore, Kyn can be regarded as a candidate onco- and immune-metabolite.

15 It is interesting to notice that the concentration of plasma gal-9 and, possibly, of  
16 plasma Kyn are consistently higher in samples related to hypopharyngeal carcinomas in  
17 comparison with other HNSCCs. One important lesson from this observation is that there is  
18 biological heterogeneity among HPV-negative HNSCCs. In the medico-scientific literature  
19 there is a strong and somehow surprising tendency to oppose only two major subsets of  
20 HNSCCs either HPV-positive or HPV-negative (Cillo, Kürten et al. 2020, Mito, Takahashi et al.  
21 2021). However, one should keep in mind the dissymmetry of these two categories. Most  
22 HPV-positive HNSCCs arise from the epithelium of the tonsils or of the base of tongue  
23 (Näsman, Du et al. 2020). On the other hand, the anatomic origins of HPV-negative HNSCCs  
24 are much more diverse including multiple sites in the rest of the oropharynx, in the oral  
25 cavity, larynx and hypopharynx. Despite the similarity of some risk factors and oncogenic  
26 events, the diversity of the cells of origin for the various types of HNSCCs probably has some  
27 influence on the phenotypes of the malignant cells as well as on host-tumor relationships.  
28 Hypopharyngeal carcinomas can nearly be seen as orphan tumors by comparison with other  
29 HNSCCs as they are less often the subject of biological investigations (Garneau, Bakst et al.  
30 2018). However, it is clear that they have specific clinical and biological characteristics. They  
31 are usually more aggressive than HNSCCs of other primary sites with the highest incidence of  
32 distant metastases at the initial stage (60%) (Kotwall, Sako et al. 1987). Genetic alterations  
33 driving their oncogenesis are also distinct with a higher frequency of 11q13 amplifications  
34 with predominant involvement of the *FGF3* and *FGF4* genes (Rodrigo, Suárez et al. 2001).  
35 There is also preliminary evidence that they have specific features with regard to invasion  
36 mechanisms with consistently high expression of MMP2 (matrix metalloproteinase-2 or 72kd  
37 type IV collagenase) (Répássy, Forster-Horváth et al. 1998). Our present data suggest that  
38 the development of hypopharyngeal carcinomas also relies on distinct mechanisms of tumor  
39 suppression with a greater involvement of gal-9 and Kyn than for HNSCCs derived from other  
40 primary sites.

1 Future investigations will have to address several questions. The first one is whether  
2 plasma gal-9 reflects an excess of production from tumor lesions, either from the malignant  
3 or the stromal cells, and with the same perspective whether the tryptophan dioxygenases  
4 inducing Kyn production are localized into the tumor lesions. The answers to these questions  
5 will require investigations on tumor fragments by immunohistochemistry. Another question  
6 is why there is a correlation between the high concentrations of gal-9 and Kyn especially in  
7 plasma samples related to hypopharyngeal carcinomas. A first answer that comes to mind is  
8 to attribute these two characteristics to a strong impregnation by  $\gamma$ -interferon, a cytokine  
9 known to stimulate, on the one hand, the expression of gal-9 and on the other hand the  
10 expression of the tryptophan dioxygenases IDO1 and IDO2 (Zhai, Ladomersky et al. 2018,  
11 Yang, Sun et al. 2021). However, we observed only a weak correlation between gal-9 and  
12 CXCL9 concentrations although CXCL9 production is known to be enhanced by  $\gamma$ -interferon.  
13 An alternative explanation would involve Myeloid-Derived Suppressor Cells (MDSCs). Indeed,  
14 MDSC expansion and maturation is known to be enhanced by gal-9 and MDSCs are known to  
15 have a strong expression of IDO1 (Dardalhon, Anderson et al. 2010, Zhai, Ladomersky et al.  
16 2018, Zhang, Huang et al. 2020). Finally, it will be interesting to investigate the contribution  
17 of gal-9 and Kyn in primary and secondary resistance to immune checkpoint inhibitors. One  
18 possible approach to address this question could rely on monitoring plasma gal-9 and Kyn  
19 concentrations in the plasma of HNSCC patients at various stages of the treatment and  
20 tumor evolution, for example at the initiation of immunotherapy and at the stage of disease  
21 progression in case of initial objective tumor responses. This would give some clues on  
22 whether high concentrations of both gal-9 and Kyn at baseline are predictive of primary  
23 resistance to ICI and whether an increase in their plasma concentration underlines the onset  
24 of a secondary resistance.

25

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## 30 **Data availability statements**

31 All data supporting the findings of this study are included in this article or its supplementary  
32 material files. Further enquiries can be addressed to the corresponding author.

## 33 **Disclosure statement**

34 PB and MD are members of a collaborative project about monoclonal antibodies  
35 neutralizing extra-cellular gal-9. This project involves P. Busson's team and HiFIBiO-  
36 Therapeutics. Otherwise, the authors declare no potential conflicts of interest.

## 37 **Authors' contribution**

1 Conception and design of the project: CE, PB, TN, CB, IB and PG. Generation and acquisition  
2 of data: BTTT, AG, SD and TN. Analysis and interpretation of the data: BTTT, AG, PB, TN, SD,  
3 MD and KB. Statistical analysis: MT with the support of FG. Writing, reviewing and editing  
4 were performed by PB with inputs from KB, MD, CE, CLT, JF, AD, CT and CB.

5

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21

## 22 **Figure Legends**

23 **Figure 1. Overall distribution and distribution by tumor categories of gal-1, -3 and -9**  
24 **concentrations in plasma samples from 83 HNSCC patients. A** Overall distribution in all  
25 samples of the cohort and comparison with healthy donors. Plasma concentrations of  
26 galectins were determined by ELISA as explained in the Materials and Methods section.  
27 Values obtained for patients and healthy donors are shown as black and red dots,  
28 respectively. For gal-1, we had 15 measurements made in samples from 5 donors (lowest  
29 and highest concentrations 7.44 and 22.81 ng/ml, respectively; median: 11.64 ng/ml). For

1 gal-3, we had 13 measurements made in samples from 5 donors (lowest and highest  
2 concentrations 1.51 and 8.70 ng/ml, respectively; median: 5.33 ng/ml). For gal-9, we had 13  
3 measurements in samples from 4 donors (lowest and highest concentrations 4.28 and 13.39  
4 ng/ml, respectively; median 6.57 ng/ml). For both patients and healthy donors,  
5 concentrations were normalized on the median concentration of the healthy donors. Fifty  
6 eight out of 83 patients, 50/83 and 19/83 patients had plasma concentrations above the  
7 highest value recorded in healthy donors for gal-1, gal-3 and gal-9 respectively. **B, C and D**  
8 Comparison of plasma galectin concentrations for five tumor categories: oropharyngeal (oro)  
9 HPV-pos and neg, oral cavity, hypopharynx, larynx. Kruskal-Wallis tests: gal-1:  $p = 0.8802$ ;  
10 gal-3:  $p = 0.7860$ ; gal-9:  $p = 0.018$ . Pairwise comparisons were made for gal-9 using Dunn's  
11 multiple comparisons test with significant differences between hypopharynx and oropharynx  
12 HPV-neg ( $p = 0.012$ ).

13 **Figure 2. Distribution of plasma galectin concentrations according to the number of**  
14 **metastatic sites.** M0: absence of any detectable metastatic site ( $n = 33$ ); M+: only one  
15 metastatic site ( $n = 20$ ); M++ : two or more metastatic sites ( $n = 30$ ). **A** Gal-1 (Kruskal-Wallis  
16 test:  $p = 0.85$ ). **B** Gal-3 (Kruskal-Wallis test:  $p = 0.45$ ). **C** Gal-9 (Kruskal-Wallis test:  $p = 0.028$  –  
17 Dunn's multiple comparisons test: M++ vs M0  $p = 0.022$ ).

18 **Figure 3. Correlations of galectin concentrations with levels of C-reactive protein (CRP) and**  
19 **CXCL9 in plasma samples from HNSCC patients.** Concentrations of gal-1, -3 and -9 and  
20 concentrations of CXCL9 were determined by ELISA as explained in the Materials and  
21 Methods section. CRP concentrations were determined using an immunoturbidimetric assay.  
22 R coefficients were calculated using the Spearman test.

23 **Figure 4. Overview and correlation mapping of the distribution of 87 metabolites detected**  
24 **by GC-MS and five selected proteins (gal-1, -3 and -9, CRP, CXCL9) in plasma samples from**  
25 **HNSCC patients.** The correlation matrix was generated using the "correlation" function of  
26 the MetaboAnalyst software (<https://www.metaboanalyst.ca>). The color scale is a function  
27 of Spearman coefficients of correlation. Areas of red colors signal biomolecules occurring  
28 with concomitant high abundance in substantial numbers of plasma samples. Five  
29 prominent clusters designated A, B, C, D, E were delimited on the basis of color contrasts  
30 and to a lesser extent dendrogram arborescence. Clusters A and B mainly consisted of free  
31 proteinogenic amino-acids including branched (valine, leucine, isoleucine) and aromatic  
32 (tryptophan, phenylalanine, tyrosine) amino-acids, proline, asparagine, serine and threonine  
33 as well as two non-proteinogenic amino-acids (citrulline and ornithine). Cluster C was  
34 heterogenous including a polyamine, putrescine, glutamine and shikimic acid, a metabolite  
35 derived from plants and microorganisms. Cluster D consisted of 3 fatty acids (oleic, linoleic,  
36 palmitoleic acids) and the related 3-hydroxybutyric acid. Like cluster C, cluster E was  
37 heterogenous containing gal-9, kynurenine (Kyn), uric acid and 4 putative plant-derived  
38 metabolites (threonic and ferulic acids, arabitol, erythritol). While gal-9 was clearly linked to

1 cluster E, just adjacent to Kyn (bold blue arrow), gal-1, gal-3, CRP and CXCL9 (thin blue  
2 arrows) were not associated to well delimited clusters.

3

4 **Figure 5. Supervised heatmap showing relative concentrations of metabolites and selected**  
5 **proteins in connection with tumor/disease categories.** This heatmap was generated using  
6 the “heatmap” function of the MetaboAnalyst software. The color scale is a function of  
7 biomolecule concentrations (m/z peak areas for metabolites and pg/ml or ng/ml for  
8 proteins, all normalized to the corresponding maximal concentration recorded in the 83  
9 sample series). For better view and reading, the heatmap was split in two parts. “Oro”  
10 stands for oropharyngeal carcinomas. Overall, according to Anova statistical analysis, none  
11 of the metabolites on this map showed a significant difference of distribution between the 5  
12 disease categories. However, it is noteworthy that a large number of metabolites (38/87)  
13 were at higher relative concentrations in plasma samples related to hypopharyngeal  
14 carcinomas. Most components of cluster E identified in Figure 4, especially one protein (gal-  
15 9; bold blue arrow) and several metabolites (kynurenine, arabitol, threonic acid; thin blue  
16 arrows, part 2)) were abundant in samples from hypopharyngeal carcinomas. In contrast,  
17 three components of cluster D (Figure 4) - linoleic, palmitoleic and 3-hydroxybutyric acid  
18 (thin blue arrows; part 1) - were abundant in samples from carcinomas of the oral cavity.

19

20 **Figure 6. Variations of plasma Kyn (kynurenine) concentrations in connection with some**  
21 **clinical and biological characteristics of HNSCCs.** **A** Comparison of the plasma Kyn  
22 concentrations according to the five tumor categories: oropharyngeal (oro) HPV-pos and  
23 neg, oral cavity, hypopharynx, larynx. Kyn concentrations were estimated from the areas  
24 under the corresponding m/z peak curves corrected according to quality control data. There  
25 was a trend towards higher concentrations for plasma samples related to hypopharyngeal  
26 and laryngeal carcinomas (Kruskal-Wallis  $p = 0.18$ ). **B** Comparisons of the ratios of  
27 Tryptophan/Kyn concentrations in the five tumor categories. **C** Distribution of plasma Kyn  
28 concentrations according to the number of metastatic sites; M0: absence of any detectable  
29 metastatic site (n = 33); M+: only one metastatic site (n = 20); M++ : two or more metastatic  
30 sites (n = 30) (Kruskal-Wallis test :  $p = 0.016$  – Dunn’s multiple comparisons test: M++ vs M0  
31  $p = 0.015$ ); **D** Correlations of gal-9 concentrations (based on ELISA) with Kyn concentrations  
32 (based on mass spectrometry) for plasma samples of the whole HNSCC cohort (83  
33 patients)(Spearman test). **E and F** Same correlations restricted to plasma samples related to  
34 hypopharyngeal (E) and laryngeal (F) carcinomas.

35

36

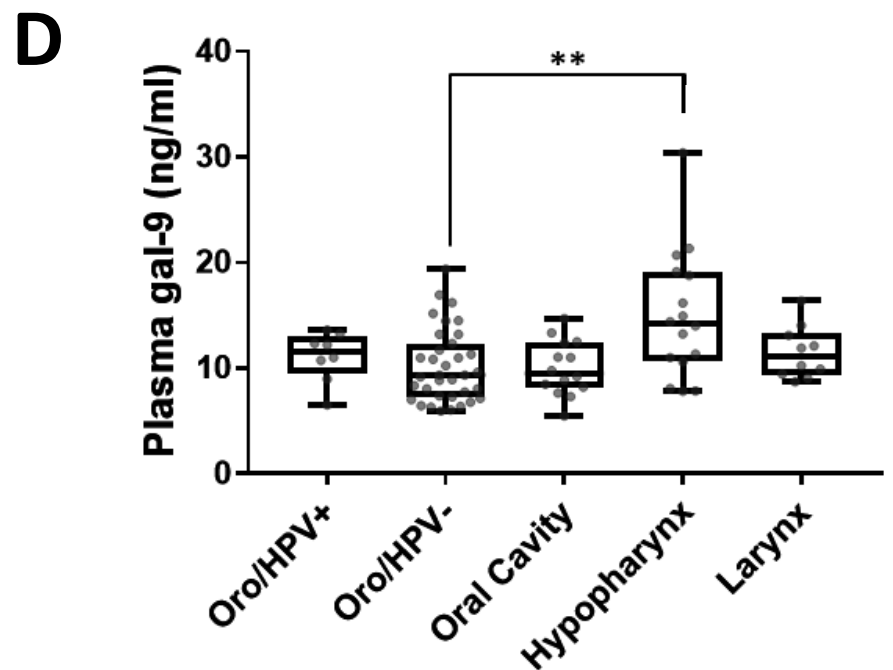
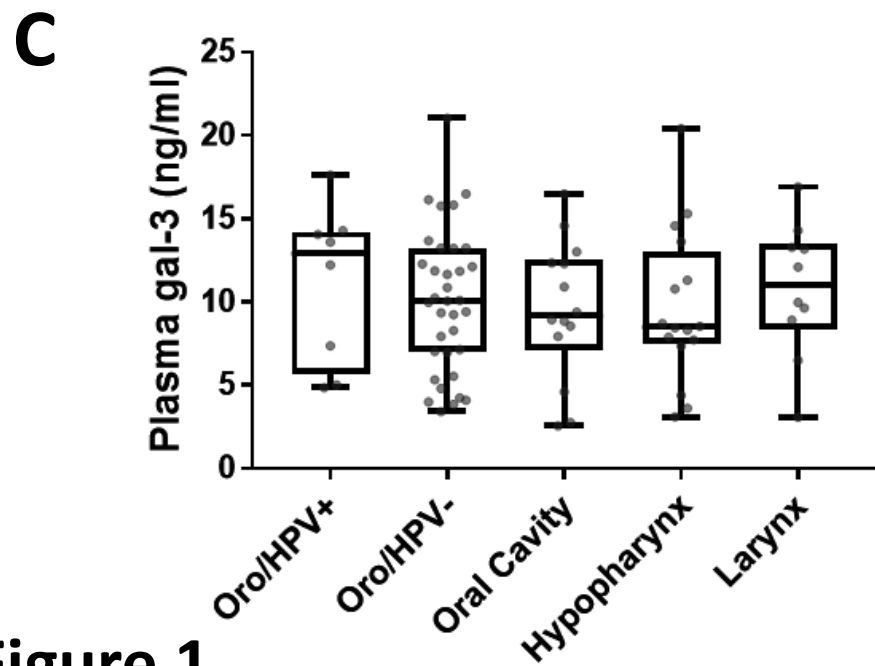
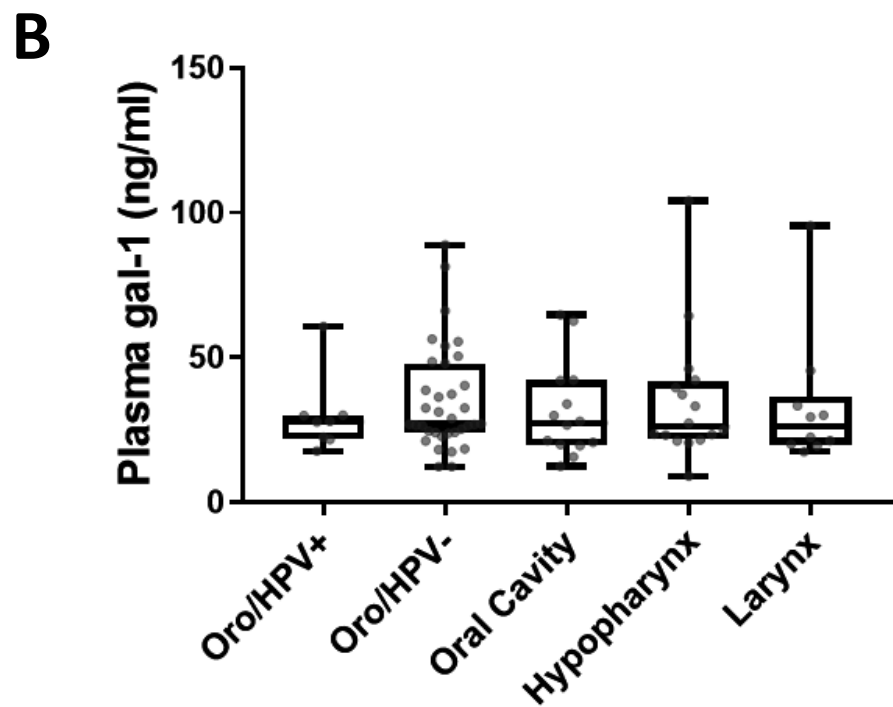
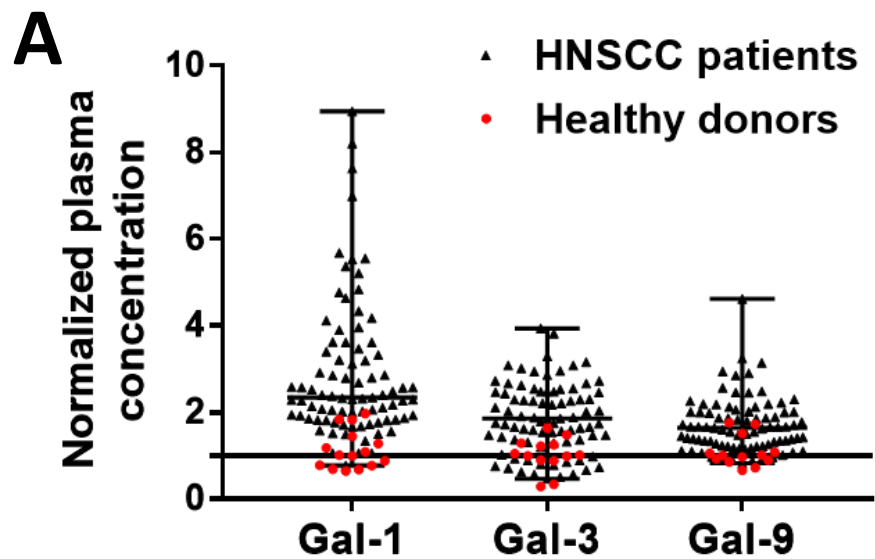
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1 **Table 1: summary of patient characteristics**

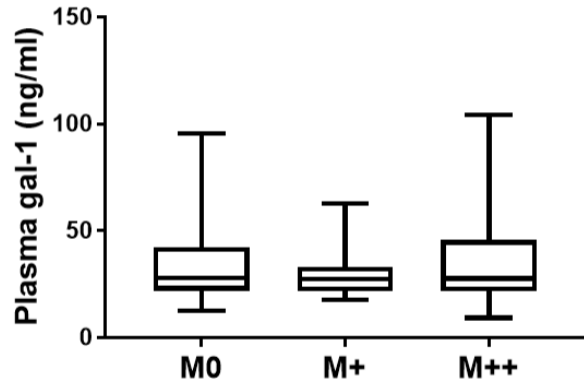
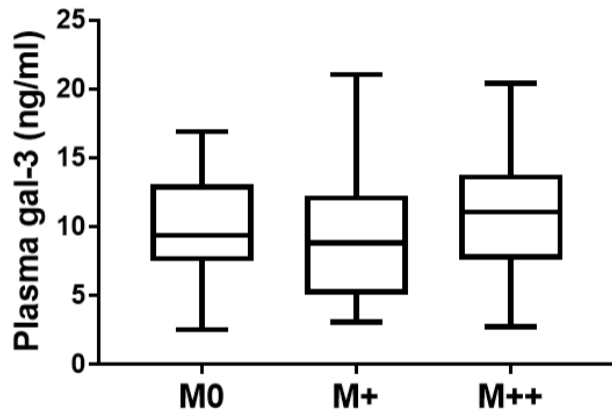
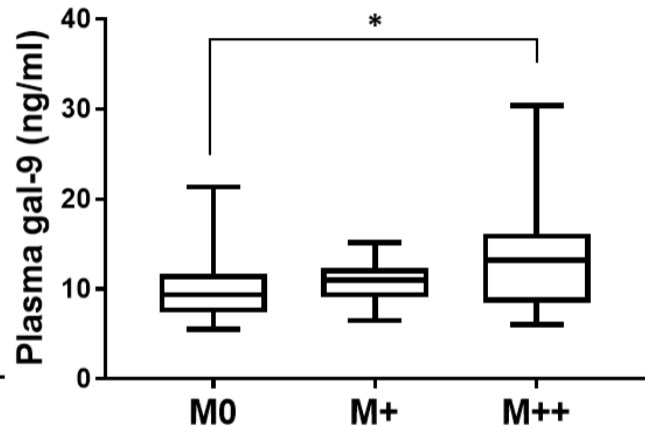
Tumor/Disease categories (1)	Details on primary tumor sites	N° of patients	Mean Age	M/F	N° of recurrences w/o distant metastases	No of patients with distant metastases
<b>HPV-positive oropharyngeal carcinomas</b>	Tonsil Base of tongue Vallecula	8	64	7/1	1	7
<b>HPV-negative oropharyngeal carcinomas</b>	Tonsil Base of tongue Vallecula	35	63	22/13	17	18
<b>Carcinomas of the oral cavity</b>	Floor of mouth Buccal mucosa Oral tongue Hard palate Oral cavity (others)	14	61	10/4	9	5
<b>Hypopharyngeal carcinomas</b>		16	64	14/2	2	14
<b>Laryngeal carcinomas</b>		10	68	8/2	4	6

(1) Based on the primary tumor site and the HPV status

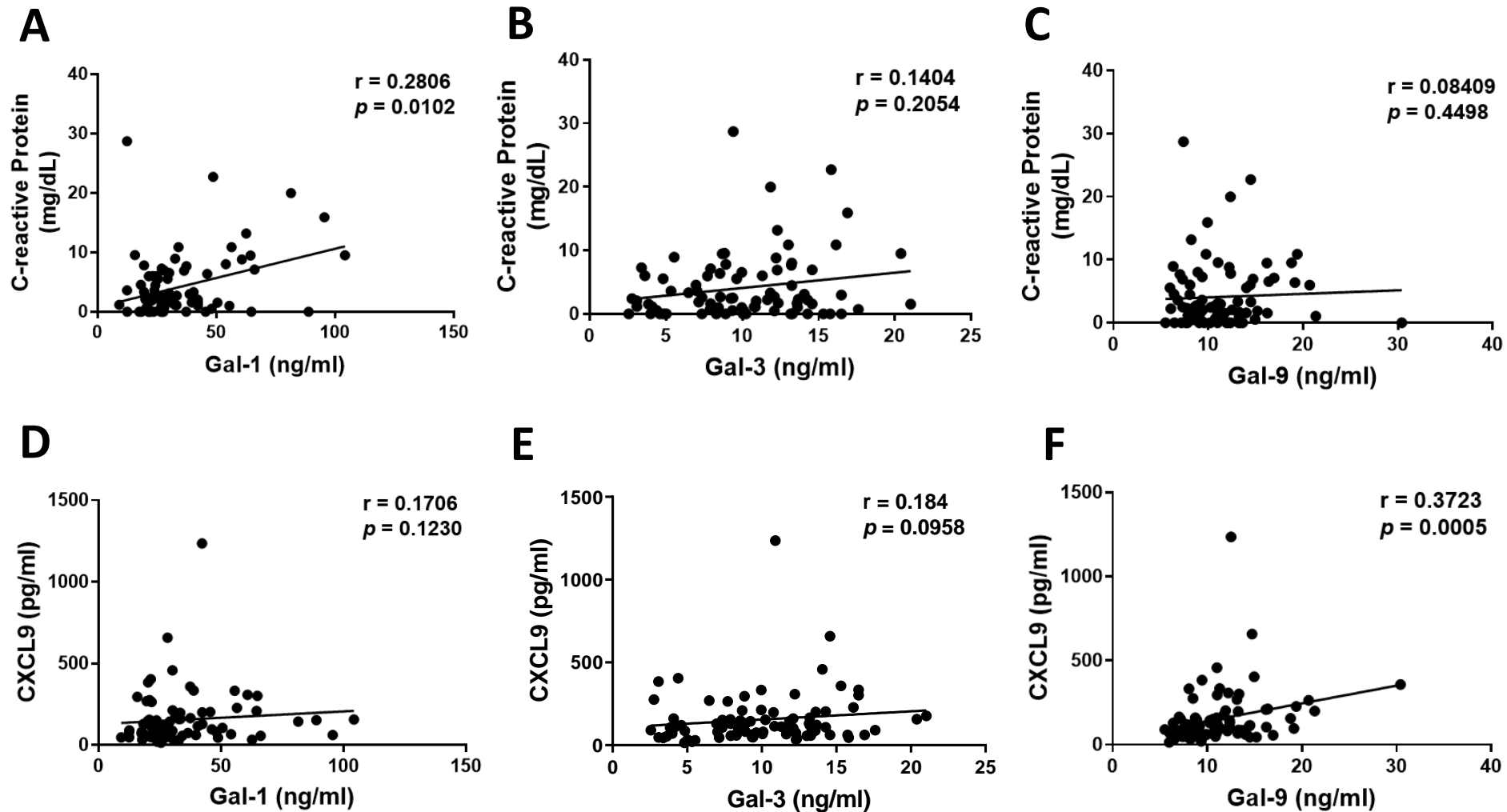
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**Figure 1**

**A****B****C****Figure 2**





**Figure 3**

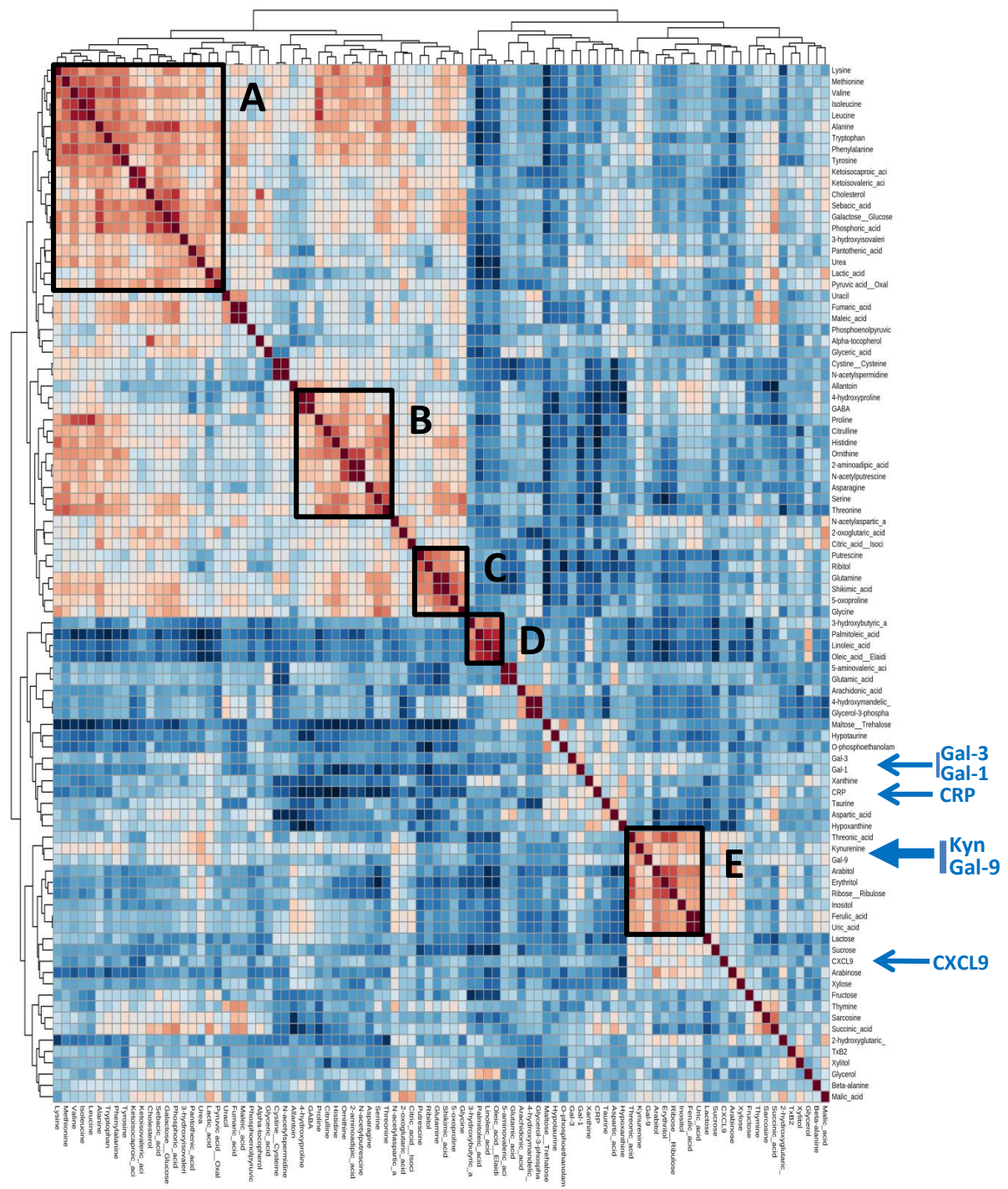
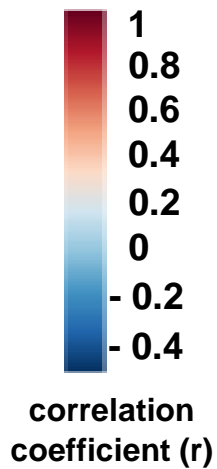
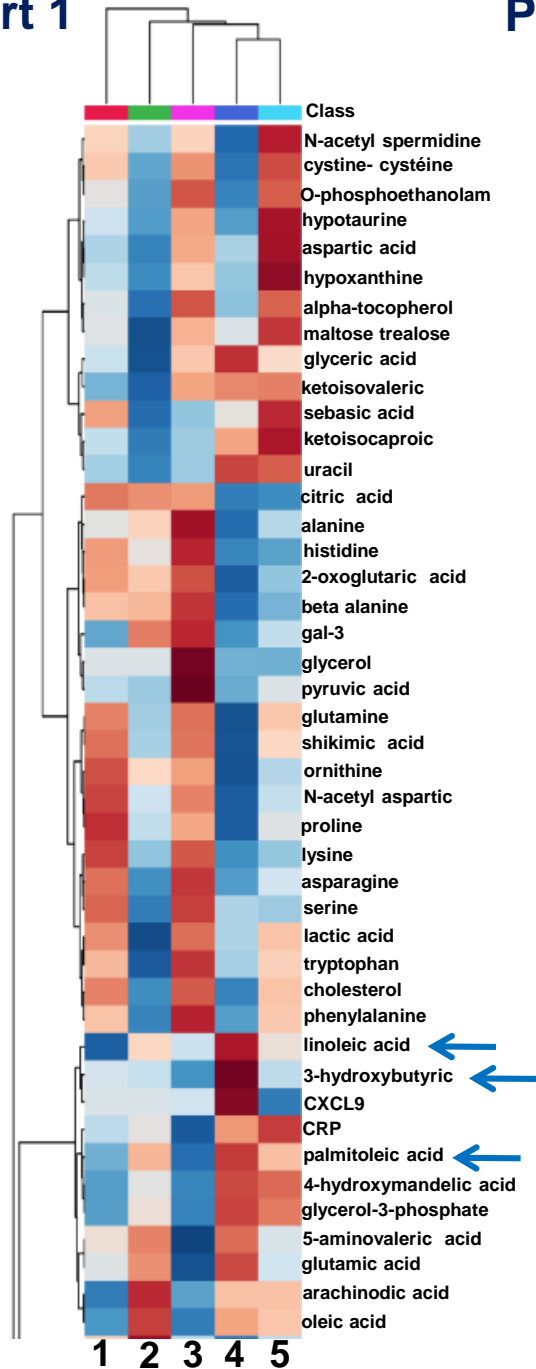
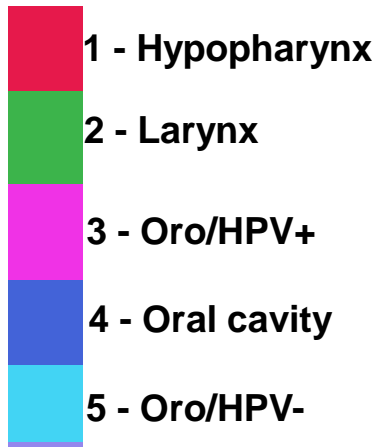
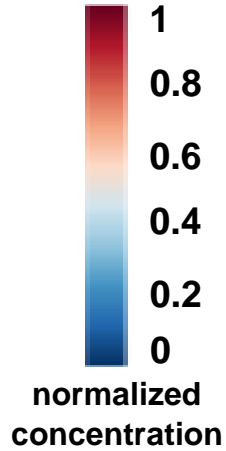


Figure 4

## Part 1



## Part 2

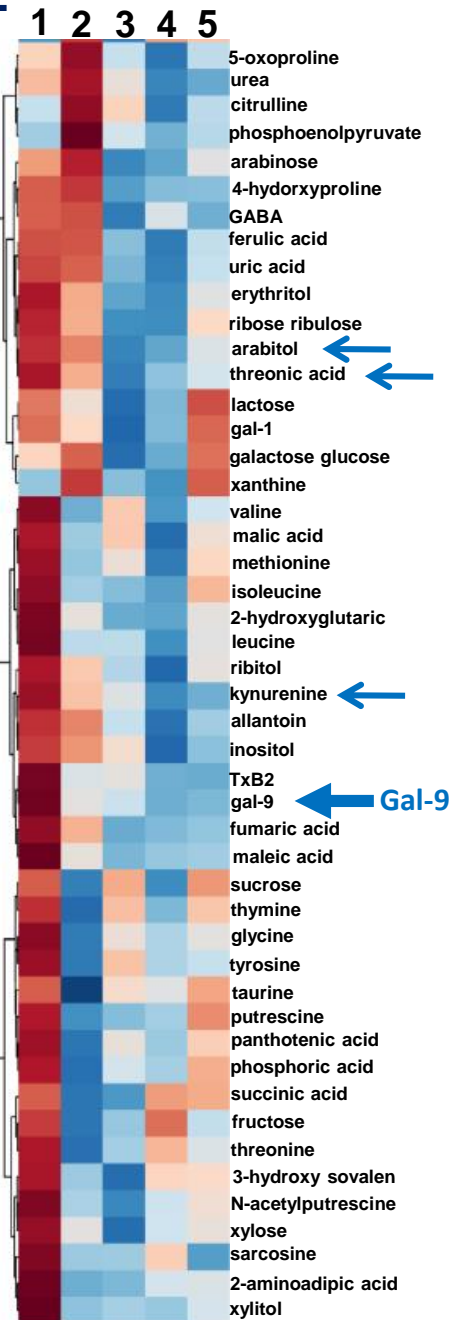
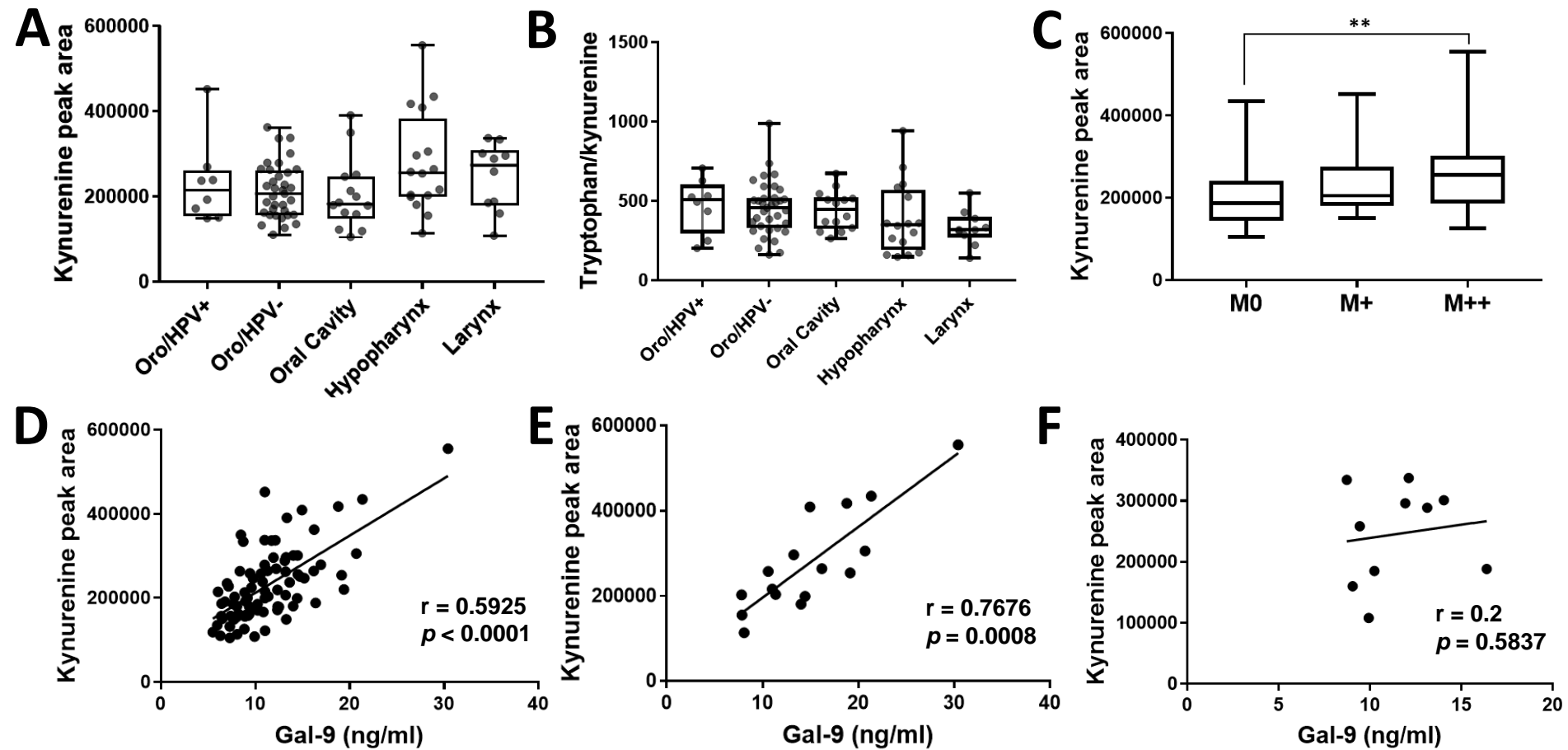


Figure 5



**Figure 6**