Extracellular histones profiles of pediatric H3K27-altered diffuse midline glioma

Running heading: extracellular histones and diffuse midline glioma

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Abstract

Background

Diffuse midline glioma, H3 K27-altered (DMG) is a fatal tumour that arises in the midline structures of the brain. When located in the pons, it is more commonly referred to as diffuse intrinsic pontine glioma (DIPG). DMG/DIPG is usually diagnosed when children are aged <10 years, and it has a median overall survival of <12 months after diagnosis. Radiological imaging is still the gold standard for DIPG diagnosis while the use of biopsy procedures led to our knowledge on its biology, such as with the identification of the canonical histone H3K27M mutation. However, the need to improve survival encourages the development of non-invasive, fast and inexpensive assays on biofluids for optimizing molecular diagnoses in DMG/DIPG. Here, we propose a rapid, new, imaging and epigenetics based approach to diagnose DMG/DIPG in the plasma of pediatric patients.

Methods

A total of 20 healthy children (mean age: 10.5 years)and 24 children diagnosed with DMG/DIPG (mean age: 8.5 years) were recruited. Individual histones (H2A, H2B, H3, H4, macroH2A1.1 and macroH2A1.2), histone dimers and nucleosomes were assayed in biofluids by means of a new advanced flow cytometry ImageStream(X)-adapted method.

Results

We report a significant increase in circulating histone dimers and tetramers (macroH2A1.1/H2B vs control: p-value<0.0001; macroH2A1.2/H2B vs control: p-value<0.0001; H2A/H2B vs control: p-value<0.0001; H3/H4 vs control: p-value =0.008; H2A/H2B/H3/H4 vs control: p-value<0.0001) and a significant downregulation of individual histones (H2B vs control: p-value<0.0001; H3 vs control: p-value<0.0001; H4 vs control: p-value<0.0001). Moreover, histones were also detectable in the cerebrospinal fluid (CSF) of DMG/DIPG patients and in the supernatant of SF8628, OPBG-DIPG002 and OPBG-DIPG004 DMG/DIPG cell lines, with patterns mostly similar to each other, but distinct compared to blood plasma.

Conclusions

In summary, we identified circulating histone signatures able to detect the presence of DMG/DIPG in biofluids of children, using a rapid and non-invasive ImageStream(X)-based imaging technology, which may improve diagnosis and benefit the patients.

Key points

- We devised propose a rapid, new, imaging and epigenetics based approach to diagnose DMG/DIPG in the plasma of pediatric patients, based on imaging flow cytometry.
- Compared to healthy children, DMG/DIPG pediatric patients had a significant increase in circulating histone dimers and tetramers and a significant downregulation of individual histones in their blood.Histones were also detectable in the cerebrospinal fluid (CSF) of DMG/DIPG patients and in the supernatant of three DMG/DIPG cell lines, with patterns mostly similar to each other, but distinct compared to blood plasma.

1. Introduction

Brain tumors are the most prevalent solid tumors in childhood and young adulthood and stand as a primary contributor to cancer-related morbidity and mortality [1]. Brain tumors constitute around 20% of all childhood cancers, with a worldwide incidence of approximately 3 to 5 cases per 100,000 live births [2,3]. High-grade gliomas (HGGs) make up approximately 12% of pediatric brain tumors and are associated with a particularly dismal prognosis [4]. Within this

category, diffuse midline glioma (DMG) - H3 K27-altered is characterized by an exceedingly aggressive phenotype and it originates in the midline brain structures, particularly the brainstem, thalamus, and spinal cord. Formerly referred to as diffuse intrinsic pontine glioma (DIPG) when located in the pons, DMG/DIPG tumors are not amenable to surgical resection due to their invasive nature and the neuroanatomical regions they affect. While tumor resection can serve both diagnostic and therapeutic purposes for low-grade gliomas, in DMG/DIPG this is not feasible due to tumor location. Until 2016, the classification of brain tumors was based mainly on histology, where tumors were classified according to their common morphologic and histopathological features with different putative cells of origin and according to different stages of development [5]. In 2016, key molecular findings have been incorporated in the definition of new tumor entities described in the fourth edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (WHO CNS4) [5]. Accordingly, the DMG-K27M mutant variant, causing a global reduction in the intra-tumoral levels of H3 lysine 27 trimethylation (H3K27me3) is frequently observed in DMG/DIPG. In normal cells, trimethylation is mainly established by the H3K27-specific histone methyltransferase enhancer of zeste homolog 2 (EZH2) within the Polycomb Repressive Complex 2 (PRC2). In DMG/DIPG, H3K27M typically results in histone H3 hypomethylation on K residues and ultimately leads to an epigenetic deregulation of cellular processes due to the inactivation of PRC2 through an interaction between EZH2 and the mutant histone [6,7]. The fifth edition of the WHO Classification of Tumors of the CNS (WHO CNS5), published in 2021refined the definition of CNS tumor entities [8,9] and, in light of the latest molecular discoveries [10,11], DMG can be divided into three subtypes depending on their molecular characteristics, and/or location: DMG H3K27M (the classical type with the H3K27M mutation), the DMG H3 wild-type EZHIP overexpressed and the DMG with EGFR alterations with or without H3K27M mutation [8]. The relative prevalence of the three subtypes is

estimated at ~75%[12-14], ~5[15, 16], and ~5-10% [17], respectively, of all DMG cases. Regardless, DMG is invariably a terminal illness, since it has a 5-year survival rate of <1%[18]. Although the majority of patients show some improvement in their symptoms after focal intensity-modulated radiation therapy (IMRT) to the primitive tumor, DMG usually progresses quickly, and the median time from tumor progression to death is usually only few months. H3K27M mutation correlates with a poorer clinical outcome [19,20] and can be used as a criterion for enrolment in clinical trials [20]. The detection and monitoring of molecular alterations is likely to be critical for the screening, the clinical management and the design of clinical trials for pediatric brain tumors, overcoming the limitations of magnetic resonance imaging (MRI) and of histology [21-24].

In this respect, liquid biopsies are emerging as a promising platform. Biofluids such as blood and cerebrospinal fluid (CSF) may contain small amounts of circulating tumor cells, cell-free DNA (cfDNA), fragmented peptides, and intact proteins. In order to be suitable as clinically useful biomarkers, they must be highly tumor-specific and present in detectable concentrations. Cell-free tumor DNA (ctDNA) is detected in high levels in plasma and CSF from pediatric and adult patients with advanced cancer and has been associated with poor prognosis [25-27]. However, pre-analytical factors such as temperature and storage conditions affect the integrity of ctDNA and currently fully established standardized methods of liquid biopsy specimens processing are lacking [28,29]. Conversely, tumor-derived and tumor-associated proteins are released in the extracellular space upon secretion or cell death, and can be detected in biofluids. The detection of proteins in plasma and CSF is a promising alternative approach for diagnostics, monitoring, and prognosis of malignancies and neurological disorders [30,31].

Histones are highly basic proteins organized in an octameric core with DNA wrapped around to form the nucleosome, the basic repeating unit of chromatin [32]. Elevated nucleosome levels were detected and used as a diagnostic tool in several adult cancers and in obesity [33-36].

Besides the “canonical” histones, ~20 variants were described, mostly of H2A and H3, in human somatic cells [37]. The differences among the variants are related to their unique temporal pattern of chromatin deposition during the cell cycle [37]. The variants macroH2A1 and macroH2A2 are the largest in nature [37] and we have shown that macroH2A1 isoforms play a pivotal role in modulating stem cell differentiation and cancer [38-43]. It is unknown whether nucleosomes or individual diverse circulating histone patterns may be used as new biomarkers for HGGs. High resolution imaging, based on ImageStream(X) imaging flow cytometry, has been used to detect and quantify the expression of multiple biomarkers on circulating blood and cancer cells, with high speed, reliability, and low costs [44]. We recently developed an ImageStream(X)-based method to identify a circulating histone signature in children and adults affected by fatty liver disease [45,46]. In a recent pilot study, we have also found that histones can be detected in the CSF of children affected by pediatric brain tumors [47]. In this study, we optimized an ImageStream(X)-based method to compare circulating histones in retrospective biofluids (blood plasma, CSF) of children with DMG/DIPG to healthy subjects, providing a new potential tool applicable in DMG/DIPG diagnosis and prognosis.

2. Materials and Methods

2.1 Patients and biofluids

Bio-banked blood samples of 20 healthy individuals were used as controls. The samples were stored at Bambino Gesù Children's Hospital from March 2018 to June 2021 and included in the EuPNAFLD protocol (Ethical Committee of Bambino Gesù Children’s Hospital, 1774_OPBG_2019). Peripheral blood (from 24 DMG/DIPG patients) and CSF (from 9 DMG/DIPG patients) samples were also collected at the Bambino Gesù Children’s Hospital during the standard clinical practice (Ethical Committee of Bambino Gesù Children’s Hospital,

2712_OPBG_2021). Peripheral blood samples were collected in serum-separating tubes, allowed to clot 1 hour at room temperature and centrifuged 10 minutes at 1,000xgat 4ºC to obtain serum; alternatively, they were collected in lithium heparin-coated vials, centrifuged at 5,000xg for 10 minutes at 4ºC to obtain plasma. Serum and plasma samples were stored at -80°C for further analysis. CSF samples were collected by lumbar puncture. After the laboratory examination, residual CSF samples were centrifuged at 1300 rpm for 5 min at room temperature to remove cells and the supernatant was stored at -80°C for further analysis. Written informed consent was obtained from the patient or, if younger than 18 years, from patient's parents or legal guardian in compliance with the relevant ethical regulations, including the Declaration of Helsinki. Patients' demographics, anthropometrical and biochemical data were withdrawn from electronic medical records.

2.2 Cell cultures

Diffuse intrinsic pontine glioma (DIPG) cell line (SF8628), harboring histone H3.3 was purchased from Sigma Aldrich/Merck (cat. no. SCC127M; St. Louis, MO, USA). The SF8628 cells were grown as suggested by supplier in Dulbecco's Modified Eagle Medium (DMEM)–High-Glucose media 4.5 g/l (Sigma Aldrich, St. Louis, MO, USA). The media were supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, USA) containing 100 IU/mL of penicillin and 100 µg/mL of streptomycin (P/S; Sigma Aldrich, St. Louis, MO, USA) and 2 mM L-Glutamine (Sigma Aldrich, St. Louis, MO, USA). Cells were incubated at 37 °C in a humidified 5% CO2 atmosphere. The medium was changed every 2 days; conditioned media was saved and stored at -80 °C for later analysis, and the cells were sub-cultured using TrypLE Express (Gibco, Ireland) when reaching 90% confluence. OPBG-DIPG002 (harboring H3.3 K27M mutation) and OPBG-DIPG004 (harboring H3.1 K27M

mutation) were derived from DMG/DIPG primary tumors at Bambino Gesù Children's Hospital, and they were previously described and characterized. References and methods are available at: https://cancermodels.org/data/models/ICCS-OPBG/OPBG-DIPG002 and https://cancermodels.org/data/models/ICCS-OPBG/OPBG-DIPG004.

2.3 ImageStream(X) protocol optimization

To measure the levels of histones we used multispectral imaging flow cytometer ImageStream MkII (Luminex Corporation). To define the appropriate experimental set up samples from blood, CSF, or cell supernatant were immunostained with a single antibody. Laser power was set not to detect any saturated pixels by detecting the number of saturated pixels in an image using "Raw Max Pixel" and feature "Saturation Count" properties (available in the IDEAS® statistical analysis software package (Amnis Corporation, USA). Pixel intensities become saturated and cannot be quantified after 4095 pixels, since the camera measures pixels between 0 and 4095 (12 bit). The defined optimal laser power for each antibody (laser 488 nm–5 mW, laser 561 nm–20 mW, laser 642–5 mW) was used for the multichannel assays.Tocorrect the data from multiparameter assays for spectral overlap a spectral crosstalk matrix was created by using single-colorcontrol samples and applied to the image files.Image-based algorithms available in the IDEAS® statistical analysis software package (Amnis Corporation, USA) were used to analyze the resulting compensated image files.

2.4 ImageStream(X) detection of histone complexes in the DMG/DIPG blood, cerebrospinal fluid and cell supernatants

The following combination sets of antibodies (Tables 1-3) were used to immunostain samples from healthy individuals:

Table 1.

Primary antibody	Company and catalogue number of primary antibody	Secondary antibody	Company and catalogue number of secondary antibody
anti-histone H2A	Abcam, Ab18255	Goat anti-rabbit IgG H&L-Alexa Fluor® 488	Thermo Fisher Scientific, A-11008
anti-histone H2B	Abcam, Ab134211	Goat anti-chicken IgY H&L-Alexa Fluor® 594	Thermo Fisher Scientific, A-11042
anti-histone H3	Abcam, Ab12079	Donkey anti-goat IgG H&L Alexa Fluor® 555	Thermo Fisher Scientific, A-21432
anti-histone H4	Abcam, Ab31830	Goat anti-mouse IgG H&L-Alexa Fluor® 647	Thermo Fisher Scientific, A-21235

Table 2.

Primary antibody	Company and catalogue number of primary antibody	Secondary antibody	Company and catalogue number of secondary antibody
anti-macroH2A1.1	Cell Signaling Technology, 12455S	Goat anti-rabbit IgG H&L-Alexa Fluor® 488	Thermo Fisher Scientific, A-11008
anti-histone H2B	Abcam, Ab134211	Goat anti-chicken IgY H&L-Alexa Fluor® 594	Thermo Fisher Scientific, A-11042
anti-histone H3	Abcam, Ab12079	Donkey anti-goat IgG H&L Alexa Fluor® 555	Thermo Fisher Scientific, A-21432
anti-histone H4	Abcam, Ab31830	Goat anti-mouse IgG H&L-Alexa Fluor® 647	Thermo Fisher Scientific, A-21235

Table 3.

Primary antibody	Company and catalogue number of primary antibody	Secondary antibody	Company and catalogue number of secondary antibody

anti-macroH2A1.2	Cell Signaling Technology, 4827S	Goat anti-rabbit IgG H&L-Alexa Fluor® 488	Thermo Fisher Scientific, A-11008
anti-histone H2B	Abcam, Ab134211	Goat anti-chicken IgY H&L-Alexa Fluor® 594	Thermo Fisher Scientific, A-11042
anti-histone H3	Abcam, Ab12079	Donkey anti-goat IgGH&L Alexa Fluor® 555	Thermo Fisher Scientific, A-21432
anti-histone H4	Abcam, Ab31830	Goat anti-mouse IgG H&L-Alexa Fluor® 647	Thermo Fisher Scientific, A-21235

The following combination sets of antibodies (Tables 4-6) were used to immunostain samples from DMG/DIPG pediatric patients, supernatants of OPBG-DIPG cell lines, and supernatants of SF8628 DMG/DIPG cell line:

Table 4.

Primary antibody	Company and catalogue number of primary antibody	Secondary antibody	Company and catalogue number of secondary antibody
anti-histone H2A	Abcam, Ab18255	Goat anti-rabbit IgG H&L-Alexa Fluor® 488	Thermo Fisher Scientific, A-11008
anti-histone H2B	BioLegend, 606302	Goat Anti-Rat IgG H&L - AlexaFluor® 594	Abcam, ab150160
anti-histone H3	LS Bio, LS-B6334-125	Donkey Anti-Sheep IgG H&L - Alexa Fluor® 555	Abcam, ab150178
anti-histone H4	Abcam, Ab31830	Goat anti-mouse IgG H&L-Alexa Fluor® 647	Thermo Fisher Scientific, A-21235

Table 5.

Primary antibody	Company and catalogue number of primary antibody	Secondary antibody	Company and catalogue number of secondary antibody
anti-macroH2A1.1	Cell Signaling Technology, 12455S	Goat anti-rabbit IgG H&L-Alexa Fluor® 488	Thermo Fisher Scientific, A-11008

anti-histone H2B	BioLegend, 606302	Goat Anti-Rat IgG H&L - AlexaFluor® 594	Abcam, ab150160
anti-histone H3	LS Bio, LS-B6334-125	Donkey Anti-Sheep IgG H&L - Alexa Fluor® 555	Abcam, ab150178
anti-histone H4	Abcam, Ab31830	Goat anti-mouse IgG H&L-Alexa Fluor® 647	Thermo Fisher Scientific, A-21235

Table 6.

Primary antibody	Company and catalogue number of primary antibody	Secondary antibody	Company and catalogue number of secondary antibody
anti-macroH2A1.2	Cell Signaling Technology, 4827S	Goat anti-rabbit IgG H&L-Alexa Fluor® 488	Thermo Fisher Scientific, A-11008
anti-histone H2B	BioLegend, 606302	Goat Anti-Rat IgG H&L - AlexaFluor® 594	Abcam, ab150160
anti-histone H3	LS Bio, LS-B6334-125	Donkey Anti-Sheep IgG H&L - Alexa Fluor® 555	Abcam, ab150178
anti-histone H4	Abcam, Ab31830	Goat anti-mouse IgG H&L-Alexa Fluor® 647	Thermo Fisher Scientific, A-21235

Sample preparation: for each combination set 50-µl of blood plasma/CSF from DMG/DIPG patients was incubated overnight at 4 °C with a combination of 4 primary antibodies. Of note, due to discontinuation by the production company, different antibodies against histone H2B and histone H3 were used in the staining of samples from DMG/DIPG pediatric patients, supernatants of OPBG-DIPG cell lines, and supernatants of SF8628 DMG/DIPG cell line, compared to the antibodies against histone H2B and histone H3 used in the staining of plasma from healthy controls. The antibody combination sets were first prepared by adding the respective primary antibodies in phosphate buffer (pH 7.4) in a 1:50 ratio. The following day, the sample was incubated with secondary antibodies for 2 h at room temperature. As with the

primary antibodies, combination sets with 4 fluorescent secondary antibodies were prepared by diluting the antibodies in phosphate buffer - pH 7.4 in a ratio of 1: 100). The sample was incubated for 2 h at room temperature with the respective combination set of antibodies.

Image acquisition: for each immunostained CSF sample, 10,000 objects were collected using: 1) excitation laser 488 nm (5 mW) for Alexa Fluor® 488; fluorescence was collected in channel two (505–560 nm), 2) excitation laser 561nm (20mW) for Alexa Fluor® 555 and Alexa Fluor® 594; fluorescence was collected in channel three (560-595 nm) and channel four (595-642 nm), respectively, 3) excitation laser 642 nm (5mW) for Alexa Fluor® 647; fluorescence was collected in channel five (642–745 nm). Channel one was used for the bright field image and channel six for the laser scatter image.

The following population gating was applied to identify fluorescence-stained objects within all measured objects: a) focused objects, b) objects with fluorescence.

2.5 Histone signature

A logistic regression has been trained to obtain an histone-based signature for the DMG/DIPG patients and we have also retrieved the coefficient distribution (obtained via Bootstrap) for each variable; variables have been scaled before training; logistic regression has been L1 regularized in order to automatically exclude non-informative variables. The whole model has been implemented using scikit-learn package (version 1.5.1).

2.6 Statistical analyses

All statistical analysis has been conducted in Python by using the SciPy library (version 1.11.3). Single histones and histones complexes abundance of different experiments have been averaged and log-transformed. Mann-Whitney U test has been used to compare abundances among different groups, while Wilcoxon rank test has been used to compare different tissue

abundances within the same group. Boxplots have been generated through matplotlib (version 3.8.1). Power has been estimated via bootstrap simulations.

3. Results

3.1 Individual histones are decreased, while histone complexes are increased, in the blood plasmaof children with DMG/DIPG

Pediatric CNS tumor patients would benefit highly from minimally invasive molecular profiling of the tumor and molecularly driven monitoring of tumor response and progression. Therefore, we sought to determine if the single histones (H2A, H2B, H3, H4, macroH2A1.1, macroH2A1.2), and histone complexes may be detectable in the blood of DMG/DIMG patients. ELISA assays can detect nucleosomes or individual histones in biofluids; however, a real-time high throughput detection of multiple histones is warranted. Here, we have used a protocol that we have previously optimized [45-47] (workflow is shown in Figure 1), consisting of a multi-channel flow imaging methodology on ImageStream(X) and image single histone staining on different channels. We analyzed the 4 canonical histones (H2A, H2B, H3, H4) and 2 large variants of histone H2A (macroH2A1.1 and macroH2A1.2), in the blood of 20 healthy children and of 24 children affected by DMG/DIPG. Basal characteristics of healthy children are reported in Table 7, while those of DMG/DIPG children are reported in Table 8. Early studies have indicated that histone dimers may serve as a stable intermediate in histone assembly, and no trimers form [48]. Each histone type can be seen as an interchangeable subunit of a complex in which the dimer species is the most stable sub-complex [48]. In particular, *in vitro*, the histones form preferentially H2A-H2B heterodimers and H3-H4 heterotetramers [43]. The H2A/H2B dimer binds onto the H3/H4 tetramer due to interactions between H4 and H2B, in the presence of DNA [43]. For these reasons, we assayed the above-mentioned 6 individual

histones (H2A, H2B, H3, H4, macroH2A1.1 and macroH2A1.2) together with the dimers H3/H4, and the heterotetramers H2A/H2B/H3/H4. We detected all histone species, variants and complexes at different frequencies in the plasma. Figure 2 exemplifies, using specific primary and secondary antibodies, the single imaging of histones H2A, H2B, H3, H4, macroH2A1.1 and macroH2A1.2 in six different imaging channels. Interestingly, we detected a significant increase in histone dimers and tetramers (macroH2A1.1/H2B vs control: $p<0.0001$; macroH2A1.2/H2B vs control: $p<0.0001$; H2A/H2B vs control: $p<0.0001$; H3/H4 vs control: $p=0.008$; H2A/H2B/H3/H4 vs control: $p<0.0001$) and a significant downregulation of individual histones (H2B vs control: $p<0.0001$;H3 vs control: $p<0.0001$; H4 vs control: $p<0.0001$) (Figure 3). However, all non-significant differences were associated with a high type II error (power < 0.8), suggesting that a potential effect might have been detected with a larger sample size (Supplementary Table 1). We have further used these data to train a logistic regressor in order to predict the probability for a histone species to mark DMG/DIPG samples versus control samples (positive coefficient values) versus those that do not (negative coefficient values). Logistic regression coefficients are shown in the Supplementary Table 2, supporting the results shown in Figure 3.

3.2 CSF from DMG/DIPG patients and supernatants of DMG/DIPG cells exhibit distinct circulating histone patterns, compared to blood plasma

In a pilot study, we have recently shown that free histones and histone complexes are detectable with a strong signal in the CSF of 8 children affected by brain tumors, including DMG/DIPG (n=1), medulloblastoma (n=3), ependymoma (n=2) and others, using ImageStream(X) technology [47]. CSF is produced by the ependymal cells in the choroid plexus and its composition is similar to the blood plasma except for the nearly protein-free nature of the CSF, and different concentrations of electrolytes compared to blood [50]. CSF can directly contact

the brain tumor and has been demonstrated as a better source of ctDNA or cfDNA than plasma for brain tumors [51]. Moreover, there is also a bulk flow of CSF into the blood via the subarachnoid space through the arachnoid granulations –which is the route for the majority of CSF contents to reach the blood. To our knowledge, a comprehensive comparative analysis of circulating histone expression levels between blood (plasma or serum) and CSF has never been performed. For 9 of the 24 patients whose blood was analyzed by imaging flow cytometry, CSF samples were also available for analysis (Table 8). ImageStream(X) analysis uncovered dramatic and significant differences in the abundance of the following histones in the CSF vs blood plasma of DMG/DIPG patients: H2A (increased, $p<0.01$), H2A/H2B (decreased, $p<0.01$), H2A/H2B/H3/H4 (decreased, $p<0.01$), H2B (decreased, $p<0.01$), H3 (increased, $p<0.01$), H4 (increased, $p<0.01$), macroH2A1.1 (increased, $p<0.01$), macroH2A1.1/H2B (decreased, $p<0.01$), macroH2A1.1/H2B/H3/H4 (decreased, $p<0.01$), macroH2A1.2 (increased, $p<0.01$), macroH2A1.2/H2B (decreased, $p<0.01$), macroH2A1.2/H2B/H3/H4 (decreased, $p<0.01$) (Figure 4). Overall, we observed an increase in histone monomers (H2A, H3, H4) and a decrease in histone complexes (H2A/H2B, H2A/H2B/H3/H4, macroH2A1.1/H2B, macroH2A1.1/H2B/H3/H4, macroH2A1.2/H2B/H3/H4) in the CSF vs blood. The tissue/cellular origin of these circulating histones in DMG/DIPG blood or CSF, however, is unknown. Subsequently, we decided to examine the circulating histone levels produced in the supernatants of 3 DIPG cell lines: SF8628 and OPBG-DIPG002, harboring histone H3.3 K27M mutation, which occurs in ~50% of DMG/DIPG cases [46] as in our cohort (Table 8), and OPBG-DIPG004 harboring H3.1 K27M mutation, which is associated to better survival. ImageStream(X) analysis showed significant differences in the abundance of the following histones in the supernatant of DMG/DIPG cells compared to the blood plasma, which were similar to the CSF vs blood plasma comparison: H2A (increased, $p<0.05$), H2A/H2B (decreased, $p<0.05$), H2A/H2B/H3/H4 (decreased, $p<0.01$), H2B (decreased, $p<0.01$), H3

(increased, $p<0.05$), H4 (increased, $p<0.05$), macroH2A1.1 (increased, $p<0.01$), macroH2A1.1/H2B (decreased, $p<0.05$), macroH2A1.1/H2B/H3/H4 (decreased, $p<0.01$), macroH2A1.2 (increased, $p<0.01$), macroH2A1.2/H2B (decreased, $p<0.05$), macroH2A1.2/H2B/H3/H4 (decreased, $p<0.01$) (Figure 4). Thus, we observed an increase in histone monomers (H2A, H3, H4) and a decrease in histone complexes (H2A/H2B, H2A/H2B/H3/H4, macroH2A1.1/H2B, macroH2A1.1/H2B/H3/H4, macroH2A1.2/H2B/H3/H4) in the DIPG cell supernatant vs blood plasma, similar to what observed in the CSF vs blood plasma comparison (Figure 4). Accordingly, circulating histone levels were quite similar between CSF vs SF8628 cell supernatants, with only slight changes observed for H3 ($p<0.01$), macroH2A1.1/H2B/H3/H4 ($p<0.01$) and macroH2A1.2/H2B/H3/H4 ($p<0.01$) (Figure 4). OPBG-DIPG002 and OPBG-DIPG004 cell lines displayed histone profiles nearly identical, and mostly comparable to SF8628 cell line and CSF for 10 out of the 13 histone species analyzed; however, for H2A, H3 and macroH2A1.1 levels, OPBG-DIPG002 and OPBG-DIPG004 cell supernatants resembled instead the quantities present in the blood plasma (Figure 4).

Overall, these findings offer a proof-of-concept of new histone-based DMG/DIPG liquid biopsies in different biofluids (blood plasma, CSF and DMG/DIPG cell supernatant), which may enable tumor epigenetic characterization by minimally invasive means.

4. Discussion

The high mortality rate and limited therapeutic choices indicate the challenges in targeting and managing pediatric DMG/DIPG. It is of utmost importance to develop advanced diagnostic and monitoring approaches that can transform not only the early detection but also guide in therapeutic strategies and monitor therapeutic response or disease progression. This is particularly indicated for the low number of cases amenable to surgical resection or biopsy. One

of the main finding of this report is the detection of a specific signature of free histones and histone complexes, with a robust signal in the blood plasma of children affected by DMG/DIPG, using ImageStream(X) technology combining flow cytometry with high content image analysis [45]. Compared to healthy children, children affected by DMG/DIPG displayed particular elevated levels of circulating histone dimers and tetramers (macroH2A1.1/H2B, macroH2A1.2/H2B, H2A/H2B, H3/H4, and H2A/H2B/H3/H4) and a concomitant decrease in individual histones (H2B, H3, H4). Upon cell death, histones are released in the bloodstream. In live cells, several chaperones are involved in pre-nucleosomal and nucleosomal histone complexes interaction, deposition, recycling and eviction. During nucleosome disassembly, eviction of H2A/H2B tends to occur first and then H3/H4 may be removed. In particular, NAP1 and FACT are responsible for H2A/H2B [47], while ASF1A/ASF1B and NASP/HAT1 mediate H3/H4 eviction [54]. FACT also mediates the depletion of macroH2A1 from the chromatin [55]. Interestingly, FACT has been recognized as an oncogenic target for DMG/DIPG, and its inhibition reverses the effect of H3K27M and significantly prolonged survival in aggressive *in vivo* models of the disease [56].

Using proteomics approaches, Bockaj *et al.* found an increase in macroH2A1 (which is released at a decreased level in DMG/DIPG blood plasma) in H3.3K27M mutant DMG/DIPG cells, suggesting perturbed replication [57], and enrichment for histone chaperoning complexes (comprising ASF1B, NASP, FACT, and others) [58]. It is tempting to speculate that the circulating histone pattern that we observe in DMG/DIPG blood plasma samples might reflect their deposition/eviction into/from nucleosomes of dying (cancer) cells.

In DMG/DIPG, as in all CNS tumors, the optimal medium to provide detailed information on risk assessment, diagnosis, tumor classification, tumor grade, helps identify potential treatment targets, or monitor therapeutic response is CSF [59]. Isolation of liquid biopsy analytes from CSF, including ctDNA, extracellular vesicles, tumor-specific proteins and histones has been

successfully performed in previous studies [47,60]. Another major finding of our study is that the circulating histone signature of DMG/DIPG CSF is quite similar to the one present in the supernatant of DMG/DIPG cultured cell lines, suggesting that the histone composition in the CSF might reflect the shedding of DMG/DIPG cell chromatin content into it. While it is not feasible to analyze and compare the CSF of healthy subjects, in a previous study we have shown that the CSF from children affected by an unrelated pathology (blood malignancy), display a scarcer histone species abundance and diversity, compared to primary brain tumors (PBT) [47]. The diversity of circulating histone composition in the blood plasma of DMG/DIPG children, compared to their CSF, indicates that additional cell types might contribute to it, beyond DMG/DIPG cells. Nevertheless, the origin of the circulating histones in health and disease is currently unknown. A novel approach suggests that composition of cfDNA/histones complexes could be used to predict the origin of the respective DNA fragments in complex with histones. CfDNA retains epigenetic characteristics, specific to a particular tissue or cell type that might be used to infer the tissue of origin [61]. Beyond cell death, histones can be released in the extracellular space through neutrophil extracellular traps (NETs) [62]. Of note, neutrophils are negatively associated with prognosis in DMG/DIPG [63,64]. In addition, tumor derived-extracellular vesicles/exosomes [65] are candidate causes for the elevated levels of histones and histone complexes in the circulation. Together with origin of the circulating histones, the findings open many questions regarding the potential value of the circulating histone signature in the clinical care of DMG patients such as: 1) can histones be used to detect patient's response to treatment, 2) can the histone profile infer the state of disease progression, 3) how does the histone signature of DMG patients differ from the signature of paediatric low grade glioma patients with BRAF alterations [66], and 4) how do histone levels reflect the location of the tumor.

This study has potential limitations. First, it was not possible to determine whether the circulating histone signature relates to tumor location, to histone gene mutation, or to the expression of histones in the tumor itself. Second, the sample size was limited due to the low incidence of the condition, and the retrospective nature. Effect estimates on circulating histones based on future interventional and prospective observational studies are warranted.

5. Conclusions

DIPG has an extremely poor prognosis; however, advances in the diagnostic and monitoring methods are steadily improving. The limitations of the typically used radiographic imaging or tissue biopsy for diagnosis are evident and the need for liquid biopsy sampling to advance our understanding of the disease is highly anticipated. This study shows that a distinct circulating histone signature, consisting of decreased monomers and increased histone complexes, is detected in the blood of DMG/DIPG children by using a rapid and non-invasive ImageStream(X)-based imaging technology. Distinct histone signatures can also be detected in the CSF of DMG/DIPG patients and in the supernatants of DMG/DIPG cells, mostly similar to each other, but different from blood. Histone-based molecular diagnostic kits could represent a good alternative to more expensive NGS-based approaches.

Statement and Declarations

Competing interests: All authors: D.B., L.L.P., J.F., D.K.T., S.D.B., M.R.B., A.A., A.M., J.C., T.M., and M.V. declare no competing financial interests.

Ethics approval and consent to participate: The Institutional Review Board of Bambino Gesù Children's Hospital– IRCCS, Rome, approved the collection of blood samples for this research. All participants provided informed consent before study procedures.

Consent for publication: All authors have reviewed and given consent to this submission of this manuscript.

Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability: Not applicable.

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Authors' contributions: Conceptualization: JC, ManV; Methodology: Diana Buzova; Formal analysis and investigation: DB, JF, DKT, LLP, MRB; Writing - original draft preparation: ManV; Writing - review and editing: DB, AA, MarV, ManV; Funding acquisition: JF, JC, ManV; Supervision: JC, AA, TM, MarV, ManV.

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Figures/Tables Legends

Table 1. First set of antibodies used for the immunostaining of histones in plasma samples from healthy individuals.

Table 2. Second set of antibodies used for the immunostaining of histones in plasma samples from healthy individuals.

Table 3. Third set of antibodies used for the immunostaining of histones in plasma samples from healthy individuals.

Table 4. First set of antibodies used for the immunostaining of histones in plasma samples from DMG/DIPG pediatric patients, supernatants of OPBG-DIPG cell line, and supernatants of SF8628 DMG/DIPG cell line.

Table 5. Second set of antibodies used for the immunostaining of histones in plasma samples from DMG/DIPG pediatric patients, supernatants of OPBG-DIPG cell line, and supernatants of SF8628 DMG/DIPG cell line.

Table 6. Third set of antibodies used for the immunostaining of histones in plasma samples from DMG/DIPG pediatric patients, supernatants of OPBG-DIPG cell line, and supernatants of SF8628 DMG/DIPG cell line.

Table 7. Healthy controls children data.

Table 8. DMG/DIPG children data.

Figure 1. Workflow of blood assays of a flow cytometry ImageStream(X)-adapted method.

Figure 2. Representative images, size distribution and fluorescence signal intensity from fluorescence marker of histones H2A, H2B, H3, H4, macroH2A1.1 and macroH2A1.2. ImageStream photographs show bright-field images and histone staining (fluorescence from Alexa Fluor® 488, Alexa Fluor® 555, Alexa Fluor® 594 and Alexa Fluor® 647).

Figure 3. Histones signatures expression of DIPG patients (orange) compared to the 20 control subjects (green) in blood tissue; significant differences (p-value<0.05) are highlighted with the asterisks.

Figure 4. Histones signatures of DIPG children in both blood (orange) and CSF (blue) and CTL supernatants (green); for DIPG children, the histones abundances are shown as boxplots while,

for supernatants, they are shown as means (dots) and standard deviations (lines); significant differences (p-value<0.05) between the CSF and supernatant abundances versus blood abundances are highlighted with the asterisks.

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