Inflammatory response in hematopoietic stem and progenitor cells triggered by activating SHP2 mutations potentiates leukemogenesis

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Abstract

Activating mutations in the protein-tyrosine phosphatase SHP2 are the most frequently occurring mutations in juvenile myelomonocytic leukemia (JMML) and JMML-like myeloproliferative neoplasm (MPN) associated with Noonan syndrome (NS). Hematopoietic stem and progenitor cells (HSPCs) are the disease propagating cells of JMML. Here, we explored transcriptomes of HSPCs with Shp2 mutations derived from JMML patients and a novel NS zebrafish model. In addition to major NS traits, CRISPR/Cas9 knock-in Shp2^{D61G} mutant zebrafish recapitulated a JMML-like MPN phenotype, including myeloid lineage hyperproliferation, *ex vivo* growth of myeloid colonies and *in vivo* transplantability of HSPCs. Single cell mRNA sequencing of HSPCs from Shp2^{D61G} zebrafish embryos and bulk sequencing of HSPCs from JMML patients revealed an overlapping inflammatory gene expression pattern. Strikingly, an anti-inflammatory agent rescued JMML-like MPN in Shp2^{D61G} zebrafish embryos. Our results indicate that a common inflammatory response was triggered in the HSPCs from sporadic JMML patients and syndromic NS zebrafish, which potentiated MPN and may represent a future target for JMML therapies.

Introduction

A broad spectrum of germline activating mutations in the tyrosine phosphatase SHP2, encoded by *PTPN11* has been found to cause Noonan syndrome (NS), a dominantly inherited developmental disorder from the RASopathy group affecting 1:1,500 individuals. NS is characterized by a systemic impact on development, most commonly resulting in short stature, congenital heart defects and specific craniofacial characteristics (Rauen, 2013; Tajan et al., 2018). Somatic activating mutations in *PTPN11* are the most common cause of sporadic juvenile myelomonocytic leukemia (JMML), a rare but aggressive myelodysplastic and myeloproliferative neoplasm (MPN) occurring in young children (Caye et al., 2015; Tartaglia et al., 2003). Consistently, children with NS are predisposed to developing neonatal MPN, that either regress without treatment or rapidly progress to JMML leading to early death (Kratz et al., 2005; Strullu et al., 2014). Previous studies support the view of a strong endogenous role of germline *PTPN11* mutations in the occurrence of myeloproliferative complications with the existence of high-risk mutations (Mulero-Navarro et al., 2015; Strullu et al., 2014). Given the aggressive nature of JMML and lack of therapies, a better understanding of the elusive JMML(-like MPN) pathophysiology and development of reliable preclinical models are essential.

Several lines of evidence indicate the importance of prenatal hematopoiesis such as the young age window for both NS-associated and sporadic JMML. The prenatal origin of *PTPN11* mutations in sporadic JMML suggests that JMML and NS-associated JMML-like MPN originate from fetal hematopoiesis (Behnert et al., 2021). Studying SHP2-driven JMML(-like MPN) during fetal hematopoiesis is challenging in conditional knock-in mouse models of JMML with *PTPN11* mutations, because mutant SHP2 expression is induced only postnatally (Chan et al., 2009; Xu et al., 2011) or indolent MPN is induced (Tarnawsky et al., 2017, 2018). NS knock-in mice with activating *PTPN11* mutations develop mild MPN only after 5 months of age (Araki et al., 2004, 2009).

Zebrafish (*Danio rerio*) with rapid *ex-utero* development, transparent embryos and conserved blood ontogeny emerged as a unique pediatric leukemia model that enables monitoring of leukemogenesis from its initial stages with high temporal and spatial resolution (de Pater & Trompouki, 2018; Gore et al., 2018). Furthermore, NS-associated features, such as shorter body axis length, craniofacial defects, defective

gastrulation and impaired heart looping, are recapitulated in zebrafish embryos upon transient over-expression of NS-associated protein variants (Bonetti et al., 2014; Jopling et al., 2007; Niihori et al., 2019; Paardekooper Overman et al., 2014; Runtuwene et al., 2011).

To better assess the link between dysregulated SHP2 and myeloproliferation in the context of NS, we developed and characterized a novel genetic zebrafish model of NS with Shp2-D61G mutation, an NS-associated mutation that is most frequently associated with NS/JMML-like MPN in human patients (Strullu et al., 2014; Tartaglia et al., 2001). Mutant zebrafish developed hematopoietic defects consistent with JMML-like MPN. Transcriptomic comparison of HSPCs obtained from JMML patients with somatic *PTPN11* mutations and HSPCs from mutant zebrafish harboring an NS-associated variant of Shp2, suggested common mechanisms of disease initiation in sporadic and syndromic PTPN11-driven JMML. Both data sets show a similar pro-inflammatory gene expression and an anti-inflammatory agent largely rescued the hematopoietic defects in mutant zebrafish, suggesting inflammation as potential drug target for sporadic and syndromic JMML(-like MPN). Our data show that Shp2-mutant zebrafish convincingly model the human NS-associated MPN thereby providing a valuable preclinical model for development of future therapies.

Results

Shp2^{D61G} mutant zebrafish display typical Noonan syndrome traits

To investigate early hematopoietic defects associated with NS in a vertebrate animal model, we turned to zebrafish, which represents a versatile model to study leukemogenesis and in which it is feasible to introduce mutations at will using CRISPR/Cas9-mediated homology directed repair (Tessadori et al., 2018). Certain NS mutations are more frequently associated with NS/JMML-like MPN than others. The D61G substitution in SHP2 is the most frequently occurring (Strullu et al., 2014; Tartaglia et al., 2001). Thus, we introduced the D61G mutation in zebrafish Shp2a using CRISPR/Cas9-mediated homology directed repair (**Supplementary Fig. 1a**), targeting the *ptpn11a* gene. Sequencing confirmed that the oligonucleotide used for the homology directed repair was incorporated correctly into the genome (**Fig. 1a**) and that the introduced mutation did not have a detectable effect on Shp2a protein expression (**Fig. 1b**). Prior to phenotypic analyses, the mutant lines were outcrossed twice to ensure that potential background mutations due to the CRISPR/Cas9 approach were removed.

In 32% of Shp2$^{D61G/wt}$ and 45% of Shp2$^{D61G/D61G}$ embryos at 5 days post fertilization (dpf) we observed typical Noonan syndrome traits, such as reduced body axis extension, heart edema and face deformities. Based on the extent of the phenotypes, defects were categorized as normal, mild and severe. (**Fig. 1c,d**). The observed phenotypic defects were mostly mild, but in some cases severe defects were found, including severely stunted growth, edemas of the heart and jaw, and absence of the swim bladder (**Fig. 1c,d**). A more detailed characterization of the typical NS traits showed that the body axis length was significantly reduced in Shp2^{D61G} mutant embryos at 5dpf (**Fig. 1e**). Furthermore, imaging of Alcian blue stained cartilage revealed NS-reminiscent craniofacial defects in 4dpf Shp2^{D61G} mutant embryos, characterized by broadening of the head (**Fig. 1f**), leading to an increased ratio of the width of the ceratohyal and the distance to Meckel's cartilage (**Fig. 1g**). We also assessed the general morphology and function of the mutant embryonic hearts. Whole-mount *in situ* hybridization (WISH) with cardiomyocyte (*myl7*), ventricular (*vhmc*) and atrial (*ahmc*) markers identified no obvious morphological heart defects, such as changes in heart size or heart looping, as well as heart chamber specification at 3dpf (**Supplementary Fig. 1b**). A decrease in

129 heart rate (**Fig. 1i)**, ejection fraction and cardiac output (**Supplementary Fig. 1c,d**)
130 was detected from the ventricular kymographs obtained from high speed video
131 recordings (Tessadori et al., 2012) of the Shp2^{D61G} mutant hearts at 5dpf **(Fig. 1h**).
132 Effects on cardiac function varied from embryo to embryo, which is reminiscent of
133 variable heart defects in human patients.

134 Since most of the embryos with obvious phenotypic defects did not develop a
135 swim bladder, they did not survive to adulthood. The rest of both Shp2$^{D61G/wt}$ and
136 Shp2$^{D61G/D61G}$ mutant zebrafish grew up normally and displayed rather mild defects in
137 adult stages, such as shorter body axis (**Fig. 1j**), with markedly reduced length
138 observed from the embryonic stage of 5dpf until the fully developed adults (**Fig. 1k**).
139 In 10% of Shp2^{D61G} mutants the phenotypes were more severe, with markedly
140 reduced body axis length compared to their siblings, skinny appearance and with
141 overall redness, especially in the head and gill region (**Fig. 1j**).

142 Taken together, the NS Shp2^{D61G} mutant zebrafish we established
143 phenocopied several of the typical NS traits, such as stunted growth, craniofacial
144 defects, and heart defects. Similar to differences in symptoms observed in individual
145 human patients, the severity of the defects varied among individual Shp2^{D61G} mutant
146 zebrafish.

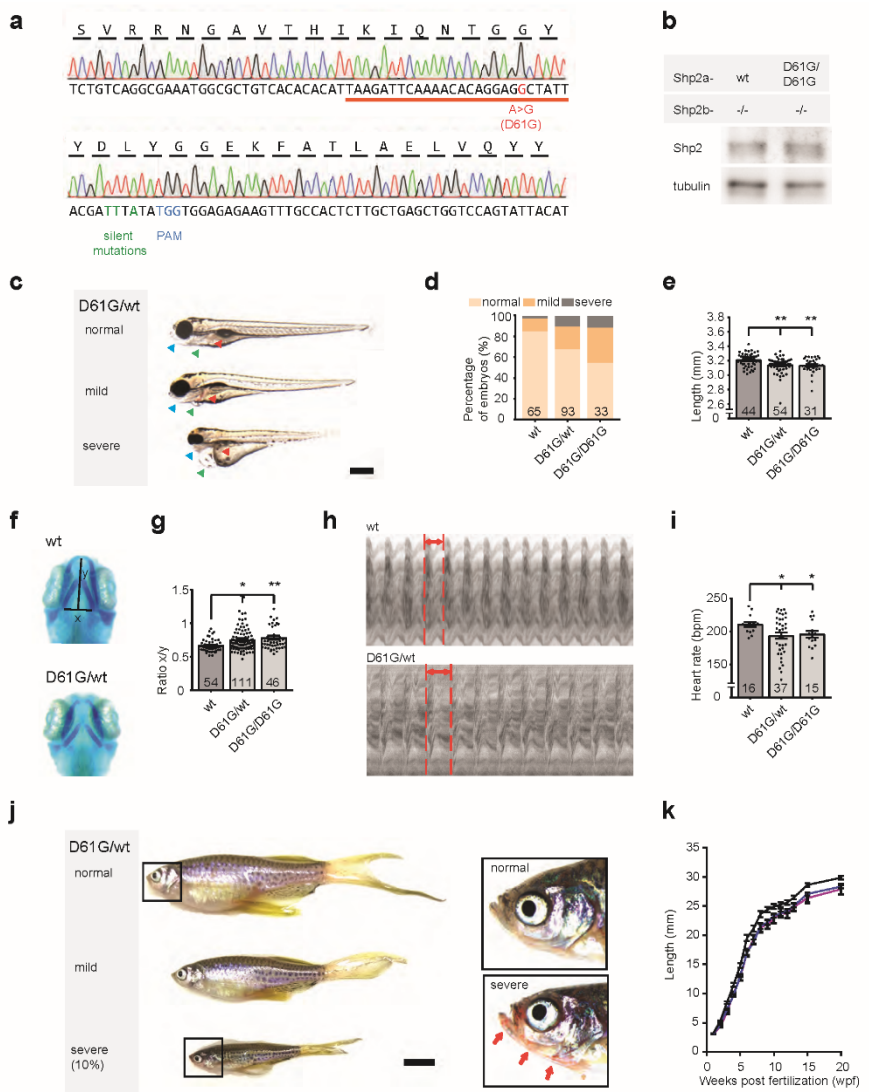

Figure 1. Shp2^{D61G} zebrafish display NS-like traits. a Sequencing trace derived from Shp2$^{D61G/D61G}$ zebrafish. Oligonucleotide sequence used to generate the model is underlined. Nucleotide substitutions for D61G mutation (red), silent mutations close to the PAM site (green) and the PAM site (blue) are indicated. **b** Immunoblot of Shp2 levels from 5 pooled Shp2a^{wt}Shp2b$^{-/-}$ or Shp2a$^{D61G/D61G}$Shp2b$^{-/-}$ embryos using antibodies for Shp2 and tubulin (loading control). **c** Representative images of typical Shp2^{D61G} zebrafish embryonic phenotypes at 5dpf. Blue arrows: jaw, green arrows: heart, red arrows: swim bladder. **d** Quantification of phenotypes of Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ embryos scored as in **c** normal, mild and severe. **e** Body axis length of Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ embryos at 5dpf. **f** Representative images of Alcian blue stained head-cartilage of 4dpf Shp2^{wt} and Shp2$^{D61G/wt}$ embryos. (x) width of ceratohyal, (y) distance to Meckel's cartilage. **g** Quantified craniofacial defects (x/y ratio). **h** Representative ventricular kymographs derived from high speed video recordings of beating hearts of 5dpf Shp2^{wt} and Shp2$^{D61G/wt}$ embryos. Red dotted lines indicate one heart period. **i** Heart rates derived from the ventricular kymographs. **j** Representative images of typical Shp2^{D61G} zebrafish adult phenotypes at 24wpf. Red arrows indicate skin redness in the jaw region. Scale bar is 0.5cm. Insets, zoom-in of boxed regions. **k** Body axis lengths of 10 Shp2^{wt}, 25 Shp2$^{D61G/wt}$ and 10 Shp2$^{D61G/D61G}$ zebrafish measured weekly between 5dpf and 20wpf of age. **d,e,g,i** Measurements originate from three distinct experiments. Number on bars: number of embryos. **e,g,i,k** Error bars: standard error of the mean (SEM), *$p < 0.05$; **$p < 0.01$, ANOVA complemented by Tukey HSD.

147 **Shp2^{D61G} zebrafish embryos develop myeloproliferative blood defect**
148 **originating in defective HSPCs**
149 Next we explored hematopoietic abnormalities in the Shp2^{D61G} zebrafish
150 during embryonic development, corresponding to prenatal hematopoietic ontogeny in
151 human. We were not able to observe any effect of the Shp2^{D61G} mutation on primitive
152 hematopoiesis in embryos at 2dpf using WISH for markers of erythroid progenitors
153 (*gata-1*), myeloid progenitors (*pu.1*) and white blood cells (*l-plastin*) (**Supplementary**
154 **Fig. 2a**). On the other hand, a significant effect of the Shp2-D61G mutation on
155 definitive hematopoiesis was observed at 5dpf (**Fig. 2**). An increased number of
156 HSPCs marked by CD41-GFP^{low} was observed in the caudal hematopoietic tissue
157 region (CHT, corresponding to the fetal liver in human) and the head kidney
158 (corresponding to the bone marrow in human) of the mutant embryos **(Fig. 2a,b)**.
159 Shp2^{D61G} mutation seems to affect both proliferation and apoptosis of HSPCs,
160 evident by an increase in CD41-GFP^{low} cells positive for phosphohistone H3 (pHis3),
161 a marker for late G2 and M phase and a decrease in Acridine orange positive cells in
162 the CHT region, marking apoptotic cells (**Supplementary Fig. 2b,c**). We observed
163 an increase of *c-myb* positive cells, marking HSPCs and of *l-plastin* positive cells,
164 marking all white blood cells, in both CHT and head kidney region of the Shp2^{D61G}
165 mutants (**Fig. 2c-f**). These latter cells appear not to be lymphocytes, since the size of
166 the *ikaros*-positive thymus, was not affected (**Supplementary Fig. 2d,e**). On the
167 other hand, the myeloid lineage was markedly expanded, evident from the increase
168 in the number of mpx-GFP positive neutrophils (**Fig. 2g,h**), and mpeg-mCherry
169 positive macrophages (**Fig. 2g,i**), in the Tg(*mpx:GFP, mpeg:mCherry*) double
170 transgenic mutant embryos. Shp2^{D61G} mutant embryos also displayed a mild
171 decrease in the number of thrombocytes, marked by CD41-GFP^{high} in the
172 Tg(*cd41:GFP, kdrl:mCherry-CAAX*) transgenic line, and number of *β-globin* positive
173 erythrocytes (**Supplementary Fig. 2f-h)**. The observed defects in all different blood
174 lineages examined here were stronger in the homozygous Shp2$^{D61G/D61G}$ than
175 heterozygous Shp2$^{D61G/wt}$ embryos, supporting a dosage effect. One of the clinical
176 hallmarks of JMML is the hypersensitivity of myeloid progenitors to GM-CSF. In
177 zebrafish, effects of stimulation by Gcsfa or Gscfb corresponds to the GM-CSF
178 stimulation in human (Svoboda et al., 2016). Compared to their wt siblings, colonies
179 developed from the CD41-GFP^{high} cells isolated from the Shp2^{D61G} zebrafish

180 embryos at 5dpf and exposed to Gcsfa were larger in size and number,
181 demonstrating an enhanced GM colony forming ability (**Fig. 2j,k)**. Finally, we tested
182 whether the observed myeloid expansion was reconstituted upon transplantation of
183 the WKM cells harvested from Shp2$^{D61G/wt}$ animals in the Tg(*mpx:GFP,*
184 *mpeg:mCherry*) background into the optically clear recipient *prkdc-/-*
185 immunodeficient zebrafish (Moore et al., 2016). Animals injected with 1x10^{5} mutant
186 WKM cells (3/5) accumulated GFP- and mCherry- positive cells near the site of
187 injection starting at 14 days and increasing until 28 days. By contrast, animals
188 injected with WKM cells from control sibling animals with wt Shp2a (5/5) lacked any
189 GFP- and mCherry- positive cells (**Fig. 2l**).
190 Our findings suggest that the Shp2^{D61G} mutant zebrafish embryos develop
191 multilineage hematopoietic defects during the definitive wave of fetal hematopoiesis,
192 which originates in the HSPCs compartment. The observed defect is reminiscent of
193 JMML-like MPN in human NS patients.

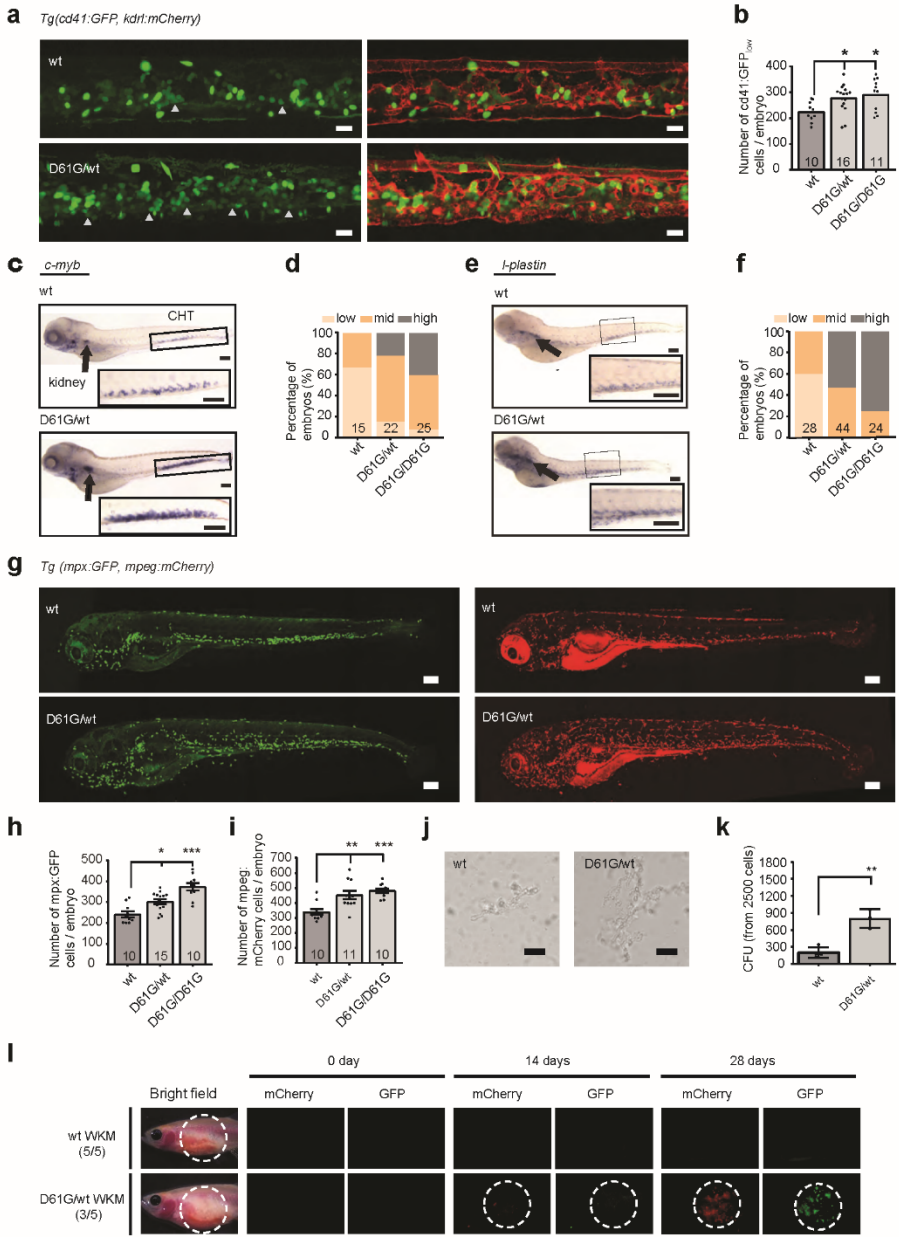

Figure 2. Shp2^{D61G} mutant zebrafish embryos display JMML-like MPN. a Representative images of the CHT region of Shp2^{wt} and Shp2^{D61G} zebrafish embryos in Tg(*cd41:GFP, kdrl:mCherry-CAAX*) background at 5dpf. *cd41:GFP^{low}* cells mark HSPCs and *cd41:GFP^{high}* cells thrombocytes. Grey arrow heads indicate *cd41:GFP^{low}* cells. Scale bar, 20µm. **b** The low intensity *cd41:GFP* positive cells in the CHT region were counted. **c** WISH of 5dpf Shp2^{wt} and Shp2$^{D61G/wt}$ embryos using *c-myb* specific probe. Head kidney (arrow) and CHT (box) are indicated; zoom-in in insert. Scale bars, 150µm. **d** Quantification of *c-myb* WISH. *C-myb* expression in Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ embryos was scored as low, mid and high. **e** WISH of 5dpf Shp2^{wt} and Shp2$^{D61G/wt}$ embryos using *l-plastin* specific probe. Head kidney (arrow) and CHT (box) are indicated and zoom-in in insert. Scale bars,150µm. **f** Quantification of *l-plastin* expression in Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ embryos scored as low, mid and high. **g** Representative images of Shp2^{wt} and Shp2^{D61G} zebrafish embryos in Tg(*mpx:GFP, mpeg:mCherry*) background at 5dpf. *Mpx:*GFP marks neutrophils and *mpeg*:mCherry macrophages. Scale bars,150µm. **h,i** Number of *mpx*:GFP and *mpeg*:mCherry positive cells per embryo. **j** Representative images of colonies developed from *cd41:GFP^{low}* cells isolated from the CHT of 5dpf Shp2^{wt} and Shp2$^{D61G/wt}$ zebrafish embryos, grown in methylcellulose with zebrafish cytokine Gcsfa for 2 days. Scale bar, 50µm. **k** Quantification of number of colonies from **j**, t-test. **l** WKM cells harvested from Shp2^{wt} and Shp2^{D61G} zebrafish in the Tg(*mpx:GFP, mpeg:mCherry*) background were injected into the peritoneum of adult *prkdc-/-* zebrafish. Recipients were monitored by fluorescence imaging. **b,d,f,h,i,k** Measurements originate from at least three distinct experiments. Number on bars: number of embryos. **b,h,i,k** Error bars represent SEM. *p < 0.05, **p<0.01, ***p<0.001. **b,h,i** ANOVA complemented by Tukey HSD.

194 **Myeloid bias is established during early differentiation of Shp2^{D61G} HSPCs**

195 In an effort to better understand the pathogenesis mechanisms in the
196 Shp2^{D61G} mutant HSPCs, single cell RNA sequencing was performed using SORT-
197 Seq on CD41-GFP^{low} cells derived from 5dpf Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$
198 zebrafish embryos in the Tg(*cd41*:*GFP*, *kdrl*:*mCherry-CAA*X) transgenic background
199 (**Fig. 3a,b**). To group cells based on their transcriptional program, unsupervised
200 clustering was performed using the RaceID3 package (Herman et al., 2018).
201 Clusters were visualized by *t*-distributed stochastic neighbor embedding (*t*-SNE) and
202 4 major clusters were further analyzed (**Fig. 3c)**. Based on the differentially
203 expressed genes and GO term analysis, cells from Cluster 1 were determined to be
204 thrombocyte and erythroid progenitors, Cluster 2 HSC-like HSPCs, Cluster 3 early
205 myeloid progenitors and Cluster 4 monocyte/macrophage progenitors (**Fig. 3c,**
206 **Supplementary Fig. 3a, Supplementary Table 1**). A small subset of cells in Cluster
207 4 represented more differentiated neutrophils (**Supplementary Fig. 3b**).

208 HSPCs of either Shp2^{wt} or mutant Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ genotypes
209 were present in all 4 major clusters, indicating that distinct HSPCs phenotypes were
210 maintained on a gene transcription level. However, the distribution of cells in clusters
211 differed among genotypes. An overrepresentation of mutant Shp2$^{D61G/wt}$ and
212 Shp2$^{D61G/D61G}$ cells was observed in the HSC-like HSPCs cluster and
213 monocytes/macrophages progenitors cluster, whereas these were underrepresented
214 in the thrombocyte and erythroid progenitors cluster **(Fig. 3d,e**). To validate this
215 observation, we investigated the expression of *pu.1* and *alas2* markers in Shp2^{D61G}
216 embryos of different genotypes by WISH. In the single cell RNA sequencing dataset,
217 expression of *pu.1* and *alas2* was upregulated in the myeloid progenitors and
218 erythroid progenitors, respectively (**Fig. 3f,i**). An increased number of *pu.1* positive
219 cells was detected by WISH in 5dpf old Shp2^{D61G} embryos compared to their Shp2^{wt}
220 siblings (**Fig. 3g,h**), whereas the number of *alas2* positive cells in Shp2^{D61G} mutants
221 was decreased (**Fig. 3j,k**).

222 Taken together, single cell RNA sequencing suggests that defects during
223 early differentiation of HSPCs to monocyte/macrophage progenitors and
224 erythroid/thrombocyte progenitors initiate the multilineage blood defect observed in
225 Shp2^{D61G} embryos, which recapitulates features of NS-JMML-like MPN.

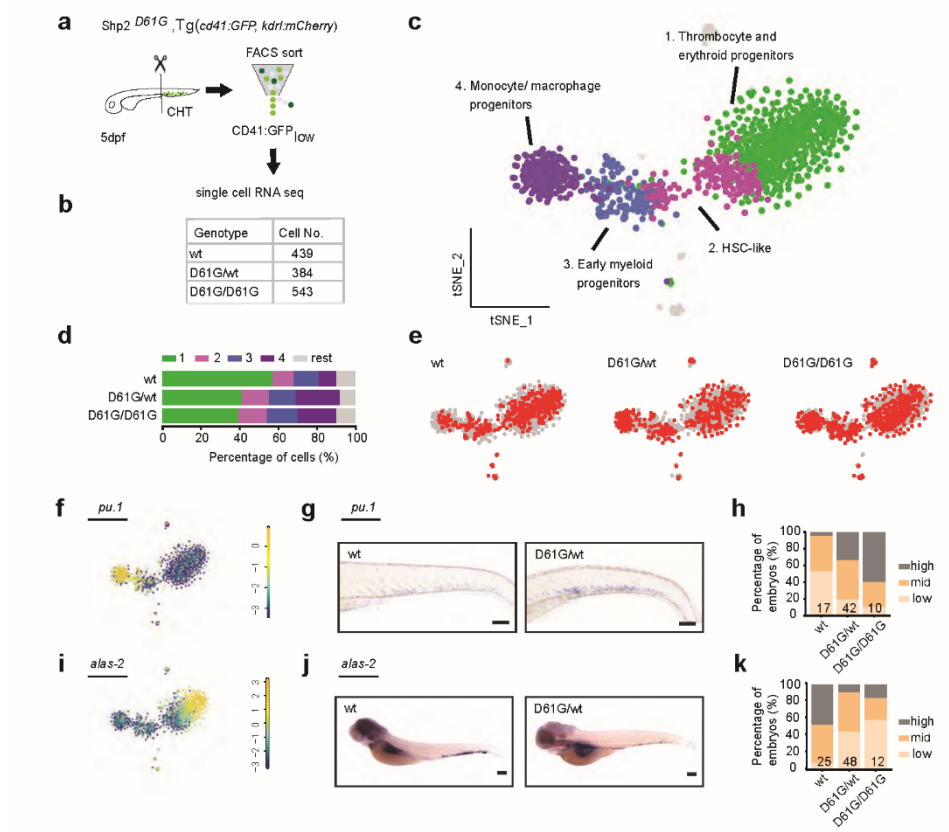

Figure 3. Single cell RNA sequencing of HSPCs reveals myeloid bias in Shp2^{D61G} embryos. a Schematic representation of the experimental procedure. At 5dpf, CHTs from Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ embryos in Tg(*cd41:GFP, kdrl:mCherry-CAAX*) background were isolated. Cells were dissociated and separated by FACS, based on *cd41:GFP^{low}* expression, prior to single cell RNA sequencing, as described in the Materials and methods section. **b** Number of cells of distinct genotypes used in single cell RNA sequencing analysis. **c** Combined tSNE map generated using the cells of all three genotypes (Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$). Single cells from 4 major clusters are marked in violet, blue, pink and green and their identities based on marker gene expression are indicated. Minor clusters are marked in grey. **d** Barplots showing the percentage of cells of Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ genotype in distinct clusters. **e** Cells of distinct genotypes Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ are visualized in tSNE maps in red. **f** tSNE maps showing log2-transformed read-counts of *pu.1*. **g** Representative images of the WISH staining for *pu.1* expression in 5dpf Shp2^{wt} and Shp2^{D61G} zebrafish embryos. Scale bar,100µm. **h** Expression of the *pu.1* marker scored as low, mid and high. **i** tSNE maps showing log2-transformed read-counts of *alas-2*. **j** Representative images of the WISH staining for *alas-2* expression in the tail region of 5dpf Shp2^{wt} and Shp2^{D61G} zebrafish embryos. Scale bar, 100µm. **k** Expression of the *alas-2* marker scored as low, mid and high. **h,k** Number on bars: number of embryos.

Excessive proinflammatory response in monocyte/macrophage progenitors of Shp2^{D61G} HSPCs

We further analyzed the cluster of monocyte/macrophage progenitors, in which we observed an overrepresentation of mutant Shp2^{D61G} cells **(Fig. 4a)**. Functional annotation of the differentially expressed genes and the GO-term enrichment analysis revealed enhanced expression of proinflammatory genes in mutant Shp2^{D61G} cells (**Fig. 4a,b, Supplementary Table 1**). Interestingly, expression of inflammation-related genes, such as *gcsfa*, *gcsfb*, *il1b*, *irg1* and *nfkbiaa,* was

234 constrained to the cells of mutant Shp2^{D61G} genotype in the monocyte/macrophage
235 progenitors cluster, whereas the monocyte marker *timp2a* was equally expressed by
236 cells of distinct genotypes (**Fig. 4b**). High expression of IL-1β was validated in
237 mutant embryos *in vivo*, in which IL-1β-eGFP positive cells were more abundant in
238 the CHT region of Shp2^{D61G} embryos than in wt embryos in the Tg(*il1b:eGFP*,
239 *mpeg:mCherry*) transgenic background (**Fig. 4c**). These results indicate that
240 Shp2^{D61G} embryos display an inflammatory response at 5 dpf, which originates in the
241 monocyte/macrophage progenitor cells.

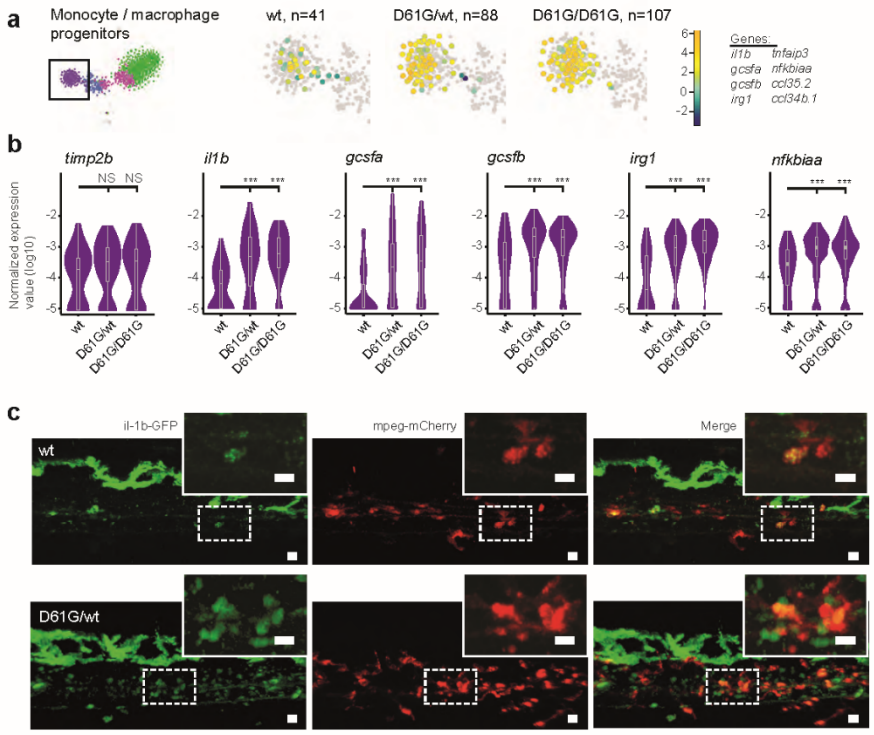

Figure 4. Inflammatory response in monocyte/ macrophage progenitors in Shp2^{D61G} embryos. **a** The monocyte/macrophage progenitors cluster (boxed on the tSNE map on left) was analyzed in detail. log2-transformed sum of read-counts of selected inflammation-related genes from the top 50 differentially expressed genes in the cells of the monocyte/macrophage progenitors cluster, with genotype and number of cells (n) indicated above. **b** Violin plots show the expression of specific genes in monocyte/macrophage progenitors of Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ genotypes. NS, not significant, ***p < 0.001, t-test. **c** *In vivo* imaging of the CHT region of Shp2^{wt} and Shp2$^{D61G/wt}$ zebrafish embryos in Tg(*il1b:eGFP*, *mpeg:mCherry*) background at 5dpf. Representative images are shown. The dashed line boxed region of CHT is zoom-in in insert. Scale bar is 10μm.

242 ***PTPN11* Somatic mutations induce inflammatory response genes in HSPCs of**
243 **JMML patients.**
244 We hypothesized that activating mutations in Shp2 may trigger an
245 inflammatory response in a similar way in sporadic JMML as we observed in
246 syndromic JMML-like MPN. To investigate this, we performed transcriptomic analysis
247 of the HSPCs compartments derived from bone marrows of sporadic JMML patients
248 with activating mutations in SHP2 (n=5) and age-matched healthy donors (n=7). The
249 SHP2 mutations in JMML HSPCs were distinct among patients (**Supplementary**
250 **Table 2**). Overall, we identified 1478 differentially expressed genes (DEGs) in
251 HSPCs from JMML patients, compared to the healthy donor HSPCs (**Fig. 5a**,
252 **Supplementary Table 3**). The functional analysis of DEGs using gene set
253 enrichment analysis (GSEA) was performed to systematically explore hallmark gene
254 signatures specific for JMML HSPCs (**Fig. 5b, Supplementary Table 4**). Strikingly,
255 altered expression of genes related to inflammation was the most prominent, such as
256 genes involved in TNFa signaling via NFKb and inflammatory response genes, which
257 were significantly enriched in JMML HSPCs (**Fig. 5b-d**). De-regulation of genes
258 related to proliferation (G2M checkpoint, MYC targets and E2F targets) was evident
259 in JMML HSPCs, suggesting their decreased quiescence (**Fig. 5b**).
260 We next studied whether the inflammatory-related gene signature overlaps
261 between HSPCs from zebrafish NS/JMML-like MPN model and human JMML. GSEA
262 revealed that human orthologs of the top 100 DEGs found in the zebrafish
263 monocyte/macrophage progenitor cells were enriched significantly in patient JMML
264 HSPCs (**Fig. 5e**). Some of the top overexpressed inflammation-associated genes in
265 JMML patients, such as TNF and MARCKSL were also overexpressed in NS/JMML-
266 like MPN mutant zebrafish embryos compared to the wt (**Fig. 5f,g**). 3D PCA analysis
267 visualized that these zebrafish signature genes were able to clearly segregate
268 HSPCs from JMML patients and healthy donors (**Fig. 5h**). These findings suggest
269 that mutant, activated SHP2 triggers proinflammatory gene expression in HSPCs
270 both in sporadic JMML patients and in our zebrafish model of syndromic JMML-like
271 MPN in a similar manner, suggesting a common underlying, endogenously driven
272 process.

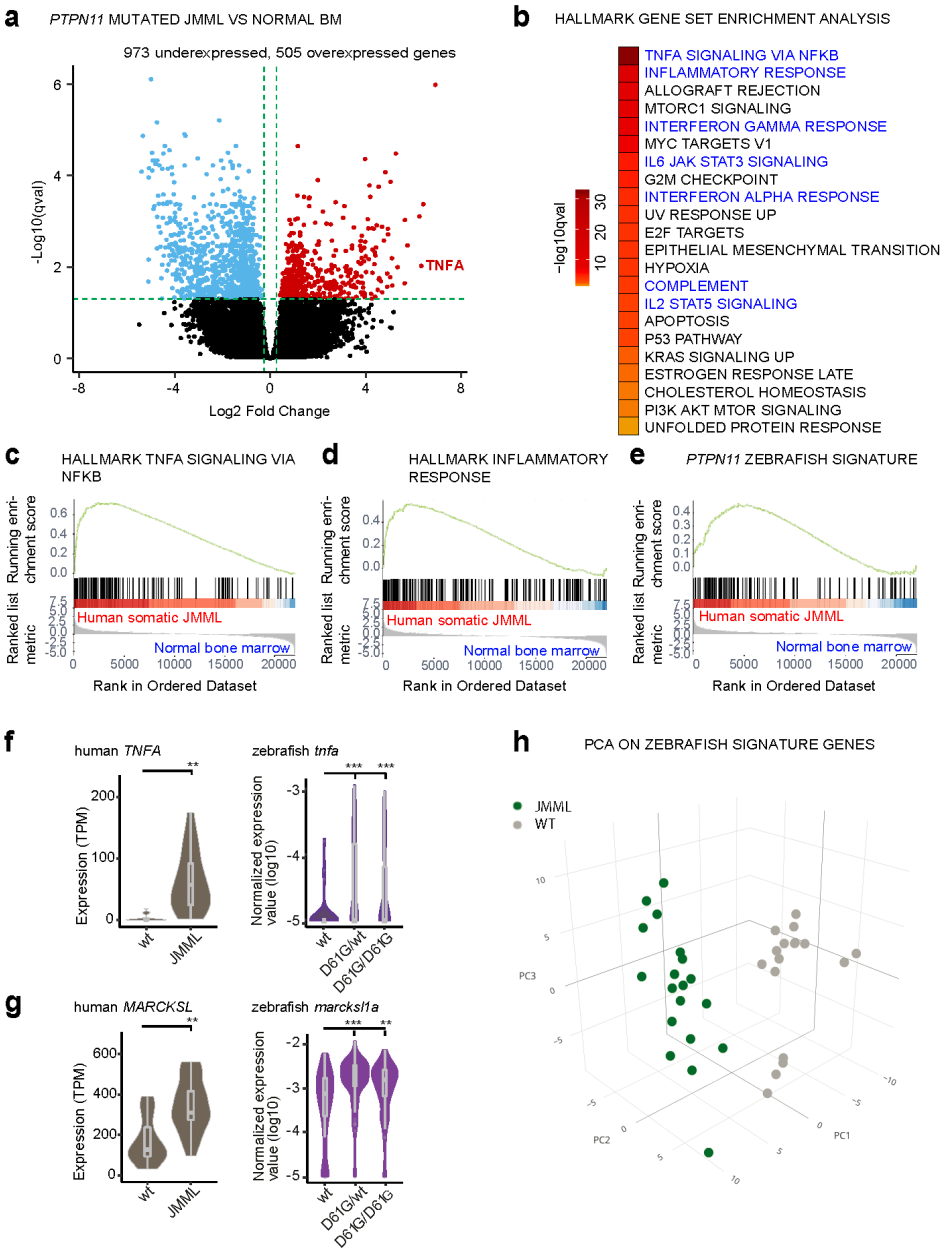

Figure 5. Similar molecular signatures in HSPCs from human JMML patients and zebrafish Shp2^{D61G} embryos. a Volcano plot of differentially expressed genes of HSPCs derived from bone marrow of JMML patients with *PTPN11* mutations (n=5) and healthy bone marrow (n=7). Underexpressed genes are marked in blue and overexpressed genes are marked in red. *TNFA* expression is highlighted. Green dashed lines indicate the significance level. **b** GSEA for the MSigDB's hallmark gene sets in HSPCs from JMML compared to normal human age-matched bone marrow. GSEA plots for TNFA_SIGNALING_VIA_NFKB (**c**), INFLAMMATORY RESPONSE (**d**), and the custom Zebrafish signature based on the top 100 human orthologous of genes upregulated in monocyte/macrophage progenitor cluster of zebrafish HSPCs (**e**). Violin plots show the expression of *TNFA* (**f**) and *MARCKSL* (**g**) in either human wt vs. JMML HSPCs, and zebrafish Shp2^{wt} vs. Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ monocyte/macrophage HSPC progenitors. **p<0.01, ***p < 0.001, t-test. **h** PCA for the 100 genes included in the custom zebrafish signature. Green dots represent *PTPN11* mutated JMML, gray ones represent normal human age-matched bone marrow. PC1: 19% of the variance, PC2: 16% of the variance, PC3: 14% of the variance.

273 **Inhibition of the proinflammatory response ameliorates the JMML-like MPN**
274 **phenotype**

275 Our results indicate that Shp2^{D61G} embryos displayed JMML-like MPN (**Fig.**
276 **2**). To investigate if pharmacological agents ameliorate the observed hematopoietic
277 defects, Shp2^{D61G} embryos were exposed to inhibitors continuously from 2 to 5 dpf
278 and subsequently *c-myb* and *l-plastin* markers were investigated by WISH (**Fig. 6a**).
279 Inhibitors of the known Shp2-associated signaling pathways were used, targeting
280 MEK (CI1040) or PI3K (LY294002) (Tajan et al., 2015). Both inhibitors led to a
281 robust reduction in expansion of both HSPCs and myeloid lineage in mutant
282 embryos (**Fig. 6b,c**), demonstrating that the NS-associated blood development
283 defect in Shp2^{D61G} embryos was largely caused by overactivation of the RAS-MAPK
284 and PI3K pathways as expected.

285 The HSPCs of Shp2^{D61G} embryos displayed a proinflammatory response (**Fig.**
286 **4**), similar to the inflammatory response in HSPCs from human patients (**Fig. 5**). To
287 investigate the role of the inflammatory response in the blood defect, we assessed
288 the effect of dexamethasone in Shp2^{D61G} embryos in parallel to the MEK- and PI3K
289 inhibitors. Dexamethasone is an anti-inflammatory agent, which is known to
290 suppress inflammatory response in the monocyte/macrophage lineage (Ehrchen et
291 al., 2019). To our surprise, dexamethasone reduced *c-myb* and *l-plastin* expression
292 to a similar extent as CI1040 and LY294002 (**Fig. 6b,c**), indicating a profound role
293 for the inflammatory response in the pathogenesis of NS-associated blood defects.
294 Taken together, we observed a proinflammatory phenotype of the macrophage/
295 monocyte lineage in Shp2^{D61G} mutant zebrafish, which is established early during
296 HSPCs differentiation. Since an anti-inflammatory agent largely rescued the
297 hematopoietic defects, we conclude that the inflammatory response in Shp2^{D61G}
298 mutant zebrafish mediates the pathogenesis of the NS/JMML-like MPN blood
299 phenotype.
300

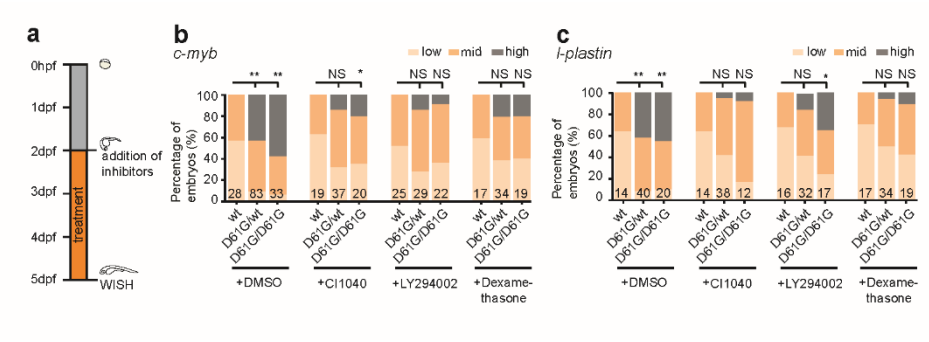

Figure 6. Anti-inflammatory treatment of zebrafish Shp2^{D61G} embryos ameliorates the JMML-like MPN phenotype. a Schematic overview of the treatments with MEK inhibitor CI1040, PI3K inhibitor LY294002 and anti-inflammatory corticosteroid dexamethasone. Embryos were continuously treated from 48hpf until 5dpf, when WISH was performed and expression of the *c-myb* (**b**) and *l-plastin* (**c**) marker was scored as low, mid and high. Measurements originate from at least three distinct experiments. Number on bars: number of embryos. NS, not significant; *p < 0.05, **p<0.01, ***p<0.001, Chi-squared test.

Discussion

To investigate the timing and pathophysiology of mutant SHP2-related myeloproliferative defects, we explored the transcriptomes of HSPCs with activating mutations in SHP2, derived from sporadic JMML patients and syndromic NS/JMML-like MPN zebrafish embryos, by bulk and single cell sequencing, respectively. Strikingly, proinflammatory gene expression was evident both in HSPCs from JMML patients and mutant zebrafish. Already early studies reported high levels of proinflammatory cytokines, such as IL-1β, TNF-α, GM-CSF, in JMML patients plasma (Bagby et al., 1988; Freedman et al., 1992). However, little is known about the cellular origin of the proinflammatory status, its pathophysiological role and therapeutic potential. The inflammatory response may be initiated in a cell autonomous way or in response to signals from the microenvironment. Dong et al. suggested that IL-1β is secreted by differentiated monocytes which get recruited upon secretion of the Chemokine, CCL3, by cells of the bone marrow microenvironment containing activating SHP2 mutations. However, the levels of CCL3 in the bone marrow of four NS patients varied (Dong et al., 2016). Furthermore, a microenvironment-driven inflammatory response would not explain high levels of cytokines in sporadic JMML, where niche cells are not mutated. On the other hand, recent reports indicate that JMML leukemia stem cells are heterogeneous but confined to the hematopoietic stem and progenitor cells (HSPCs) compartment, defining JMML HSPCs as the origin of the disease (Caye et al., 2019; Louka et al., 2021).

323 Here we identified HSPCs as the cells in which the proinflammatory status of
324 the monocyte/macrophage lineage is initiated both in JMML and NS/JMML-like MPN.
325 Hence, our data suggest that proinflammatory reprogramming of the
326 monocyte/macrophage lineage might be endogenously driven at least in part, and
327 detailed mechanisms remain to be elucidated in the future.
328 To our knowledge, the Shp2^{D61G} mutant zebrafish we developed is the first NS
329 zebrafish model carrying an activating Shp2 mutation at its endogenous locus
330 generated by CRISPR/Cas9-based knock-in technology. Strikingly, phenotypes
331 developed by the Shp2^{D61G} zebrafish corroborate closely with the phenotypes
332 displayed by human NS patients and the existing NS mouse models **(Fig. 1)**. Our
333 model presents an exciting novel tool for deciphering pathogenesis mechanisms of
334 NS with its complex traits and for finding novel therapies for this as yet poorly
335 treatable condition.
336 NS children with D61G mutation have a higher predisposition to develop
337 JMML(-like MPN), and comparably Shp2^{D61G} zebrafish embryos displayed typical
338 JMML-like MPN characteristics, such as expansion of the myeloid lineage, increased
339 sensitivity to gcsfa, mild anemia and thrombocytopenia **(Fig. 2)**. Furthermore, the
340 observed blood defect was transplantable to secondary recipients and the disease
341 originated in HSPCs, which displayed aberrant proliferation and apoptosis. Given the
342 importance of fetal hematopoiesis during JMML(-like MPN) development, the
343 zebrafish Shp2^{D61G} mutant represents a reliable and unique model for JMML-like
344 MPN, which allows us to study hematopoietic defects caused by mutant Shp2 during
345 the prenatal development for the first time. Our results suggest that the JMML-like
346 MPN defect is initiated at the CHT, which is the counterpart of fetal liver in human.
347 Here we observed expanded HSPCs, which displayed aberrant proliferation and
348 apoptosis. We further characterized the transcriptomes of HSPCs specifically
349 originating from the CHT, and studied their response to inhibitor treatments directly
350 at the CHT. Striking similarities between the patient and zebrafish HSPCs
351 transcriptomes indicate that the Shp2^{D61G} zebrafish model is an exciting preclinical
352 model for *in vivo* drug screens at relevant developmental time points, in a high
353 throughput manner.
354 The only effective treatment of JMML is allogeneic stem cell transplantation,
355 which has a high relapse rate of 50%. Hence, there is a great need for other means
356 of therapeutic intervention. The role of inflammation as one of the drivers in myeloid

357 leukemogenesis is emerging (Arranz et al., 2017; Craver et al., 2018). Here we
358 demonstrate that dampening inflammation using the glucocorticoid dexamethasone,
359 partially rescued the observed blood phenotype in Shp2^{D61G}, suggesting that the
360 inflammatory response evoked in the cells of myeloid/macrophage lineage was an
361 important driver of the NS/JMML-like blood defect and might be a potential drug
362 target for both sporadic and syndromic JMML(-like MPN). To our surprise, targeting
363 inflammation reversed the blood defect to a similar extent as the MAPK and PI3K
364 pathway inhibitors, emphasizing not only the crucial role of inflammation during
365 JMML pathogenesis, but also its strong therapeutic potential. Since anti-
366 inflammatory therapies are non-invasive and widely available, targeting inflammation
367 might represent a novel avenue for JMML treatment, either alone or in combination
368 with other therapies.
369 In conclusion, we observed striking similarities in expression patterns of
370 HSPCs from sporadic human JMML patients with an activating SHP2 mutation and
371 HSPCs from an engineered zebrafish model with an activating NS-associated
372 mutation in Shp2. Particularly genes associated with the inflammatory response
373 were upregulated and strikingly, pharmacological inhibition of the inflammatory
374 response ameliorated JMML-like MPN in the zebrafish model, suggesting this may
375 be a first step for therapeutic intervention in human patients.

Materials and methods

Zebrafish husbandry

All procedures involving experimental animals were approved by the animal experiments committee of the Royal Netherlands Academy of Arts and Sciences (KNAW), Dierexperimenten commissie protocol HI18-0702, and performed under the local guidelines in compliance with national and European law. The following zebrafish lines were used in the study: Tübingen longfin (TL, wild type), Tg(*cd41:GFP, kdrl:mCherry-CAAX*) (Hogan et al., 2009; Lin et al., 2005), Tg*(mpx:GFP, mpeg:mCherry)* (Ellett et al., 2011; Renshaw et al., 2006), TgBAC(*il1b:eGFP*)sh445 (Ogryzko et al., 2019), *ptpn11b-/-* (Bonetti et al., 2014)*prkdc-/-* (Moore et al., 2016) and the novel Shp2^{D61G} zebrafish line. Raising and maintenance of zebrafish was performed according to (Aleström et al., 2020; Westerfield, 2000). When required, pigmentation of embryos was blocked by adding phenylthiourea (PTU) (Sigma Aldrich, St. Louis, MO, USA, Ref: P7629) at a concentration of 0.003% (v/v) to the E3 medium at 24hpf.

Patient material

All children's samples were obtained after parents had given their written informed consent. Experiments were approved by the institutional review board of the French Institute of Health and Medical Research (INSERM) (IORG0003254) in accordance with the Helsinki declaration. Healthy children bone marrows were obtained from intrafamilial BM transplantation donors and used with the approval of the Institutional Review Board of "Hôpitaux Universitaires Paris Nord Val-de-Seine," Paris 7 University, AP-HP), (IRB: 00006477), in accordance with the Helsinki declaration. HSPCs fractions were FACS sorted according to immunophenotypic signature as previously described (Caye et al., 2019), from PTPN11^{mut} JMML (n=5) and healthy children bone marrow (n=7). Age, sex, mutations and fractions available for each sample (Hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs)) are indicated in Supplementary Table 2.

Generation of the Shp2^{D61G} zebrafish line

The Shp2^{D61G} zebrafish line was generated using the previously described CRISPR/Cas9-based knock-in approach (Tessadori et al., 2018). The sgRNA

409 targeting exon 3 of the *ptpn11a* gene (5'- GGAGACTATTACGACCTGTA-3') was
410 designed using the CHOP-CHOP database (http://chopchop.cbu.uib.no/), further
411 processed according to the previously published guidelines (Gagnon et al., 2014)
412 and finally transcribed using the Ambion MEGAscript T7 kit (TermoFisher Scientific,
413 Waltham, MA, USA, Ref: AMB13345). The sgRNA, constant oligonucleotide and
414 template oligonucleotide were all generated by Integrated DNA Technologies (IDT,
415 Coralville, IA, USA) as standard desalted oligos and template oligonucleotide was
416 further purified using the QIAquick Nucleotide Removal Kit (Qiagen, Hilden,
417 Germany, Ref: 28304). The oligonucleotide used for the homology repair is 59
418 nucleotides long and besides the D61G mutation, contains three additional silent
419 mutations in proximity of the PAM site. The sequence of template oligonucleotide is
420 5'-
421 GAGTGGCAAACTTCTCTCCACCATATAAATCGTAATAGCCTCCTGTGTTTTGAAT
422 CTTA-3'. Tübingen longfin wt zebrafish embryos at the one-cell-stage were injected
423 directly in the cell with 1 nl of the injection mixture containing 18.75 ng/ml of sgRNA,
424 37.5 ng/ml of template oligonucleotide and 3.6 mg/ml of Cas9 protein in 300 mM
425 KCl. Cas9 protein was a gift from the Niels Geijsen laboratory at the Hubrecht
426 Institute. The injected embryos were grown into the adulthood. F0 generation
427 zebrafish were outcrossed with the wt zebrafish. DNA extracted from the 12 distinct
428 1dpf old F1 generation embryos was screened for the correct insertion of the
429 template oligonucleotide. Screening was done by Sanger sequencing (Macrogen
430 Europe B.V., Amsterdam, The Netherland) of the 225 bp long PCR product
431 encompassing the genomic regions of the CRISPR target sites, which was
432 generated using the forward 5'-TCATCTCCTCACTAGGCGAAAT-3' and reverse
433 primer 5'- TATGTATGTGCTCACCTCTCGG-3'. The efficiency of the knock-in was
434 1.8% (1 founder zebrafish in 54 screened primary injected zebrafish). F1 generation
435 was then established from the F0 founder. F1 generation adults were finclipped and
436 sequenced for the presence of the mutation. All experiments were performed in
437 zebrafish embryos and adults from the F3 and F4 generation. For most of the
438 experiments, embryos were derived from an incross of Shp2$^{D61G/wt}$ animals. After the
439 experimental procedure, embryos were lysed and genotyped by sequencing as
440 described above. Western blotting was performed as previously described (Hale &
441 den Hertog, 2017) using the Shp2 (Santa Cruz Biotechnology, Dallas, TX, USA, Ref:
442 SC-280) and β-tubulin (Merck Millipore, Burlington, MA, USA Ref: CP06) antibodies.

443 **Phenotyping of the NS traits**

444 Body axis lengths were measured from the tip of the head to the end of the trunk in
445 the bright-field images of laterally positioned embryos, larva and adults, which were
446 anesthetized in 0.1% MS-222. Alcian blue (Sigma Aldrich, Ref: A5268) staining was
447 performed as previously described (Paardekooper Overman et al., 2014), on PTU-
448 treated 4dpf old embryos, which were anesthetized in 0.1% MS-222 and fixed in 4%
449 PFA overnight. Embryos were positioned on their back in 70% glycerol in PBS and
450 imaged with Leica M165 FC stereomicroscope (Leica Microsystems, Wetzlar,
451 Germany. Analysis was performed in ImageJ. *In vivo* high-speed brightfield imaging
452 of the embryonic hearts from PTU-treated embryos at 5dpf, which were anesthetized
453 in 0.1% MS-222 and embedded in 0.3% UltraPure agarose (Thermo Fisher
454 Scientific, Waltham, MA, USA) prepared in E3 medium containing 16 mg/ml MS-222.
455 Measurements were performed at 28°C using a Leica DM IRBE inverted light
456 microscope (Leica Microsystems) with a Hamamatsu C9300-221 high-speed CCD
457 camera (Hamamatsu Photonics, Hamamatsu, Japan). Imaging was conducted at
458 150 frames per seconds (fps) using Hokawo 2.1 imaging software (Hamamatsu
459 Photonics) for a period of 10 seconds (approximately 30 cardiac cycles). Heart rate
460 measurements and contractility parameters were analysed using ImageJ. Volumes
461 were analysed using ImageJ by drawing an ellipse on top of the ventricle at end-
462 diastole and end-systole. Averages of three measurements per heart were
463 determined. End diastolic and end systolic volume (EDV/ESV) were calculated by:
464 (4/3)*(π)*(major axis/2)*((minor axis/2)^2). Stroke volume (SV) by: EDV-ESV.
465 Ejection fraction (EF) by: (SV/EDV)*100. Cardiac output (CO) by: SV*Heart rate.

466 **Whole mount *in situ* hybridization**

467 PTU-treated embryos were anesthetized in 0.1% MS-222 (Sigma Aldrich, Ref:
468 A5040) and fixed in 4% PFA for at least 12h at 4°C. *WISH* was performed as
469 described in (Thisse et al., 1993)**.** Probes specific for *myl7*, *vhmc* and *ahmc* were
470 described in (Bonetti et al., 2014). Probes specific for *c-myb*, *l-plastin*, *pu.1*, *gata1*,
471 *ikaros*, *b-globin* and *alas-2* were described in (Choorapoikayil et al., 2014; Hu et al.,
472 2014)**.** Subsequently, embryos were mounted in 70% glycerol in PBS and imaged
473 with Leica M165 FC stereomicroscope (Leica Microsystems). Images were
474 processed in ImageJ (U. S. National Institutes of Health, Bethesda, MD, USA).
475 Abundance of the probe signal was scored as low, mild or high.

476 **Inhibitors treatment**

477 PTU-treated embryos were incubated with either 0.15μM of CI1040 (Sigma Aldrich,
478 Ref: PZ0181), 4μM LY294002 (Sigma Aldrich, Ref: L9908) or 10μM of
479 Dexamethasone (Sigma Aldrich, Ref: D4902) continuously from 24hpf until 5dpf. At
480 5dpf embryos were fixed and half of the embryos was processed for WISH using
481 probe specific for *c-myb* and the other half using probe specific for *l-plastin*. Images
482 were processed in ImageJ. Abundance of the probe signal was scored as low, mild
483 or high.

484 **Confocal microscopy**

485 All confocal imaging was performed on a Leica SP8 confocal microscope (Leica
486 Microsystems). Embryos were mounted in 0.3% agarose. Live embryos were
487 anesthetized in MS-222. Whole embryos were imaged using a 10X objective and z-
488 stack step size of 3μm, while the CHT area with 20X objective and z-stack step size
489 of 1μm. The number of CD41-GFP^{low} cells was determined by imaging the CHT of
490 the living 5dpf old embryos of the Shp2^{wt}, Shp2$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ siblings in
491 the Tg(*cd41:GFP, kdrl:mCherry-CAAX*) transgenic background, while the number of
492 CD41-GFP^{high} cells was determined by imaging whole embryos, which were fixed for
493 2h in 4% PFA prior to imaging. To determine the number of mpx-GFP and mpeg-
494 mCherry cells, whole live 5dpf old embryos of the Shp2^{wt}, Shp2$^{D61G/wt}$ and
495 Shp2$^{D61G/D61G}$ line in the Tg*(mpx:GFP, mpeg:mCherry)* transgenic background were
496 imaged. Imaris V9.3.1 (Bitplane, Zurich, Switzerland) was used to reconstruct 3D
497 images and count individual GFP and/or mCherry positive cells.

498 **Phosphorylated Histone 3 (pHis3) staining**

499 PTU-treated 5dpf old Shp2^{D61G} embryos in the Tg(*cd41:GFP, kdrl:mCherry-CAAX*)
500 transgenic background were fixed in 2% PFA overnight and stained as described in
501 (Choorapoikayil et al., 2012). Primary pHis3 antibody (1:500 in blocking buffer,
502 Abcam, Cambridge, UK, Ref: ab5176) and secondary GFP antibody (1:200 in
503 blocking buffer, Aves Labs Inc. Tigard, OR, USA, GFP-1010) were used. Embryos
504 were mounted in 0.3% agarose, their CHT was imaged using the SP8 confocal
505 microscope and 3D images were subsequently reconstructed using Imaris.

506 **Acridine orange staining**

507 PTU-treated embryos at 5dpf were incubated in 5μg/ml of Acridine orange (Sigma
508 Aldrich, Ref: A6014) in E3 medium, for 20 minutes at room temperature. They were
509 then washed 5 times for 5 minutes in E3 medium, anesthetized in MS-222 and

510 mounted in 0.3% agarose. Whole embryos were imaged with SP8 confocal
511 microscope and 3D images were reconstructed using Imaris.

512 **Colony forming assay**

513 The CD41-GFP^{low} cell population isolated from CHTs of Shp2^{wt}, Shp2$^{D61G/wt}$ embryos
514 at 5dpf was FACS sorted. 250 μl of solution containing 2 500 cells, media prepared
515 as described in (Svoboda et al., 2016)**,** and 100 ng/ml of granulocyte colony
516 stimulating factor a (Gscfa, gift from the Petr Bartunek lab, Institute of Molecular
517 Genetics, Academy of Sciences of the Czech Republic v.v.i. Prague) was plated per
518 well of a 96 well plate in a triplicate. Cells were grown in humidified incubators at
519 32°C, 5% CO_2. After 6 days colonies were imaged and counted using the EVOS
520 microscope (Termo Fisher Scientific).

521 **Transplantation experiments**

522 Zebrafish kidney marrow transplantation were performed as previously described
523 (Moore et al., 2016; Tang et al., 2014). In short, tissues were isolated from donor
524 Shp2$^{D61G/wt}$ or wild type animals in the Tg(*mpx:GFP, mpeg:mCherry*) background
525 following Tricaine (Western Chemical, Brussels, Belgium) overdose. Excised tissues
526 from dissected fish are placed into 500 µl of 0.9x PBS + 5% FBS on a 10-cm Petri
527 dish. Single-cell suspensions were obtained by maceration with a razor blade,
528 followed by manual pipetting to disassociate cell clumps. Cells were filtered through
529 a 40-µm Falcon cell strainer, centrifuged at 1,000 *g* for 10 min, and resuspended to
530 the 2 x 10^7 cells/ml. 5-µl suspension containing 10^5 kidney marrow cells were
531 injected into the peritoneal cavity of each recipient fish using a 26s Hamilton 80366
532 syringe. Cellular engraftment was assessed at 0, 7, 14, 28 dpt by epifluorescence
533 microscopy.

534 **Isolation of CD41-GFP^{low} cell population and single-cell RNA sequencing**

535 From 24hpf onwards embryos were grown in PTU-containing medium. The CHTs of
536 approximately 50 Shp2^{wt}, Shpt$^{D61G/wt}$ and Shp2$^{D61G/D61G}$ embryos in Tg(*cd41:GFP,*
537 *kdrl:mCherry-CAAX*) transgenic background at 5dpf were dissected and collected in
538 Leibovitz-medium (Thermo Fisher Scientific, Gibco, Ref: 11415049). After washing
539 with PBS0 the CHTs were dissociated with TryplE (Thermo Fisher Scientific, Gibco,
540 Ref: 12605036) for 45 minutes at 32°C. The resulting cell suspension was washed
541 with PBS0, resuspended in PBS0 supplemented with 2mM EDTA, 2% FCS and
542 0.5µg/ml DAPI (Sigma Aldrich, Ref: D9542) and passed through a 40µm Falcon cell

543 strainer. DAPI staining was used to exclude dead cells. Cells with CD41-GFP^{low}
544 positive signal were subjected to fluorescence-activated cell sorting (FACS) with an
545 influx cytometer (BD Biosciences, San Jose, CA, USA). Single-cell RNA sequencing
546 was performed according to an adapted version of the SORT-seq (Muraro et al.,
547 2016) with adapted primers described in (Van Den Brink et al., 2017). In short, single
548 cells were FACS sorted, as described above, on 384-well plates containing 384
549 primers and Mineral oil (Sigma Aldrich). After sorting, plates were snap-frozen on dry
550 ice and stored at -80°C. For amplification cells were heat-lysed at 65°C followed by
551 cDNA synthesis using the CEL-seq2 (Hashimshony et al., 2016) and robotic liquid
552 handling platforms. After the second strand cDNA synthesis, the barcoded material
553 was pooled into libraries of 384 cells and amplified using *in vitro* transcription.
554 Following amplification, the rest of the CEL-seq2 protocol was followed for
555 preparation of the amplified cDNA library, using TruSeq small RNA primers (Illumina,
556 San Diego, CA, USA). The DNA library was paired-end sequenced on an Illumina
557 Nextseq™ 500 (Illumina), high output, with a 1x75 bp Illumina kit (R1:26 cycles,
558 index read: 6 cycles, R2:60 cycles).

559 **Data analysis of single-cell RNA sequencing**

560 During sequencing, Read1 was assigned 26 base pairs and was used for
561 identification of the Ilumina library barcode, cell barcode and unique molecular
562 identifier. Read2 was assigned 60 base pairs and used to map to the reference
563 transcriptome of Zv9 Danio rerio. Data was demultiplexed as described in (Grün et
564 al., 2014). Single cell transcriptomics analysis was done using the RaceID3
565 algorithm (Herman et al., 2018), following an adapted version of the RaceID manual
566 (https://cran.r-project.org/web/packages/RaceID/vignettes/RaceID.html) using R-
567 3.5.2. In total 768 cells per genotype were sequenced for the datasets. After
568 removing cells with less than 1000 UMIs and only keeping genes that were detected
569 with al least 3 UMIs in 1 cell, 439 wt, 384 D61G/wt and 543 D61G/D61G cells were
570 left for further analysis. Batch effects observed for plates which were prepared on
571 different days was removed using the scran function. 4 major clusters and 5 minor
572 clusters were identified. The minor clusters contained 130 cells in total and were
573 excluded from further analysis for statistical reasons. Differential gene expression
574 analysis was done as described in (Muraro et al., 2016) with an adapted version of
575 the DESseq2 (Love et al., 2014). GO term enrichment analysis for differentially

576 expressed genes of each major cluster was performed using the DAVID
577 Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/).

578 **RNA sequencing and differential gene expression analysis of the patient**
579 **material**

580 Libraries were prepared with TruSeq® Stranded Total RNASample preparation kit
581 (Illumina) according to supplier's recommendations. Briefly, the ribosomal RNA
582 fraction was removed from 1µg of total RNA using the Ribo-Zero™ Gold Kit (Illumina).
583 Fragmentation was then achieved using divalent cations under elevated temperature
584 to obtain approximately 300bp pieces. Double strand cDNA synthesis was performed
585 using reverse transcriptase and random primers, Illumina adapters were ligated and
586 cDNA library was PCR amplified for sequencing. Paired-end 75b sequencing was then
587 carried out on a HiSeq4000 (Illumina). Quality of reads was assessed for each sample
588 using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). A subset
589 of 500,000 reads from each Fastq file was aligned to the reference human genome
590 hg19/GRCh37 with tophat2 to determine insert sizes with Picard. Full Fastq files were
591 aligned to the reference human genome hg19/GRCh37 with tophat2 (-p 24 -r 150 -g 2
592 --library-type fr-firststrand) (Kim et al., 2013). We removed reads mapping to multiple
593 locations. STAR was used to obtain the number of reads associated with each gene
594 in the Gencode v26lift37 annotation (restricted to protein-coding genes, antisense, and
595 lincRNAs). Raw counts for each sample were imported into R statistical software using
596 the Bioconductor DESeq2 package. Extracted count matrix was normalized for library
597 size and coding length of genes to compute TPM expression levels. Differential gene
598 expression analysis was performed using the Bioconductor limma package and the
599 voom transformation. To improve the statistical power of the analysis, only genes
600 expressed in at least one sample (TPM >0.3) were considered. A qval threshold of <
601 0.05 and a minimum fold change of 1.2 were used to define differentially expressed
602 genes. Due to the imbalance between male and female samples, sex differences were
603 adjusted with the function model.matrix from the stats package. RNAseq data analysis
604 was performed with Galileo® v1.4.4, an interactive R shiny application.
605 Representations of the gene expression levels were performed with the library ggplot2
606 and R 4.0.2.

607 **Pathway enrichment analysis (GSEA) and Principal component analysis (PCA)**

608 Gene set enrichment analysis was performed by clusterProfiler::GSEA function using
609 the fgsea algorithm. Gene list from the differential analysis was ordered by decreasing

610 log2 (fold change). Hallmark classes Gene sets from the MSigDB v7.2 database were
611 selected keeping only gene sets defined by 10-500 genes. The p-values were adjusted
612 by the Benjamini-Hochberg (FDR) procedure. The custom zebrafish signature was
613 built from the human orthologous genes of the 100 most overexpressed zebrafish
614 genes based on the log2 (Fold Change) derived from the monocyte/macrophage
615 progenitor cluster of the zebrafish HSPCs single cell sequencing dataset. The
616 Bioconductor edgeR package was used to import raw counts into R, and compute
617 normalized log2 CPM (counts per millions of mapped reads) using the TMM (weighted
618 trimmed mean of M-values) as normalization procedure. The normalized expression
619 matrix from the 100 most overexpressed genes (based on log2FC in the zebrafish
620 dataset) was used to classify the samples according to their gene expression patterns
621 using principal component analysis (PCA). PCA was performed by FactoMineR::PCA
622 function with “ncp = 10, scale.unit = FALSE” parameters.

623 **Statistical analysis**

624 Data was plotted in GraphPad Prism 7.05 (GraphPad Software Inc., San Diego, CA,
625 USA), except for the violin plots of gene expression, which were plotted in R using
626 ggplot2(Wickham, 2009). Statistical difference analysis was performed using the
627 one-way ANOVA supplemented by Tukey’s HSD test in GraphPad Prism 7.05,
628 except for the gene expression differences in single cell RNA sequencing data,
629 where T-test was performed in Rstudio 1.1.463 (Rstudio, Boston, MA, USA), and the
630 treatment WISH experiments, where Chi-squared test was performed in GraphPad
631 Prism 7.05. Significant difference was considered when $p<0.05$ (*$p<0.05$, **$p<0.01$,
632 ***$p<0.001$, NS=non significant).

Author Contributions

MS Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization, **SBF** Investigation, Formal analysis, Data Curation, Methodology, Writing - Review & Editing, **FP** Investigation, Formal Analysis, Methodology, Writing - Review Editing, **CY, QY** Investigation- performed transplantation experiments, Data Curation, Formal analysis, Methodology, Writing - Review & Editing**, MS** Investigation, Formal Analysis, Methodology**, SMK** Investigation- provided assistance with heart function analysis, Methodology, Resources, Writing - Review & Editing**, JB** Conceptualization, Supervision, Writing - Review & Editing**, DML** Conceptualization, Supervision, Writing - Review & Editing**, HC** Conceptualization, Supervision, Writing - Review & Editing, **JDH** Conceptualization, Supervision, Funding acquisition, Project administration, Writing - Original Draft

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The authors declare no competing interests.

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