remission after a few cycles of chemotherapy (Shapira *et al*., 2018). Thus, restriction of pre-harvesting chemotherapy may have serious medical consequences and cannot be recommended based on the data presented in this study.

The other conclusions reached by the authors are similarly problematic. The authors state that they observed no increased activation of primordial follicles in ovarian tissue exposed to chemotherapy based on morphometric assessment of follicle populations and FOXO3A immunostaining. However, more than half of the samples used for histological counting were from frozen/thawed tissue and not fresh embedded tissue. The process of freezing/thawing alters follicle morphology (Demirci *et al*., 2002, Rimon *et al*., 2005), preventing accurate assessment of follicle stage and atresia. Studies show that after freezing/thawing ~30% of detected follicles are not viable (Gandolfi *et al*., 2006; Campos *et al*., 2011). Additionally, analysis was conducted on a pooled data base of tissue from patients exposed to high, low and no alkylating agent chemotherapy. Given the different outcomes of each of these treatment groups on follicle populations, a combined assessment is invalid. Furthermore, immunostaining for FOXO3A was performed on ovaries removed between 14 and 35 days after treatment (from only three treated patients), a time frame long after any change in FOXO3A expression would be evident. As a result of these methodological errors, the authors cannot draw any conclusions from this data regarding follicle activation after chemotherapy.

In summary, we feel that the authors' conclusions, in particular the recommendation to perform OTC before initiation of any chemotherapy, are not supported by these results and may have critical medical consequences.

Conflict of interest

None.

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Reply: Impact of first-line cancer treatment on follicle quality in cryopreserved ovarian samples

Sir,

On behalf of all co-authors, we thank M. Shapira and colleagues for their valuable criticism that has enabled us to raise public awareness about the impact of inclusion criteria and timing of fertility preservation in relation to therapy exposures in pediatric patients. It is of utmost importance for us that the correct message is conveyed from our study (Pampanini *et al.*, 2019).

Shapira *et al.* wrote: 'the authors define all first-line chemotherapy received by their treatment group as low-risk in terms of gonado-

toxicity, and as such incorrectly describe their results as reflecting the damage caused to the ovary by exposure to low-risk chemotherapy.'

In Nordic Countries, pediatric patients are offered fertility preservation if they are at very high risk of POI (>80%) due to the planned treatments (allogenic/autologous hematopoietic stem cell transplantation (HSCT) or radiotherapy with ovary in the field). This is in line with the guidelines on fertility preservation of the Nordic Society of Paediatric Haematology and Oncology (NOPHO 2013).

We have used the term 'low-risk' patients to refer to all the patients that did not fulfill these inclusion criteria and that were not eligible for fertility preservation. In our Materials and Methods section, we state that this group includes patients exposed to low-, intermediate- and high-risk treatments. However, we acknowledge that the term 'low-risk' when referring to eligibility for fertility preservation might be misleading.

Our present report gives a realistic picture of the variability of treatment exposures in pediatric patients. After the indication for ovarian cryopreservation is established, the procedure is often delayed for different reasons, related to the hospital logistics, to the health operators or often to the patient's conditions. For example, there may be a need to stabilize the patient, to reach molecular remission or to combine the operation with another procedure under general anesthesia, such as central venous catheter implantation before HSCT.

The impossibility of determining sample size and the heterogeneity of conditions are intrinsic limitations of retrospective clinical studies. We verified and controlled the differences in cancer therapy exposures by running correlation analysis between atresia and cyclophosphamide equivalent dose (CED)/isotoxic dose equivalents (DIE).

We are aware that the use of ovarian tissue exposed to chemotherapy in fertility preservation is well-established and has proven to be safe and effective in terms of restoration of endocrine and reproductive ovarian function. However, it should be noted that these data come from adult patient series. The ability of chemotherapy-exposed ovarian tissues from prepubertal pediatric patients to restore fertility upon reimplantation is yet unproven. Moreover, the majority of studies that report successful reimplantation of ovarian tissues harvested from adult patients, do not report CED exposure. It would be interesting to know the extent of exposure to alkylating agents in these cases to determine more precisely the impact of chemotherapy exposure on the functionality of the ovarian tissue. It is for example not known if the short-term functionality of the reimplanted tissue (i.e. ~7 years) (Donnez and Dolmans, 2017) is influenced by the damage induced by pre-harvesting chemotherapy exposure. Studies that elucidate this are also lacking.

Moreover, studies focusing on cryopreservation of *in vitro* matured oocytes as an additional technique to ovarian tissue freezing for fertility preservation in pediatric female cancer patients have shown that exposure to chemotherapy significantly reduces the number of collected oocytes and the amount of *in vitro* matured oocytes, concluding that 'further studies are needed on the fertility-restoring potential of oocytes from pediatric and prepubertal patients, especially after exposure to chemotherapy' (Abir *et al.*, 2016, Abir *et al.*, 2008).

Pre-harvesting chemotherapy treatment is fundamental in leukemias, where complete remission is associated with a lower risk of ovarian contamination. However, as reimplantation of ovarian tissue is still highly questionable for these patients, due to the high risk of reseeding the disease, the impact of chemotherapy exposure on different fertility-restoring techniques, such as *in vitro* maturation, should also be taken into account.

In the present study, we show that increased atresia and decreased function of ovarian tissue correlated significantly with increasing cumulative doses of chemotherapy exposure. Since all exposed/not exposed samples used for histological counting were processed in the same way (i.e. all were frozen/thawed samples fixed in Bouin), any freeze-thaw effects on the extent of atresia should be equal between the groups and thus negligible. Although our cohort contained samples stored in different fixatives either freshly or after freezing and thawing, different sample types were not mixed in the assays.

Our conclusion on the lack of follicle activation after exposure to chemotherapy was based on the comparison of the ratio of growing/total follicles between exposed and not exposed patients. This is a widely used and solid morphological measure to assess follicle activation. Indeed, a similar morphological measure of growing/dormant follicles was used by Meirow and coworkers to evaluate follicle activation in response to chemotherapy (Kalich-Philosoph *et al.*, 2013).

The long-time frame for FOXO3a assessment, which may have prevented us from observing an earlier activation, was clearly acknowledged as a limitation in the study.

In conclusion, it is far from our intention to convey the message that patients at low risk of infertility should be offered fertility preservation, as we wholly agree with the leading principles that unnecessary interventions should be avoided and that resources should always be correctly allocated. Our final message is that, when indication to perform fertility preservation is established, harvesting of the tissue should be done as early as possible to avoid unnecessary exposure to further chemotherapy drugs. The present study is the first report clearly showing that adverse effects on ovarian follicles correlate with increasing exposure to chemotherapy. This is a clear signal to the clinicians involved in the care of these patients that ovarian cryopreservation should not be unnecessarily delayed once the indication is established. We hope to have clarified the most burning issues presented by Shapira and colleagues, and we once again thank them for having raised this important discussion.

Conflict of interest

None.

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