Looking for the Most Suitable Orthotopic Retinoblastoma Mouse Model in Order to Characterize the Tumoral Development

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Submitted: February 28, 2017
Accepted: May 14, 2017
Citation: Lemaitre S, Poyer F, Marco S, et al. Looking for the most suitable orthotopic retinoblastoma mouse model in order to characterize the tumoral development. Invest Ophthalmol Vis Sci. 2017;58:3055–3064. DOI:10.1167/iovs.17-21760

PURPOSE. Because retinoblastoma therapies have many adverse effects, new approaches must be developed and evaluated on animal models. We describe orthotopic xenograft models of retinoblastoma using different strains of mice, suitable for this purpose.

METHODS. Human retinoblastoma tumors were established on immunodeficient mice by subcutaneous engraftment of tumors from enucleated eyes. The orthotopic model was obtained by subretinal injections of suspension cells into the right eye of immunodeficient (Swiss-nude, severe combined immunodeficiency [SCID]) and immunocompetent mice (C57BL/6N, B6Albino). In vivo tumor growth was monitored by fundus and spectral-domain optical coherence tomography (SD-OCT) imaging and compared with histology.

RESULTS. Retinal and vitreal tumor growth was achieved both in immunocompetent and immunodeficient strains after the subretinal injection of tumor cells. The best tumor engraftment rate was obtained in the SCID mice (68.8%). No tumor growth was observed in the C57BL/6N strain. Chronic retinal detachment may occur in most strains after the subretinal injection, in particular the Swiss-nude strain, which exhibits retinal degeneration.

CONCLUSIONS. The setting up of an orthotopic mouse model depends mainly on the choice of the xenografted mouse strain. Severe combined immunodeficiency mice (an immunodeficient strain) achieved the best tumor engraftment rate (68.8%). However, intraocular tumor growth was also satisfactory (50%) in the immunocompetent strain B6Albino, and this strain will allow to exploit the immune response after a tumor treatment. Both of these strains may therefore be recommended when setting up orthotopic retinoblastoma xenografts.

Keywords: retinoblastoma, orthotopic xenografts, mouse models, fundus and SD-OCT imaging

Retinoblastoma is the most common primary intraocular malignancy in children. Substantial progress has been made over the last decades in the management of this cancer, leading to a decrease of mortality from retinoblastoma in developed countries to less than 5%.1 Thus, in addition to the primary goal of retinoblastoma treatment, which is to save the life of the patient (if left untreated this tumor can spread to the orbit or the central nervous system and metastases can occur), the secondary goal of eye and vision preservation becomes a priority, especially in children with bilateral disease. Eye-sparing therapies like external beam radiotherapy and chemotherapy may have severe adverse effects. Externalbeam radiotherapy has been widely used as a conservative treatment modality for retinoblastoma until the 1990s, when studies showed that it was associated with an increased risk of secondary cancers in survivors.2 Therefore, chemotherapy combined with focal retinal treatment is currently the primary conservative treatment of retinoblastoma in children.3 Nevertheless, in order to minimize the risk of complications due to treatment, the development of new therapeutic approaches is still required. For this purpose, studies on animal models are a compulsory step before considering clinical trials. Because of their availability, mice are the species most widely used in modeling intraocular primary malignancies although rats and rabbits have also been used because of the larger size of their eyeballs.4,5 Nowadays, two different sorts of mouse models are available for retinoblastoma: transgenic mice and xenografts. Transgenic mice bear spontaneous bilateral retinal...
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In Vivo Imaging
Pupils were dilated with a drop of tropicamide 1% (Mydriaticum; Laboratoire Thea, Clermont-Ferrand, France) and a drop of phenylephrine chloride 2.5% (Neosynephrine; Laboratoire Europharma, Monaco, Monaco). Mice were then anesthetized by an intraperitoneal injection of the mixture of ketamine and xylazine. Both eyes were examined using the Micron IV rodent imaging system (Phoenix Research Labs, Pleasanton, CA, USA) after the instillation of moisturizing drops (Goniovisc; Hubs Pharmaceuticals, Rancho Cucamonga, CA, USA).

Imaging of the right eye of the mice was repeated every week after the subretinal injection in order to have a longitudinal follow-up of tumor development. Given that the precise area of tumor growth was not known in advance, the whole retina of the mouse was scanned during each imaging session.

Retinal Thickness Measurement
For each strain of mice, spectral-domain optical coherence tomography (SD-OCT) images of the normal retina in the left eye were performed and used as control. Images from the right eye were also obtained in some mice prior to the injection of tumor cells. In order to compare retinal thickness between the strains only the images of the retina surrounding the optic nerve were used. They were acquired in both the vertical (superior and inferior) and horizontal (nasal and temporal) axes. Measure of the total retinal thickness 500 μm from the center of the optic nerve was done on these images using a home-developed macro (available from authors on request) for the ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).11 Four measures were done for each eye in the nasal, temporal, superior, and inferior sides of the optic nerve. The measurements were done in five mice from each strain and the mean thicknesses (±SEM) were calculated and compared. Due to tissue shrinking during the fixation protocol for histology, in vivo measurements on SD-OCT images were considered more reliable than measurements of retinal thickness on histologic specimens.12 Therefore, these last measures were not performed.

Histology
Animals were euthanized by cervical dislocation and enucleation of both eyes was performed. Eyes were fixed overnight by immersion in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) before paraffin-embedding and sectioning. Eyes were sectioned using a microtome (Leica RM2235 microtome; Leica Microsystems, Nanterre, France) into 7 or 10 μm sections, which were stained with haematoxylin and eosin (H&E).

Tumor Engraftment Rate After the Subretinal Injection
Even if histology is the gold standard method for tumor diagnosis, it is not always necessary in ophthalmic oncology. In many cases, diagnosis can be made on the clinical features of the ocular mass. In particular, a documented tumor growth on successive clinical examinations is a criterion used to differentiate malignant lesions from benign ones.13

Tumor engraftment rate is the percentage of engrafted eyes where tumor growth occurred. In this study, histologic analysis and/or documented growth on fundus and SD-OCT were used to make the difference between eyes where tumor growth had

METHODS

Animals
Female Swiss-nude, severe combined immunodeficiency (SCID), B6Albino, and C57BL/6N mice aged 5 weeks were purchased from Charles River Laboratories (Saint-Germain sur l’Arbresle, France). Swiss-nude and SCID strains are immunodeficient whereas B6Albino and C57BL/6N strains are immunocompetent. Animals were housed on a 12-hour light-dark cycle with access to food and water ad libitum. Animals were killed by cervical dislocation at the end of the experiment or for ethical reasons if proptosis occurred, and both eyes were enucleated.

Ethics Statement
All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional guidelines and local ethics committee regarding animal experimentation.

Setting Up of Xenografts
A panel of retinoblastoma subcutaneous PDX has been established at the Institut Curie. Four retinoblastoma lines (Rb-102, Rb-109, Rb-111, and Rb-200) were used in previous studies to evaluate photodynamic therapy responses on subcutaneous xenografts.6,8,10 Due to the rapid growth obtained in ~6 weeks of the Rb-200 tumor line on subcutaneous models, we used only this retinoblastoma PDX to establish the orthotopic model in this study.

The preparation of the Rb-200 cell suspension and the orthotopic xenograft procedure were previously described.9 Briefly, animals were anesthetized by intraperitoneal injection of ketamine (10 mg/mL) and xylazine (1.2 mg/mL) and a volume of 2 μL of culture medium (Dulbecco's Modified Eagle Medium [DMEM]; Life Technologies, Saint-Aubin, France) containing 10,000 cells/μL was injected only in the right eye of each mouse using a 32-G Hamilton syringe (Reno, NV, USA). A prehole was performed with a 25-G needle near the limbus prior to the introduction of the Hamilton syringe; this Hamilton syringe, which had a blunt tip 32-G needle was then introduced into the eye until it touched the sclera, the injection was finally performed. A transient change of the pupillary reflection showed that the injection caused a retinal detachment and that the culture medium was injected in the subretinal space. All the procedures were made using a binocular lens. A drop of tobramycine (Tobrex; Alcon, Fort Worth, TX, USA) was put into the eye after the injection.

In the present study, we report our results on the tumor engraftment and growth in an orthotopic patient-derived xenograft (PDX) model obtained using immunodeficient and immunocompetent mouse strains. Mouse eyes were monitored in vivo using an eye imaging system for rodents after the subretinal injection of tumor cells.

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RESULTS

Normal Left Eyes (Control)

Retinal Thickness. Retinal thickness is known to be different when measured on in vivo SD-OCT sections and ex vivo histologic ones because the extraction and treatment of samples can be responsible for variation in histology determinations. Therefore, retinal thickness was measured in vivo using the SD-OCT images in the four different strains of mice (3 albino strains Swiss-nude, SCID, and B6Albino, and 1 pigment-ed strain C57BL/6N). Due to homozygous presence of the recessive retinal degeneration 1 (rd1) mutation of the Pde6b gene, Swiss-nude mice have a retina almost twice thinner than SCID, B6Albino, and C57BL/6N mice on SD-OCT analysis (Table 1). On SD-OCT analysis, mean retinal thickness was 84 µm for the Swiss-nude strain, while the other strains had mean retinal thicknesses comprised between 189.6 µm (SCID) and 223.3 µm (C57BL/6N).

Retinal Individual Layers. In SCID, B6Albino, and C57BL/6N mice, SD-OCT imaging and histologic analysis of the retina show that all the usual retinal layers are present (from inside to outside: ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, outer limiting membrane, inner and outer segments of the photoreceptors, retinal pigmented epithelium, and Bruch’s membrane, Fig. 1).

The retinas of Swiss-nude mice lack the outer plexiform and outer nuclear layers, as well as the photoreceptor outer segments. Missing retinal layers in the retina of Swiss-nude mice seem to be responsible for the difference in retinal thickness observed between Swiss-nude mice and the three other mouse strains (Fig. 1).

As expected on histologic analysis, the choroid was pigmented in the C57BL/6N strain, unlike the choroid of the other strains that are albino.

Injected Right Eyes (Orthotopic Engraftment)

Tumor Engraftment Rate After the Subretinal Injection. Tumor growth was achieved both in immunodeficient (Swiss-nude and SCID) and immunocompetent strains (B6Al-
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TABLE 2. Tumor Engraftment Rate

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<thead>
<tr>
<th>Mouse Strain</th>
<th>Tumor Engraftment Rate</th>
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<tbody>
<tr>
<td>Swiss-nude</td>
<td>28.6% (2/7)</td>
</tr>
<tr>
<td>SCID</td>
<td>68.8% (11/16)</td>
</tr>
<tr>
<td>B6Albino</td>
<td>50% (4/8)</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>0% (0/11)</td>
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Number of mice with tumors on histologic analysis/number of mice with available histology is shown in parenthesis for the four strains of mice.

... and 4. Histologic analysis confirmed retinoblastoma in all these cases.

**Tumor Growth Imaging.** Examples of tumor growth in Swiss-nude, SCID, and B6Albino mice are given in Figures 2, 3, and 4. Histologic analysis confirmed retinoblastoma in all these cases.

Figure 2 shows the longitudinal follow-up of retinal tumor growth in a Swiss-nude mouse. At day 13 after the subretinal injection of tumor cells nothing is visible either on fundus examination or on the corresponding SD-OCT scan. A small retinal thickening is visible on the SD-OCT scan at day 20 (white arrow) and it progressively increases in size on the following scans at days 27, 34, and 41. This retinal mass is barely visible on all the fundus images, especially on those showing the early stages of tumor growth.

Tumor growth in a SCID mouse is shown in Figure 3. A preretinal seed is visible at day 13 after the injection of tumor cells. It then increases in size and tumor growth in the adjacent retina can be seen on the SD-OCT scan at day 27. On fundus examination the tumor is visible only at later stages (days 27, 34, and 41) as a white inferior retinal mass. Spectral-domain OCT is less precise for the visualization of the tumor at day 41 because of a shadowing effect due to tumor thickness.

In Figure 4, an intravitreal tumor in a B6Albino mouse is shown (fundus, SD-OCT, and corresponding histologic section). It is well seen on the fundus as a white lesion, which masks the underlying retina. Despite the shadowing effect, the corresponding SD-OCT scan is helpful to localize the lesion into the vitreous. A retinal thickening is visible next to the intravitreal lesion on the same SD-OCT scan, however histologic analysis showed that it is an area of retinal detachment and not a tumor (unlike the mass in the vitreous, which is retinoblastoma).

**Injection Site.** The injection site can be localized on SD-OCT imaging but it isn't necessarily the precise retinal area where a tumor growth will occur. Tumor growth can theoretically occur in any area of the retinal detachment induced by the subretinal injection. The retinal scar located at the injection site can also be found on the histologic sections. The absence of documented tumor growth on SD-OCT and the absence of tumor cells on histology allow discriminating between a scar lesion and an active growing tumor (Fig. 5).

**Histologic Analysis.** Histologic analysis confirmed retinoblastoma tumor growth in some of the engrafted eyes like in the three previous examples (Swiss-nude in Fig. 2, SCID in Fig. 3, and B6Albino in Fig. 4). Central necrosis was a common feature of orthotopic tumors (similar to human retinoblastoma), but no calcifications were found. No rosettes were observed, unlike human retinoblastoma and the transgenic LHBetaTag mouse model. When processing the samples for histologic analysis, retinal detachment is a frequent artifact (as can be seen in Fig. 1).

**Limitations of Orthotopic Models**

**Media Opacity.** Transparency of ocular tissues makes fundus and SD-OCT examination possible. Any media opacity is therefore a limitation for in vivo follow-up of tumor growth using a retinal imaging system (Micron IV device). Mice lose their blink reflex under general anesthesia and despite the use of artificial tears the cornea may dry causing a corneal opacity. In other cases, media opacity is a consequence of the subretinal injection, which is an invasive procedure. Traumatic hyphema, cataract, or intravitreal hemorrhage are some of the possible complications that may impede the retina examination. Tumor growth in the vitreous or in the anterior chamber can also prevent the SD-OCT light penetration to the investigated tissues.

In the present study, four mice were excluded due to total cataract after the injection (2 B6Albino and 2 Swiss-nude mice). Two other mice had hyphema after the injection (1 Swiss-nude and 1 C57BL/6N mouse) but fundus and SD-OCT follow-up were possible. One SCID mouse had tumor growth in the anterior chamber during follow-up, which eventually prevented the visualization of the fundus; histologic analysis confirmed tumor growth in both the anterior chamber and the vitreous (data not shown).

**Retinal Detachment After a Subretinal Injection.** Our orthotopic xenograft procedure consists of a subretinal injection of tumor cells leading to a temporary retinal detachment. In our experience, the retina is usually reattached within the first 48 hours after the subretinal injection. In some cases a chronic rhegmatogenous retinal detachment occurs, the retinal hole resulting at the injection site. It can be seen on fundus examination, SD-OCT scan, and histologic sections (Fig. 6). In our series, Swiss-nude mice are particularly prone to the occurrence of a chronic rhegmatogenous retinal detachment (35% of cases). Chronic rhegmatogenous retinal detachment is less frequent in the other strains; only 14% to 15% occurred in B6albino and SCID mice and no chronic retinal detachment was observed in C57BL/6N mice (Table 5). This difference may be due to the fact that the retina is thinner in Swiss-nude mice compared with the other strains.

In vivo follow-up of tumor growth is difficult when there is a chronic bulous retinal detachment because some of the retinal folds may be hard to distinguish from a malignant retinal lesion. What makes the difference between the two is the absence of tumor growth in the case of retinal folds during the follow-up.

**DISCUSSION**

**Heterotopic Versus Orthotopic Xenograft Models Of Retinoblastoma**

Heterotopic subcutaneous retinoblastoma models are often used because subcutaneous tumors are easier to establish than orthotopic ones and their growth can be monitored in vivo with a simple caliper and/or with different imaging modalities (like ultrasound, computed tomography, or magnetic resonance imaging). In addition, subcutaneous tumors in mice provide more material for analysis than orthotopic intraocular tumors. Subcutaneous tumor growth can be achieved in any immunodeficient mice (nu/nu or SCID) but it is best achieved in SCID mice, which lack both B and T lymphocytes. In order to improve engraftment in nude mice, large tumor cell inocula may be necessary (>10^6 cells). Nude mice treated with immunosuppressive drugs (cyclophosphamide) have also better subcutaneous tumor engraftment rates. 7,15 We do not use immunosuppressive drugs in our mouse models because...
FIGURE 2. Follow-up of tumor growth on fundus and SD-OCT scans in a Swiss-nude mouse after the subretinal injection of tumor cells at day 13 (a), day 20 (b), day 27 (c), day 34 (d), day 41 (e), and the corresponding histology image (f). There is no visible tumor on fundus examination during the follow-up but the corresponding SD-OCT scans show the intraocular tumor even at its early stages of growth (b–e). The arrow in (b) shows the beginning of tumor growth. There is a size increase of the retinal lesion over time. On the final histologic examination the diagnosis of retinoblastoma is confirmed and there is an area of central necrosis (N) in the retinal tumor corresponding to a central hyperreflective area on the SD-OCT scan (e, f). Scale bar: 100 μm.
FIGURE 3. Follow-up of tumor growth on fundus and SD-OCT scans in a SCID mouse after the subretinal injection of tumor cells at day 15 (a), day 20 (b), day 27 (c), day 34 (d), day 41 (e), and the corresponding histology image (f). At early stages no tumor can be seen on fundus examination but preretinal seeds can be seen on the SD-OCT scans of the retina (a, b). These lesions increase in size progressively and may invade the retina (c). At later stages of tumor growth a white retinal mass (T) can be seen on fundus examination (c-e). On the corresponding SD-OCT scans there is a posterior shadowing effect due to the thickness of the lesion, which impedes the visualization of the deepest layers (e). Scale bar: 100 μm.
mice, which are immunologically normal. Thus, taking into consideration that orthotopic models are more reliable than subcutaneous ones for the evaluation of treatment efficacy and therefore differences in angiogenesis mechanisms between the two strains (C57BL/6 and B6Albino) could partly explain, and therefore differences in angiogenesis. Angiogenesis is one of the hallmarks of cancer, and therefore differences in angiogenesis mechanisms between the two strains (C57BL/6 and B6Albino) could partly explain, with the differences in immune response, the disparity in tumor engraftment rates that were observed in our study.

The suspension of retinoblastoma cells used to generate an orthotopic xenograft model can come either from a retinoblastoma cell line or from a patient-derived tumor (fresh retinoblastoma or PDX). Both reproduce the histologic features of human tumors and can be used to evaluate new therapies (e.g., the use of intravitreal chemotherapy is effective both in the human disease and the orthotopic mouse model22). We preferred the use of cells coming from subcutaneous PDX because these cells may better reproduce the features of human tumors compared with cells in culture (immortal cell lines do not accurately replicate the primary cells due to clonal selection).

**Intraocular Retinoblastoma Mouse Models**

Two sorts of intraocular retinoblastoma mouse models are currently available: genetically engineered mice and orthotopic xenograft mouse models.20,21 Both reproduce the histologic features of human tumors and can be used to evaluate new therapies (e.g., the use of intravitreal chemotherapy is effective both in the human disease and the orthotopic mouse model22). However, each model has its own advantages and disadvantages, making them complementary for the study of retinoblastoma. Thus, transgenic mice have spontaneous bilateral murine retinal tumors and therefore are good models for the study of retinoblastoma tumorigenesis, while orthotopic xenograft models have the advantage of the use of human
cells, leading to responses to therapies which may be more similar to the response in human retinoblastoma.

Human retinoblastoma can be either solid (retinal tumors) or viscous (vitreous tumors). In transgenic models tumors arise from the retina, whereas in orthotopic xenograft models tumor growth can be retinal and/or intravitreal, depending on the injection site (subretinal or intravitreal injection of tumor cells). We observed that tumor growth is more frequent in the vitreous, even if the injection was subretinal, due to the reflux of tumor cells into the vitreous cavity. Intravitreal tumor growth in mice can be useful if the goal of a study is to evaluate new therapies for vitreal seeds. However, if only retinal tumors are needed, transgenic models may be more suitable than the orthotopic ones because an intravitreal tumor growth may impede visualization of the retina on fundus and SD-OCT examination.

In children, retinoblastoma diagnosis and follow-up rely mainly on fundus examination. Spectral-domain OCT is helpful in retinoblastoma for the follow-up of tumors and diagnosis of treatment-induced retinopathy but retinoblastoma classifications do not take into account this imaging modality. We find that fundus examination in mice is not accurate enough to visualize all the retinal tumors and to monitor their growth (Table 4). An additional imaging device is needed and both SD-OCT and ultrasound examinations could be used. The Micron IV imaging device provides simultaneous visualization of the fundus and its corresponding SD-OCT scan, whereas an ultrasound examination of the eye must be performed separately, which can be time-consuming. The Micron III device has already been used to monitor tumor growth in transgenic retinoblastoma mice (LHBetaTag mice) and we find similar results on the orthotopic xenograft model.

**Table 3. Occurrence of Chronic Retinal Detachment After a Subretinal Injection in the Four Strains of Mice.**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Chronic Retinal Detachment in Xenografted Mice</th>
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<tbody>
<tr>
<td>Swiss-nude</td>
<td>35% (6/17)</td>
</tr>
<tr>
<td>SCID</td>
<td>15% (6/40)</td>
</tr>
<tr>
<td>B6Albino</td>
<td>14% (2/14)</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>0% (0/11)</td>
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</table>

Number of mice with chronic retinal detachment/number of engrafted mice is shown in parenthesis. The presence of a retinal detachment was evaluated both on fundus and SD-OCT images. Histology was not used for this purpose because the retina is frequently detached at the end of the paraffin-embedding process that is artefactual.

**Chronic Retinal Detachment**

We hypothesize that chronic retinal detachment is more frequent in Swiss-nude mice because their retina is twice as thin than the other strains due to retinal degeneration, making...
it more prone to persistent retinal holes. Chronic retinal detachment after a subretinal injection of tumor cells can be an obstacle to correct in vivo follow-up of tumor growth. Mouse strains that exhibit retinal degeneration (including Swiss-nude mice) should therefore be avoided if subretinal injections of tumor cells are to be performed.

CONCLUSIONS

A retinoblastoma orthotopic xenograft model can be obtained after the subretinal injection of tumor cells. Tumor growth is achieved both in immunocompetent and immunodeficient mice. Retinal degeneration is present in some mouse strains, including the Swiss-nude strain; this must be taken into account when choosing a mouse strain for setting up an orthotopic retinoblastoma model. Intraocular tumor growth is achieved with the best engraftment rate in the immunodeficient SCID strain. However, tumor engraftment was also satisfying in the immunocompetent B6Albino strain, which may result in a mouse model closer to the human disease and should therefore be preferred. We also show in the present study that fundus examination and SD-OCT imaging are complementary imaging modalities for in vivo follow-up of tumor growth in the retinoblastoma orthotopic xenograft model. Our model should be very helpful to evaluate new therapies for retinoblastoma.

Acknowledgments

The authors thank Sophie Leboucher from the histology plateau (Institut Curie, Orsay, France) for technical assistance in histology.

This work was partially presented at the ARVO (Association for Research in Vision and Ophthalmology) 2016 (poster) and the EVER (European for Vision and Eye Research) 2015 meetings.

Supported by grants from the nonprofit French organization Retinostop (Paris, Ile-de-France, France), and a MD-PhD fellowship from Institut Curie (SL, Paris, Ile-de-France, France).

Disclosure: S. Lemaitre, None; F. Poyer, None; S. Marco, None; P. Fréneaux, None; F. Doz, None; I. Aerts, Canon (F); L. Desjardins, None; N. Cassoux, None; C.D. Thomas, None

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