ABSTRACT
When production of bispecific antibodies requires the co-expression and assembly of three or four polypeptide chains, low expression of one chain can significantly limit assembly and yield. λκ bodies, fully human bispecific antibodies with native IgG structure, are composed of a common heavy chain and two different light chains, one kappa and one lambda. No engineering is applied to force pairing of the chains, thus both monospecific and bispecific antibodies are secreted in the supernatant. In this context, stoichiometric expression of the two light chains allows for maximal assembly of the bispecific antibody. In this study, we selected a λκ body with suboptimal characteristics due to low kappa chain expression. Codon optimization to increase expression of the kappa chain did not improve bispecific yield. Surprisingly, progressive introduction of non-optimal codons into the sequence of the lambda chain resulted in lowering its expression for an optimal tuning of the relative distribution of monospecific and bispecific antibodies. This codon de-optimization led to doubling of the λκ body yield. These results indicate that assembly of different proteins into a recombinant complex is an interconnected process and that reducing the expression of one polypeptide can actually increase the overall yield.

Optimizing assembly and production of native bispecific antibodies by codon de-optimization

Giovanni Magistrelli, Yves Poitevin, Florence Schlosser, Guillemette Pontini, Pauline Malinge, Soheila Josserand, Marie Corbier, and Nicolas Fischer

Novimmune SA, Plan-les-Ouates, Geneva, Switzerland

Introduction

Since the development of recombinant DNA technologies, efficient expression and purification of recombinant proteins have transformed not only basic and applied research, by providing access to a multitude of tools, but also enabled the use of biologics for therapy.1-5 Quality and yield of the recombinant protein are critical parameters when considering industrial scale manufacturing for therapeutic usage. Although several organisms can be considered for recombinant protein expression, mammalian cells are often preferred for proteins that require post-translational modifications. Numerous strategies have been developed to increase the yield of recombinant proteins obtained from expression in mammalian cells.6,7 Various factors, such as gene copy number, transcriptional control elements, mRNA stability, translational efficiency, codon usage and codon order, influence the level of gene expression.8-17 DNA sequence optimization often includes removal of repeated sequences, killer motifs and splice sites, reduction of GC content (guanine-cytosine content) and optimization of codon usage for tRNA (tRNA) frequency in a given organism, all aiming at maximizing translation and stability of mRNA (mRNA).18-20 A variety of software are available for synthetic gene design, as well as computational procedures to evaluate the effect of codon optimization.21-26 Using such strategies, increases in protein yields have been reported.27-31 However, although the final protein encoded by an optimized DNA sequence has the same amino acid composition, the modifications that are introduced might alter proper protein folding, solubility and activity by modifying the dynamics of protein translation.32,33 Indeed, low frequency codons located at certain positions can be important to slow down translation and avoid ribosome ‘traffic jams’ or to pause translation for correct folding of the nascent polypeptide and membrane targeting of cell surface proteins.11,12,34,35

An additional level of complexity arises when expressing protein complexes composed of different polypeptides. In this situation, over- or under-expression of one or several polypeptides can lead to a decrease in production of the final protein complex and the accumulation of partially assembled complexes. Monoclonal antibodies, which are composed of four disulfide-linked polypeptides (two identical heavy chains and two identical light chains), represent a relevant example given their importance both as research tools and as a class of drugs. Production of several grams per liter can be routinely achieved using fed-batch fermentation of stable Chinese hamster ovary (CHO) cell lines.35,36 The correlation between the level of light chain expression and antibody titer, as well as the presence of free light chains in the culture supernatant, suggest that the heavy and light chains are not expressed or assembled in a stoichiometric manner.77,38 The situation is even more complex for the expression of bispecific antibodies that often require the expression of three or four different polypeptides and the
The formation of heterodimers to incorporate two specificities within a single molecule. We have described previously a native bispecific antibody format, the $\kappa\lambda$ body, which is composed of two identical heavy chains and two different light chains, one kappa and one lambda.\(^{39}\) The co-expression of these three polypeptides leads to the assembly of three different molecules, two monospecific antibodies (one with two kappa light chains, IgG$\kappa\kappa$, and one with two lambda light chains, IgG$\lambda\lambda$) and the bispecific $\kappa\lambda$ antibody (IgG$\kappa\lambda$), which can be effectively purified using three steps of affinity chromatography using resins that specifically bind to 1) the Fc of the heavy chain; 2) the constant kappa; and 3) the constant lambda domains. As each affinity step is specific to one of the chains of the $\kappa\lambda$ body, the process ensures high purity and quality of the purified product.\(^{39}\)

A benefit of this approach is that $\kappa\lambda$ bodies have the structure of a native human IgG and do not incorporate any mutations or foreign sequences. Thus, $\kappa\lambda$ bodies maintain the favorable properties of IgGs that are particularly suitable for therapeutic use. As homodimer and heterodimer formation is dependent on the random assembly of heavy and light chains, the proportion and final yield of the $\kappa\lambda$ body in the supernatant are influenced by the relative expression of the two light chains. In this study, we investigated the possibility of tuning the relative expression of the chains to maximize $\kappa\lambda$ body expression by modifying the sequence encoding the different antibody chains. We demonstrate that by combining sequence optimization and de-optimization, the relative expression of the three antibody forms can be precisely optimized.

**Results**

Over 300 $\kappa\lambda$ bodies have been produced in our laboratory and, in most cases, expression of the light chains is sufficiently balanced so that the bispecific $\kappa\lambda$ body is the most abundant molecule secreted in the supernatant (Fig. 1A). However, in some instances expression of the light chains can be significantly unbalanced, which affects the distribution of the three antibody forms and reduces the yield of $\kappa\lambda$ body (Fig. 1B). K11L7–2 is a bispecific $\kappa\lambda$ body targeting hCD47 and hCD19.\(^{39}\)

The heavy chain and the two light chains of K11L7–2 were transiently expressed in mammalian cells using a single vector with three independent promoters. All three IgG forms (IgG$\kappa\kappa$, IgG$\kappa\lambda$, IgG$\lambda\lambda$) were purified from the culture supernatant via affinity chromatography. Separation by electrophoresis of the polypeptides revealed that for this candidate, the lambda light chain was significantly over-represented (90%) compared with the kappa light chain (Figs. 2A and B). The difference in band intensity suggested that more IgG$\lambda\lambda$ was secreted. This result was confirmed by isoelectric focusing (IEF) gel analysis, which showed that the IgG$\lambda\lambda$ was the most abundant form instead of the bispecific IgG$\kappa\lambda$ and only a minor amount of IgG$\kappa\kappa$ was present (Fig. 2C). Thus, K11L7–2 was used as an example of a $\kappa\lambda$ body with suboptimal characteristics in terms of bispecific antibody yield. The aim was to modify the relative expression of the two light chains to maximize assembly and yield of the bispecific IgG$\kappa\lambda$.

**Generation of K11L7–2 variants and characterization in transient transfections**

We first generated a panel of 6 variants of K11L7–2 containing different combinations of antibody heavy (VHCH) and light (VKCK K11 and VLCL L7–2) chains encoded by sequences optimized for expression in mammalian cells (Table 1; constructs 1 to 6). All DNA sequences encoded the same amino acid sequence. These constructs were used for transient
transfections in PEAK cells, and the content of the culture supernatant was then purified and characterized. While the total IgG productivity remained unaltered (Fig. 2D), increase in light chain abundance correlated with sequence optimization (Figs. 2B and C). Indeed, the over-representation of the lambda light chain was further increased in the K11L7–2 variants 1 and 3 containing an optimized sequence for the lambda light chain. The situation remained unchanged when both light chains were optimized as in variants 2 and 4. Finally, optimization of the kappa light chain sequence in variants 5 and 6 lead to an increase of assembled kappa chain. However, even in variants 5 and 6 in which the kappa chain was optimized, the IgGλλ remained the major form secreted (Fig. 2C).

To improve this situation, a second strategy aimed at reducing the expression of the highly expressed lambda light chain was explored. Three new variants of K11L7–2 were generated, each containing the same optimized heavy and kappa chain sequences as in variant 5 combined with genes encoding the lambda light chain that were de-optimized to various degrees. The de-optimization was achieved by including codons less frequently used in CHO cells. The variants, i.e., constructs 7, 8 and 9, respectively, contained increasing numbers of non-optimal codons (Table 1; see materials and methods and Fig. S1-4 for details). The different IgG forms isolated from the supernatant of PEAK cells transiently transfected with K11L7–2 and the variants 4, 5 and 7 to 9 were analyzed by IEF (Fig. 3A). A reduction of IgGλλ was observed with increasing levels of lambda light chain codon de-optimization. Inversely, IgGkk was increasingly more abundant in variants 7, 8 and 9, respectively. The bands corresponding to the bispecific IgGkλ were also more intense for variants 7, 8 and 9 compared with the original K11L7–2 construct or the optimized variants 4 and 5 (Fig. 3A). The overall productivity was unaffected, with total IgG concentrations ranging between 20 and 30 μg/mL (Fig. 3B).

To better quantify the relative abundance of the three forms, hydrophobic interaction chromatography (HIC) was performed on these samples (Fig. 3C). The integration of the three distinct peaks indicated that the bispecific content was significantly increased, from 22% in the initial K11L7–2 to 43% for variant 8 (Fig. 3D and Table 2). This data confirmed the IEF analysis indicating that codon de-optimization of the lambda light chain sequence had an effect on the distribution of the three IgG forms secreted in the culture supernatant. Interestingly, although the IgGκκ/IgGλλ ratio varied significantly in variants 7, 8 and 9 (0.38, 1.07 and 2.27, respectively), the

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**Table 1.** Constructs generated with optimized and de-optimized sequences for the heavy, lambda light and kappa light chains. WT: wild type sequence; Opt.: codon optimized sequence for mammalian cell expression; Opt. (VK): optimization of the variable kappa region only; De-opt.-1, –2, –3: sequences that were increasingly de-optimized for mammalian cell expression.

| Constructs | VKCK | K11 | VHCH | VLCL | L7–2 |
|------------|------|-----|------|------|------|
| K11L7–2    | WT   | WT  | WT   | WT   |     |
| 1          | WT   | Opt.| WT   | Opt. |
| 2          | Opt. | WT  | Opt. |
| 3          | WT   | Opt.| Opt. |
| 4          | Opt. | Opt.| Opt. |
| 5          | Opt. | Opt.| WT   |
| 6          | Opt. (VK) | Opt.| WT   |
| 7          | Opt. | Opt.| De-opt.-1 |
| 8          | Opt. | Opt.| De-opt.-2 |
| 9          | Opt. | Opt.| De-opt.-3 |
Figure 3. IgG expression after transfection in PEAK cells of K11L7–2 as well as the codon optimized and de-optimized variants. (A) Isoelectric focusing analysis of the purified total IgG samples, the top and bottom bands represent the IgG_k and the IgG_λ, respectively, the intermediate band corresponds to the IgG_kλ (κλ body). (B) Total IgG concentration in the culture supernatant. (C) Analysis by HIC-HPLC of purified total IgG. Peaks corresponding to each of the three forms are indicated. (D) Distribution of the three IgG forms analyzed by HIC-HPLC. pI: Isoelectric Point; L: Ladder.

Figure 4. Analysis of IgG ratio and yields in the supernatant of stable CHO pools transfected with K11L7–2 and the codon optimized or de-optimized variants. Each symbol represents an independent pool. Open symbols indicate the two highest IgG_kλ producing pools for each variant. (A) Percentage of different IgG forms in total IgG. (B) Total IgG and IgG_kλ titers in the supernatants.
Table 2. Total IgG and κλ body yields obtained from the supernatants after transient transfections of PEAK cells.

| Constructs | Total IgG Yield (μg/mL) | κλ body Yield (μg/mL) | κλ body % by HIC |
|------------|------------------------|----------------------|-----------------|
| K11L7–2    | 29.3                   | 5.4                  | 21.6            |
| 4          | 23.2                   | 2.4                  | 14.4            |
| 5          | 28.6                   | 5.8                  | 29.4            |
| 7          | 29.3                   | 8.8                  | 38.1            |
| 8          | 29.2                   | 8.9                  | 42.9            |
| 9          | 20.0                   | 6.2                  | 39.7            |

Proportion or IgGκλ remained comparatively stable (38%, 43% and 40%, respectively). This observation is in line with the fact that when one of the light chains is less expressed, the assembly of the monospecific IgG form incorporating two copies of the less available light chain is more affected than the bispecific form incorporating a single copy of the less abundant chain. Thus, within a certain range, unbalanced expression of the two light chains has a low impact on the assembly of the IgGκλ. This observation supports the fact that for most κλ body candidates expressed, the proportion of the bispecific form ranges between 35% and 50% without any codon optimization.

Expression in stably transfected CHO cells

We then investigated whether the results obtained using transient transfections would also apply to expression in stable CHO cells that represent a more relevant situation for large-scale production of therapeutic antibodies. K11L7–2 as well as variants 4, 5, 7, 8 and 9 were transfected into CHO cells. Between 4 and 13 stable pools were selected and expanded for each construct. Productivity and IgG species distribution were evaluated for fed-batch fermentation conditions. After total IgG purification by Protein A affinity chromatography, relative abundance of the three IgG forms was determined by HIC (Fig. 4A). Similar to transient expression conditions, the IgGκκ/IgGκλ ratio increased with the level of codon de-optimization of the lambda chain and the proportion of IgGκλ increased to reach a maximum of about 40% in variants 7 and 8 (Fig. 4A). Interestingly, the drastic reduction of lambda chain expression in variant 9 leads to a decrease in the abundance of IgGκλ. This observation indicates that the lambda chain expression became limiting for bispecific antibody assembly. The average total IgG titer measured in the supernatant increased in variants containing a de-optimized lambda chain (Fig. 4B). This increase in total IgG titer combined with a significantly larger proportion of IgGκλ lead to the generation of CHO cell pools with an increase in IgGκλ titer for variants 7 and 8 (Fig. 4B). Indeed, when considering the two best CHO stable cell pools for each construct, the yield of bispecific IgGκλ doubled in variant 8 (721 and 678 μg/mL) as compared with the K11L7–2 (348 and 365 μg/mL; Table 3). Purification of the bispecific IgGκλ was performed using the three sequential affinity chromatography steps (Fig. 5A). The purified bispecific IgGκλ had similar aggregate levels and equivalent binding to their targets (data not shown). Total IgG isolated after the Protein A step and purified κλ body were analyzed on an Agilent Bioanalyzer and by IEF (Fig. 5B and C). The intensity of the bands

![Figure 5. Analysis of total IgG and purified IgGκλ (κλ body) expressed by stable CHO cells.](image-url)
corresponding to the light chains (Fig. 5B) or the intact IgG species (Fig. 5C) confirmed the HIC data indicating that the assembly of the two light chains was more evenly distributed in variants 7 and 8, and lead to the highest level of IgGκλ secretion in the cell culture supernatant.

**Discussion**

Optimal production of hetero-multimeric complexes requires appropriate expression and assembly of each polypeptide component of the complex. Antibodies are composed of two identical heavy chains and two identical light chains that assemble along the secretory pathway in mammalian cells. The importance of the relative expression of these two chains has been highlighted in previous studies showing that excess production of the light chain correlates with higher IgG expression and also to the secretion of free light chain in the supernatant.7,13,27,28,30-32,43 We have been widely described.7,13,27,28,30-32,43 It has been shown that light chain pairing with the heavy chain is required for folding of the CH1 domain of the heavy chain and subsequent secretion.40-42 Thus, light chain transcription and translation can be a limiting step to optimal IgG production.

Bispecific antibody formats that require the co-expression and assembly of three or four different polypeptides are even more dependent on appropriate expression levels to achieve maximal expression. The κλ body format does not involve any engineering to force correct pairing of heavy and light chains, and thus leads to the secretion of a mixture of three different IgG forms from which the κλ body can effectively be purified. An equivalent expression of the two light chains is in principle the optimal situation to reach maximal IgGκλ production. Relatively balanced expression appears to be quite common as most of 300 κλ bodies expressed so far show 40–50% of bispecific form without any optimization. However, it is possible that a κλ body having a biologic activity of particular interest might present large differences in light chain expression levels, and thus not represent a suitable candidate for development. To simulate this potential situation, we used K11L7–2 in this proof-of-concept study because this candidate presented with an excess of lambda chain expression and only 20–25% κλ body secretion.

Codon optimization for expression in different organisms has been widely described.7,13,27,28,30-32,43 We first attempted to re-establish a more balanced expression of the two light chains by optimizing the sequence encoding the less expressed kappa light chain, but this approach did not improve the situation. We then considered the opposite approach and tested the possibility of reducing the expression of the lambda light chain by incorporation into the coding sequence different degrees of less favorable codons for expression in mammalian cells. As motifs known to alter mRNA structure or stability were avoided during sequence optimization, we attribute the effects observed to the modification of codon content. This approach enabled the progressive reduction of lambda light chain expression leading to a decrease of IgGκλ and concomitant increase of IgGκκ and IgGκλ production. Maximal IgGκλ productivity was achieved when the expression of both light chains was relatively balanced. The results suggest an interconnected system in which light chains compete for assembly with the common heavy chain. Thus, tuning down the abundance of one chain not only reduces its incorporation into the IgG forms, but also increases the incorporation of the other chain.

Importantly, by de-optimizing the codon usage in the coding sequence of the K11L7–2 lambda light chain, we could double the yield of bispecific κλ body in the supernatant of stable CHO pools. Similar results were obtained with the variants in transient expression using PEAK cells and with stable CHO pools. Thus, the evaluation of optimized variants in transient transfections appears to be predictive, facilitating development of stable pools and cell lines with a limited number of preselected optimized variants. Beyond κλ bodies and bispecific antibodies, the codon de-optimization approach described here is likely to be applicable to a wide range of protein complexes composed of different polypeptides.

**Materials and methods**

**Codon optimization and de-optimization**

Optimal and non-optimal codon sequences for mammalian cells were generated by GeneArt® (GeneOptimizer™ software) on the heavy chain, the kappa and the lambda light chains. Inhibitory motifs (such as possible splice sites) have been removed in all sequences. During the optimization process the following sequence motifs were avoided: internal TATA-boxes, chi-sites, ribosomal entry sites, AT-rich or GC-rich sequence stretches, RNA instability motifs, repeat sequences, RNA secondary structures, splice donor and acceptor sites. To reduce the lambda chain expression, three different levels of de-optimization were applied by progressive introduction of codons used less frequently. All sequences are shown in Figs. 1–3 and the codon quality distribution for each de-optimized sequences shown in Fig. S4 For variant 9, the parameters have been modified in such a way that the result leads to a nearly inverted codon usage table. For variants 7 and 8, the parameters were more relaxed.

**Plasmid generation**

The common heavy chain, the kappa and the lambda light chains previously generated were cloned into a single pNOVI expression vector containing three expression cassettes under the transcriptional control of the human cytomegalovirus promoter.

First, second and third promoters drove, respectively, the expression of the kappa light chain, which binds to hCD47, the common heavy chain, and the lambda light chain, which targets hCD19.

Different candidates were obtained by cloning the optimized and de-optimized chains in the wild type plasmid of K11L7–2 (Table 1).

**Transient IgG expression in PEAK cells**

Transformed Human Embryo Kidney monolayer epithelial cells (PEAK cells; Edge Bio) were maintained in 5% CO2 at 37°C in a humidified atmosphere in DMEM (Thermo Fisher Scientific, Waltham, MA) containing 10% FCS (Sigma-Aldrich, Waltham, MA).
Transient transfections were performed using a mix containing 30 μg of DNA and 42 μL of Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific) in 1.4 mL of DMEM for 10 7 cells per T175 flask in 50 mL of complete DMEM.

IgG expression was measured using the Octet RED96 with Protein A-coated biosensors (Pall ForteBio, Menlo Park, CA). According to antibody concentration, supernatants were harvested 7 to 10 d after transfection.

**Stable pool generation in CHO-S cells**

CHO-S (Thermo Fisher Scientific) cells were routinely cultured in suspension at 2×10⁶ cells/mL in CD CHO medium (Sigma-Aldrich) supplemented with 6 mM L-glutamine in Erlenmeyer flasks. Cell culture was performed at 37°C with 5% CO₂ and 85% relative humidity at 140 rpm. For stable transfection, CHO-S cells in exponential growth phase were resuspended at 1.43×10⁷ cells/mL in CD CHO medium without glutamine and mixed with 40 μg of linearized DNA in an electroporation cuvette of 0.4 cm (Bio-Rad, Hercules, CA).

After electroporation by single pulse of 300 V, 900 μF and infinite resistance using Gene Pulser Xcell™ Electroporation Systems (Bio-Rad), cells were immediately added in 50 mL of CD CHO medium without glutamine and distributed in two 6-well plates at 4 mL/well or in two T-75 flasks and placed in humidified 10% CO₂ incubator set at 37°C. The following day, L-methionine sulfoximine (MSX) (Sigma-Aldrich) was added to the culture at 50 μM final concentration for transfected cell selection. After 4 to 5 weeks of growth under selection pressure, pools were assessed for their IgG productivity, and transferred to Erlenmeyer flasks and amplified in suspension.

**Productivity evaluation by fed-batch overgrow culture**

The productivity of CHO pools was assessed by fed-batch overgrow culture evaluation. Cells were inoculated at 3×10⁶ cells/mL in 50 mL of CD CHO medium supplemented with 300 mg/L of L-cysteine, 120 mg/L of L-tyrosine, 50 μM MSX and fed with 15 mL CD CHO EfficientFeed™ B Liquid Nutrient Supplement (Thermo Fisher Scientific) at day 1. The glucose level was monitored using the GlucCell Glucose Monitoring System (CESCO Bioproduct), and adjusted when necessary. Cells were harvested at day 15 post inoculation or when viability had dropped below 75%. IgG quantitation were measured by Octet.

**IgG purification**

After 7 to 10 and 15 d of antibody production for PEAK cells and CHO cells, respectively, the supernatant was harvested, clarified by centrifugation 10 min at 2000 rpm and filtered on a 0.22 μm membrane (Merck Millipore, Darmstadt, Germany). Total IgGs were purified by one affinity chromatography step using the FcXL resin (Thermo Fisher Scientific) or the MabSelect™ SuRe™ resin (GE Healthcare, Chicago, IL) for PEAK and CHO supernatant, respectively. A serum-containing medium is used for production in PEAK cells; bovine IgGs do not bind to the FcXL resin, while they bind to the MabSelect™ SuRe™ resin. Then, two additional affinity chromatography steps were required to isolate the κλ body and eliminate the two monospecific mAbs: one purification on the KappaSelect resin (GE Healthcare) to eliminate the IgGκ and one purification on the LambdaFabSelect resin (GE Healthcare) to get rid of IgGκx.

An appropriate amount of MabSelect™ SuRe™ or FcXL resin was washed three times with phosphate-buffered saline (PBS) and resuspended in PBS (Sigma-Aldrich). The resin was added to the supernatant and the mix was incubated overnight at 4°C and 15 rpm. Samples were centrifuged at 2200 rpm for 10 minutes to collect the resin and the flow through was discarded. The resin was washed with PBS and transferred on SigmaPrep™ spin column (Sigma-Aldrich). Elution was performed with glycine 50 mM at pH 3.0. Following purification, the total IgG and the κλ body were formulated into 25 mM histidine, 125 mM NaCl at pH 6.0, by desalting on Amicon Ultra-4 centrifugal filters with membrane Ultrace 50 kDa (Merck Millipore) previously equilibrated with formulation buffer.

The final antibody concentration was evaluated by Nanodrop®.

**Characterization of purified antibodies**

Monospecific antibodies and κλ body distribution and integrity was assessed by electrophoresis, IEF, HIC-HPLC and SEC-HPLC.

Purified IgGs were analyzed by electrophoresis in denaturing and reducing conditions. The Agilent 2100 Bioanalyzer was used with the Protein 80 kit as described by the manufacturer (Agilent Technologies, Santa Clara, CA). The distribution of the different formats of IgG (IgGκ, IgGκκ and IgGκλ) was determined by isoelectric focusing (pH 7–11 IsoGel agarose plates, Cambrex, East Rutherford, NJ) and HIC-HPLC analysis using ProPac HIC-10 column (Dionex, Sunnyvale, CA). A gradient of mobile phase A (0.001 M phosphate buffer + 1 M ammonium sulfate, pH 3.5) from 85 to 35% and a growing gradient of mobile phase B (0.001 M phosphate buffer + acetonitrile 10%, pH 3.5) from 15% to 100% were applied. A blank was performed with mobile phase A, pH 7.0.

Aggregate and fragment levels were determined by SEC-HPLC with a Biosep-SEC-s3000 column (Phenomenex, Torrance, CA) using a 200 mM sodium phosphate, pH 7.0 mobile phase.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**ORCID**

Florence Schlosser http://orcid.org/0000-0001-8194-8132

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