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# Quality by design to define critical process parameters for mesenchymal stem cell expansion

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## Abstract

Stem cell-based therapeutic products could be the key to treat the deadliest current pathologies, ranging from neuro-degenerative to respiratory diseases. However, in order to bring these innovative therapeutics to a commercialization stage, reproducible manufacturing of high quality cell products is required. Although advances in cell culture techniques have led to more robust production processes and dramatically accelerated the development of early-phase clinical studies, challenges remain before regulatory approval, particularly to define and implement science-based quality standards (essential pre-requisites for national health agencies). In this regard, using new methodologies, such as Quality By Design (QBD), to build the production process around drug quality, could significantly reduce the chance of product rejection. This review-based work aims to perform a QBD approach to Mesenchymal Stem Cell (MSC) manufacturing in standard two-dimensional flasks, using published studies which have determined the impact of individual process parameters on defined Critical Quality Attributes (CQA). Along with this bibliographic analysis, parameter criticality was determined during the two main manufacturing stages (cell extraction and cell amplification) along with an overall classification in view of identifying the Critical Process Parameters (CPP). The analysis was performed in view of an improved standardization between research teams, and should contribute to reduce the gap towards compliant Good Manufacturing Practice (cGMP) manufacturing.

*Keywords:* Quality by design, Mesenchymal stem cells, Critical process parameters

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## 1 Introduction

Advanced Therapy Medicinal Products (ATMP's) are a class of bio-pharmaceuticals comprised of innovative therapeutics such as gene therapies, tissue engineered products or cell therapies. Notably, the development of genetically engineered T-cells (CAR-T) and stem cell products have triggered a recent wave of applications. For example, over twice as many clinical trials using Mesenchymal Stem Cells (MSC) were registered by the US National Library of Medicine between 2011 and 2015. These cutting-edge products were shown to address a wide range of indications such as bone/cartilage, heart, neuro-degenerative, immune / autoimmune diseases Trounson and McDonald [2015]. More recently, MSC-based products have been shown to be efficient in cases of acute respiratory distress syndrome (ARDS) for example for the treatment of COVID-19 patients Metcalfe [2020], Moll et al. [2020]. However, the distribution of these products by phase was shown not to evolve, indicating the products were not moving out of the clinical pipeline (only 7% of clinical trials passed onto phase 3) Trounson and McDonald [2015], Trounson et al. [2011]. A rapid search of the NIH database (<https://clinicaltrials.gov/>) for MSC-based clinical trials in 2020 seems to indicate a similar distribution, possibly due to incomplete quality standards during early development phases. For example, 73% of interviewed companies producing ATMP's have faced manufacturing and quality assurance issues which may be related to inconsistencies between the laboratory and scale-up phase or issues with quality standard definitions Ten Ham et al. [2018]. Although great progress has been made since 2011 to understand and develop MSC therapies, several lead trials have either undergone early termination or failed to meet the requirements for phase progression Parekkadan and Milwid [2010]. Thus, it seems that important bottlenecks still remain to produce high quality MSC's and pass onto commercial development of these therapies. In this regard, using new tools and methodologies, such as the Quality By Design (QBD) approach, may be an essential foundation to help products achieve commercialization.

## 1. Defining a quality-based approach for cGMP manufacturing

### 1.1. The Quality By Design approach

QBD is a scientific and risk-based approach to product development which is currently flourishing in the pharmaceutical industry Rathore and Winkle [2009]. Using this methodology brings the definition of quality standards early in the process development stages. In this mindset, the QBD method begins by defining the desired product characteristics from a patient and clinical perspective : the Quality Target Product Profile (QTPP) (Figure 1). These requirements are then used to define Critical Quality Attributes (CQA's) which will be necessary and sufficient to guarantee product efficacy and safety. Each CQA should be routinely tested (with rapid, sensitive and reliable procedures Gad [2008]) and stability should be maintained within a defined range Yu et al. [2014]. Once the quality attributes are defined, it is important to understand how the production process will impact these quality attributes. In this regard, the Critical Process Parameters (CPP's) will be determined as well as their defined ranges. Working within these ranges ( *i.e.* the design space) should ensure consistent product quality regardless of the set of parameters used. During routine manufacturing, Ongoing Process Verification (OPV) should then be put in place according to a defined control strategy of these CPP's in order to easily detect shifts or abnormalities which may impact the quality of the final drug product.

### 1.2. Identifying Critical Process Parameters

In order to define and compare the criticality of process parameters, an approach inspired by the Risk Priority Number (RPN) was proposed. The method has typically been used for Failure Mode and Effect Analysis (FMEA) Xiao et al. [2011], Sellappan N [2013], Certa et al. [2017], Khandagade et al. [2013], Harms et al. [2008], Seely and Haury [2005] in order to prioritize potential failures based on their Severity (S), Occurrence (O) and ease of Detection (D). For this, scores between 1 and 10 are defined according to a predefined scale suggested by the IEC 60812 for each factor (S, O and D) and are multiplied to obtain the RPN number. Issues with the highest RPN value are therefore the most critical and should be addressed first.

Using this basis, an "impact score" was determined to evaluate the expected impact of each process parameter on each CQA. A similar strategy has been proposed by the CMC Biotech working group using a five-level scale to evaluate the impact of CQA's in a monoclonal antibody production process case study Group [2009]. Although the attribution of a given impact score was based on available literature and scientific knowledge, it remains, by nature, a subjective quantification. In this study, the scale proposed was reduced to three risk levels for an easier and less subjective assessment (Table 1). Admittedly, three-level hierarchies are common in literature for criticality evaluations Mitchell [2014]. When no literature was available, an impact score of 1.1 was set to account for uncertainty, considering a low parameter impact if little literature exists. Based on RPN calculations in FMEA's, a RPN number for each QTPP attribute was calculated by the product of its factors. However, since not all QTPP attributes were defined by the same number of parameters, a correction factor was applied on all RPN calculations for a better comparability between results. Lastly, an overall RPN calculation was defined, calculated through the average of the RPN calculations of all QTPP attributes in order to define the most critical parameters for the overall quality of cells and to present an overview of the production process as a whole.

## 2. Using Quality By Design for Mesenchymal Stem Cell Manufacturing

### 2.1. Defining the Quality Target Product Profile (QTPP)

In order to understand their therapeutic potential, two categories of cell-based medicinal products using MSC's will be described, on the basis of existing reviews Sharma et al. [2014], Lipsitz et al. [2016] and similar QBD examples Rathore [2009] (Table 2). To begin with, MSC's exhibit tropism for sites of tissue damage Spaeth et al. [2008] and generate local regenerative environments (secretion of anti-inflammatory factors Eggenhofer and Hoogduijn [2012], stimulation of local cell-mediated repair processes Caplan and Correa [2011]). In addition, MSC's have interesting immunomodulatory properties by activating and/or modulating the maturation of various immune cells including T-Lymphocytes Di Nicola et al. [2002], B lymphocytes Corcione et al. [2006], Natural Killer (NK) cells Spaggiari et al. [2006] and dendritic cells Zangi et al. [2009]. However, evidence suggests that these properties may intrinsically depend on the micro environment in which the cells are grafted Han et al. [2019]. For example, excess cytokine production (growth factors, chemokines etc.) or an uncontrolled immunosuppressive effect can stimulate tumor growth Amariglio et al. [2009] or favour an inflammatory response Li et al. [2012]. As a result, sufficient purity and quality of these products will be required for their successful development. In addition, their properties should be preserved as well as their capacity to adapt *in-vivo* to avoid adverse effects.

78 On the other hand, MSC's are able to differentiate into different tissues of mesodermal lineages such  
79 as bone, cartilage, tendon, muscle or fat cells Caplan and Correa [2011]. It has also been suggested that  
80 MSC's can differentiate into ectodermal lineage cells such as neurons or epithelium cells, but also endodermal  
81 lineage cells such as hepatocytes Han et al. [2019]. As a result, cells can be used for *ex-vivo* tissue engineering  
82 using adapted scaffolds such as extracellular matrix scaffolds Agmon and Christman [2016], or on polymeric  
83 scaffolds Caplan [2007]. In this case, the specific and controllable differentiation potential needs to be  
84 maintained. The differentiation may also be of interest *in-vivo*, although MSC differentiation in the acute  
85 phase of injury is unlikely and regeneration is most-likely mediated by their trophic function or cell-cell  
86 contact rather than differentiation.

## 87 2.2. Defining Critical Quality Attributes (CQA's)

88 Building upon previous quality-based approaches for MSC manufacturing Martin et al. [2017], the at-  
89 tributes which affect the QTPP defined in Table 2 are proposed as well as suggested acceptance criteria for  
90 these parameters, mandatory per FDA's Code of Federal Regulations (21-CFR) (Table 3). The acceptance  
91 criteria proposed are based on literature data but should, without any doubt, be adapted to individual  
92 applications.

### 93 2.2.1. Dosage Strength

94 A generalized optimal dose without adverse side-effects is not expected since likely depends on disease,  
95 route of administration, frequency and other parameters Sharma et al. [2014]. It is therefore likely that the  
96 number of cells required at the end of the expansion process will vary and, ideally, the process should keep  
97 flexibility in this regard. The dosage strength will depend, from a process perspective, on cell quantity and  
98 cell viability throughout the process.

99 **Cell Quantity (Qua.)** Final cell quantity can be directly obtained by viable cell counting of the detached  
100 cells after expansion. During the expansion process, this parameter is intrinsically linked to the different step  
101 yields (from initial cell extraction to final detachment phase) as well as the final concentration. Although  
102 it would be interesting to perform the cell culture process in concentrated conditions to reduce equipment  
103 volume, it should be kept in mind that an increased concentration may cause the aggregation of cells or an  
104 increased viscosity which may impact the lot-to-lot homogeneity of the final product or cause needle clogging  
105 during patient administration Amer et al. [2017].

106 **Cell Viability (Via.)** Viability can easily be determined after cell detachment using exclusion staining  
107 such as trypan blue or flow cytometry techniques. In order to achieve robust and cost-effective large-scale  
108 manufacturing, it is important to maintain a high level of viability, typically greater than 90 % all throughout  
109 the process. In certain exceptional cases, a lower limit of 70 % can be acceptable for the finished product  
110 Gad [2008].

### 111 2.2.2. Therapeutic Potency

112 Potency is the specific ability of a product to effect a given result when administered according to defined  
113 posology. This characteristic is influenced by 3 target attributes: identity, differentiation capacity and *in-vivo*  
114 effect for which testing should comply with 21CFR section 600.3 recommendations.

115 **Identity (Id.)** The cells produced during and after expansion must have maintained the characteristics of  
116 MSC's, at the risk of expanding the wrong cell type. In 2006, the International Society for Cellular Therapy  
117 (ISCT) proposed a list of cell markers which can identify MSC's Dominici et al. [2006] and which continues to  
118 be used. It should be noted that there are debates concerning the robustness of ISCT cell markers for MSC  
119 characterization Martin et al. [2017] and that additional RNA's or proteins could be used to complete the  
120 criteria proposed Billing et al. [2016], Mindaye et al. [2013]. In this mindset, the review of the 20 most-used  
121 production markers in 66 MSC-based product submissions to the FDA calls for caution regarding the need  
122 for further characterization, especially for non-bone marrow derived MSC's Mendicino et al. [2014]. It is  
123 likely that the definition proposed is neither exhaustive nor universal.

124 **In-vitro differentiation capacity (Dif.)** MSC differentiation capacity was separated from identity mark-  
125 ers for clarity in the analysis. Although some quantitative descriptions have been proposed for MSC differen-  
126 tiation Vishnubalaji et al. [2012], they globally remain rare. Most articles qualitatively evaluate the capacity  
127 of a sample to undergo differentiation and the proportion of cells which have the tri-lineage differentiation  
128 capacity is, in most cases unknown. However, since routine testing of clonal populations would require long  
129 analysis and delay product release, this CQA has been evaluated in a qualitative manner (ie, sub-populations  
130 of the final cell culture sample must be able to differentiate into the three lineages and variations between  
131 conditions may be visually defined).

132 **Expected in-vivo effect (Viv.)** The last quality attribute impacting potency is the capacity of the cells  
133 to exert their appropriate *in-vivo* effect. Functionality assays are typically measured through *in-vitro* co-

134 cultures with T-cells, for example, but tests should ultimately depend on the target therapy Gad [2008].  
135 Although this attribute is rarely measured, information was included when possible.

### 136 2.2.3. Product quality

137 In order to keep a homogeneous nomenclature between sections, the term drug quality will be used accord-  
138 ing to its use in ICH Q8 guidelines (*i.e.* characteristics required to avoid adverse effects after administration  
139 such as sterility, purity and stability of the product).

140 **Genetic stability (Gen.)** Many studies have shown an increased senescent state after an *ex-vivo* culture of  
141 MSC's, for which replication is limited by the Hayflick limit and telomere length Hayflick [1964]. Generally,  
142 senescent cells display a characteristic large, flattened morphology and are characterized by an irreversible G1  
143 growth arrest involving the repression of genes that drive cell cycle progression and the up-regulation of cell  
144 inhibitors like p53/p21 (primarily due to telomere dysfunction and DNA damage) and p16/RB (oncogenes,  
145 chromatin disruption and various stresses Sethe et al. [2006]). Senescent cells secrete factors that are pro-  
146 inflammatory Kletsas et al. [2004], stimulate tissue aging and tumorigenesis Krtolica and Campisi [2003],  
147 Han et al. [2019]. Although data suggests a higher genetic stability of MSC's compared to other cell sources  
148 such as Embryonic Stem Cells (ESC) or induced Pluripotent Stem Cells (iPSC) Kim and Park [2017], more  
149 routine and thorough genetic phenotyping would be required to further understand these possibly critical  
150 effects. It is still unclear, however, how such data would be interpreted, and what level of genetic abnormality  
151 would be required to disqualify a cell line for clinical use Vazin and Freed [2010]. For simplification, these  
152 problematics have been regrouped into a qualitative attribute called genetic stability.

153 **Cell Purity (Pur.)** The last quality attribute concerns the absence of contamination during the process.  
154 Ideally, sterility testing should be performed after each critical manipulation step during cell culture in  
155 order to detect fungal, bacterial or mycoplasma contamination of the cells. The presence of contaminations  
156 compromises the safety of therapeutic products. Furthermore, the final cell product should be free of other  
157 cell contaminants which could have adverse effects *in-vivo*. For example, the presence of 25-50% of non-  
158 MSC cells within the cell population causes a dose-dependant diminution of differentiation potential *in-vivo* and  
159 *in-vitro* Caplan [2007]. Although this amount of contamination may seem high, and purity is expected to  
160 be higher than 75% at the end of cell culture, it should be kept in mind that, after clinical applications, the  
161 injected MSC's will be diluted with host connective tissue, at a factor which is difficult to predict.

### 162 2.3. Defining Critical Process Parameters (CPP's)

163 Maintaining high quality standards within a production facility requires a rigorous understanding of the  
164 interaction between process parameters and the impact of their potential deviation on product quality (*i.e.*  
165 defined CQA's). In the case of MSC culture, the most common manufacturing technique is composed of two  
166 main stages, each with a set of co-dependent parameters.

#### 167 2.3.1. Cell Extraction

168 To begin with, MSC's are extracted from a suitable donor and an appropriate tissue source. Although the  
169 majority of clinical trials have used cells isolated from Bone Marrow aspirations (BM), extraction protocols  
170 remain invasive and painful. Alternative tissue sources include discarded Umbilical Cords (UC) Margossian  
171 et al. [2012] or lipoaspirate-derived Adipose Tissue (AT) Aust et al. [2004] which offer an easily accessible,  
172 low cost and pain-free source of MSC's. Independently of tissue source, the extracted cell population has  
173 typically been composed of only a small proportion of MSC's (less than 0.05% in the case of BM Guo et al.  
174 [2006]). As a result, cells need to be carefully selected to obtain a homogeneous initial population of MSC's  
175 Han et al. [2019], Tong et al. [2011], Sharma et al. [2014], Oedayrajsingh-Varma et al. [2006], Guo et al.  
176 [2006], Gentry et al. [2007]. The question of which tissue source or extraction method might be optimal  
177 for any given clinical situation is, however, not yet clearly understood due to important quality variations  
178 reported in literature Han et al. [2019], Sharma et al. [2014], De Ugarte et al. [2003], Vishnubalaji et al.  
179 [2012], Yang et al. [2014], Planat-Benard V. et al. [2004]. Lastly, the extraction phase typically ends with a  
180 cryopreservation step for logistic purposes (expansion of the cells is rarely performed at the site of extraction)  
181 Ikebe and Suzuki [2014]. Studies generally tend to show that cryopreservation has little impact on CQA's  
182 and that traditional cryopreservation techniques can be considered robust for pre-clinical trials Steigman  
183 et al. [2008].

184 Overall, the most noteworthy characteristic, at this stage, is the considerable donor-to-donor and intra-  
185 population heterogeneity Christ et al. [2017], Phinney [2012], Vangsness et al. [2015] which is particularly  
186 critical for allogenic therapies since each donor brings a "one to many" associated risk in case of un-detected  
187 infection or functional abnormality Group [2014]. In this regard, donor age seems to be the most important  
188 factor Baker et al. [2015], for example through a decreased quantity of cells extracted was generally lower  
189 in older patients Yang et al. [2014] possibly due to a reduced plastic attachment Kretlow et al. [2008] or to  
190 a lower initial titer of MSC's Caplan [2007] (see Table 4). Evidence also suggested that expression levels

191 of MSC markers may vary upon age Stolzing et al. [2008] as well as an altered differentiation potential  
192 ('adipogenic switch' described in most studies Sethe et al. [2006], Meunier et al. [1971]). In addition, age  
193 seemed to reduce cell growth Baxter et al. [2004], increase doubling times Yang et al. [2014], Choudhery  
194 et al. [2014], reduce replicative lifespan Zaim et al. [2012], Baxter et al. [2004] and increase genetic instability  
195 Baxter et al. [2004], Stolzing et al. [2008]. Since donor age is the only parameter which scored a RPN value  
196 greater than 9 (see Figure 2), it can be considered as the only CPP at this stage. However, the combination of  
197 parameters as a whole, for example patient illness such as diabetes Pierdomenico [2011] or obesity Tencerova  
198 et al. [2019], make it difficult to predict, at a large scale, how many cells can initially be extracted for each  
199 donor and whether the quality and purity of these cells will meet the requirements for clinical applications.

### 200 2.3.2. Cell Amplification

201 After the initial extraction phase, cells undergo an amplification phase using mono-layer flasks (tissue  
202 flasks) of different sizes Ikebe and Suzuki [2014] in order to expand the initial cells to a reproducible target  
203 concentration for administration. This method of expansion is well documented in literature Chen et al.  
204 [2013] but labour intensive due to the required manual passaging of cells between different containers. Fur-  
205 thermore, the risk of contamination increases with the number of container systems and the required number  
206 of passages. This risk can be reduced by limiting the number of containers (through cell stack systems for  
207 example Fekete et al. [2012]), using fully automated processes Thomas et al. [2007] or developing fully closed  
208 production systems Hanley et al. [2014], Zanini et al. [2020], although these options are not yet fully ex-  
209 plored in clinical facilities. Furthermore, in most clinical trials, cells were expanded using defined basal cell  
210 culture media, composed of all the elements required for cell growth such as an energy source (glucose often  
211 supplemented with L-glutamine), essential amino-acids and specific ions (calcium, magnesium, potassium,  
212 sodium etc.) Ikebe and Suzuki [2014]. In addition, these chemically defined media were, in most studies,  
213 completed with 5 to 20% bovine serum or xeno-free alternatives such as Human Platelet Lysate (HPL) Gad  
214 [2008], which provides complementary factors such as hormones, substrate-attachment molecules or binding  
215 proteins Caterson et al. [2002], Schallmoser et al. [2007]. Notably, the choice of supplementation type and  
216 concentration has been shown to impact bone formation capacity Kuznetsov et al. [2000] and immunomod-  
217 ulatory effects Abdelrazik et al. [2011], Bernardo [2010], Nasef et al. [2007]. In parallel, seeding density has  
218 been at the center of various studies Ikebe and Suzuki [2014] but results remain controversial, leading to low  
219 overall RPN scores. The only parameters which scored RPN ratings over 9 (and may therefore be considered  
220 as critical) seem to be the amount of population doublings that cells undergo and the oxygen concentration  
221 used during cell culture (see Figure 2).

222 Cell culture duration can typically be calculated using either the number of passages (usually performed  
223 using enzymatic detachment such as trypsination) or the number of population doublings (PD) that the  
224 cells undergo. The population doublings, rather than the amount of passages, should be considered when  
225 comparing studies due to high variability between research teams in seeding densities and confluence before  
226 harvest. This renders the comparison between cells at a same passage difficult Wagner et al. [2010]. In this  
227 regard, the number of population doublings will determine the maximum amount of cells that can be obtained  
228 through cell culture since proliferation typically remains within the Hayflick limit of around 20-60 population  
229 doublings Wagner et al. [2009], Rombouts and Ploemacher [2003], Schallmoser et al. [2010], Charbord et al.  
230 [2011], Bonab et al. [2006], Noer et al. [2007], Kim et al. [2009]. The most described reason for senescence  
231 during cell culture is a reduced telomere length throughout culture (50 - 100 bp/PD) Sethe et al. [2006],  
232 Bonab et al. [2006], Baxter et al. [2004] associated with very low levels of telomerase activity indicating  
233 cells are not "repairing" the shortened telomeres Kim et al. [2009]. It can be noted that the shift towards  
234 senescence may be gradual over time (possibly though the progressive regulation of genes Schallmoser et al.  
235 [2010], Wagner et al. [2009, 2008] and early signs of senescence (for example through change in morphology  
236 or an increased doubling time) have been observed as early as a few days after culture Banfi et al. [2000],  
237 Larson et al. [2008]. In addition, increase of population doublings has also been associated with a loss of  
238 multipotency Kim et al. [2009], Wagner et al. [2010] as early as 18-20 population doublings Banfi et al. [2000]  
239 as well as reduced immunosuppressive capacities Nasef et al. [2007] or an increased tumorigenicity Kim et al.  
240 [2009].

241 MSC's are naturally present in tissues for which  $dO_2$  concentrations can vary between 1 and 7 % of  
242  $O_2$  saturation depending on the host tissue and tissue vasularization Chow et al. [2001], Fehrer et al.  
243 [2007]. The most common belief is that *ex-vivo* expansion should be performed in conditions close to the  
244 physiological environment from which the cells were extracted, since the amount of oxygen available for cells  
245 will have an impact on cell metabolism. For example, an increased growth has been observed in hypoxic  
246 conditions (1% v/v  $O_2$  in the head-space) Hung et al. [2012] as well as an increased lifespan (3% v/v  $O_2$   
247 in the head-space) Estrada et al. [2012], Fehrer et al. [2007] compared to normoxic conditions. Some authors  
248 propose that cells have a higher susceptibility to cell death at elevated  $O_2$  concentrations for which viability  
249 is reduced Fehrer et al. [2007]. In addition, the differentiation potential seems to be impacted Hung et al.

250 [2012], Zhou et al. [2005], Fehrer et al. [2007] as well as the cell secretome Fehrer et al. [2007] and genetic  
251 aberrations Estrada et al. [2012], Li and Marbán [2010]. These studies tend to show the quality of cells  
252 produced in normoxic conditions may not meet the requirements for clinical use and this parameter should  
253 be continuously monitored. Furthermore, it should be kept in mind that, in the case of culture in T-flasks,  
254 the oxygen concentration available for the cells is difficult to predict as depends on medium height, density  
255 of cells, oxygen consumption, and the incubator characteristics and the concentration is also influenced by  
256 the multiple re-perfusion of room air oxygen concentrations during medium changes, passaging or other  
257 manipulations Csete [2005].

258 Furthermore, it can be noted that there are vast controversies in the published data when evaluating  
259 the impact of these parameters independently. The review of existing data seems to suggest that there are  
260 important interactions between parameters (for example oxygen concentration and nutrient requirements,  
261 or cell age and the impact of population doublings) which are still difficult to predict or analyze, but may  
262 have a critical impact on the quality of the MSC products.

### 263 2.3.3. Overview of the cell culture process

264 Overall process parameter criticality during MSC cell culture was evaluated to identify the most critical  
265 parameters throughout the process (see Figure 3). The data suggests that the most critical parameters (as  
266 a whole, and assuming an identical weight for all of the CQA's) are cell source, donor age, supplementation  
267 type, oxygen concentration and the amount of population doublings that the cells undergo during this phase.  
268 These parameters all exceed the global average RPN limit of 4. In order to meet requirements for quality  
269 specifications, these parameters should typically be controlled during the expansion phase, have science-  
270 based specifications, and their impact should clearly be defined. It is noteworthy, however, that it is difficult  
271 to predict how various independent parameters interact during MSC production processes, in particular with  
272 regard to deviation accumulations. As a result, using additional tools such as Design Of Experiments (DOE)  
273 to detect and understand these interactions may be required to further define the design space of quality  
274 based MSC manufacturing.

## 275 3. Future Challenges

276 Although great progress has been made to understand and develop cell expansion in two dimensional  
277 flasks, these manufacturing processes are not economically viable to supply the predicted requirements for  
278 clinical research of cell therapies and tissue engineering products Rowley [2018]. For larger scale manu-  
279 facturing, new automated technologies Thomas et al. [2007] with greater yields per lot and which reduce  
280 equipment occupation space within production facilities, will be required Merten [2015]. In this regard,  
281 trends are shifting towards 3D cultures using scalable bioreactors Godara et al. [2008], Hoch and Leach  
282 [2014] which are already extensively used in the pharmaceutical industry and are generally well known (on-  
283 line monitoring, maintenance procedures, cleaning validation, etc.). In order to do so, the adherent MSCs  
284 are no longer cultured in planar conditions but using new scaffolds such as microcarriers, hollow fibre or  
285 packed bed systems. In addition, the possibility of a fully closed system significantly reduces contamination  
286 risks, equipment volume, and will ultimately reduce costs of GMP production Zanini et al. [2020]. We should  
287 however note that data concerning MSC cell culture in bioreactors still remains scarce and fine-tuning of pro-  
288 cess parameters to increase quality and yield is still in early stages of research. Only one clinical trial using  
289 bioreactor-based expansion of MSCs seems to have been published (NCT00919958 Prather et al. [2009], key  
290 words "Mesenchymal Stem Cell" and "Bioreactor" in <https://clinicaltrials.gov/>). Understanding the  
291 physical and biological mechanisms as well as their kinetic evolution during the different cell culture stages  
292 will help unlock current bottlenecks towards quality driven protocols. Using similar QBD methodologies,  
293 online monitoring data, and modelization techniques (reviewed in Wyrobnik et al. [2020]) during early-phase  
294 process development could be the key to a successful scale-up process and approved commercialization by  
295 health authorities.

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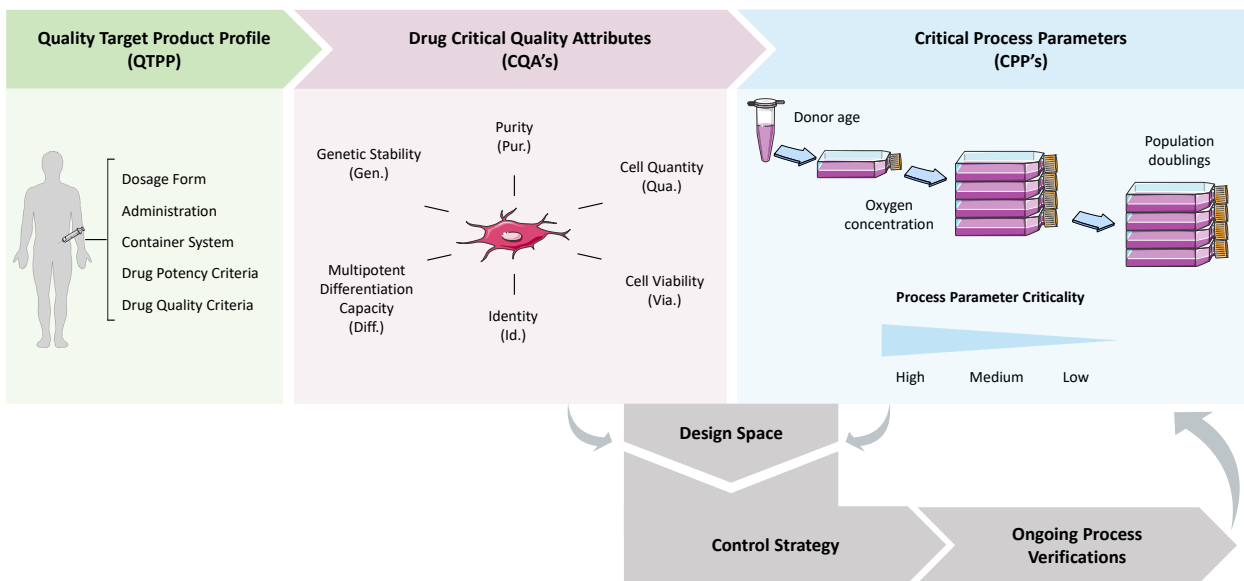
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636 **Author contributions statement**

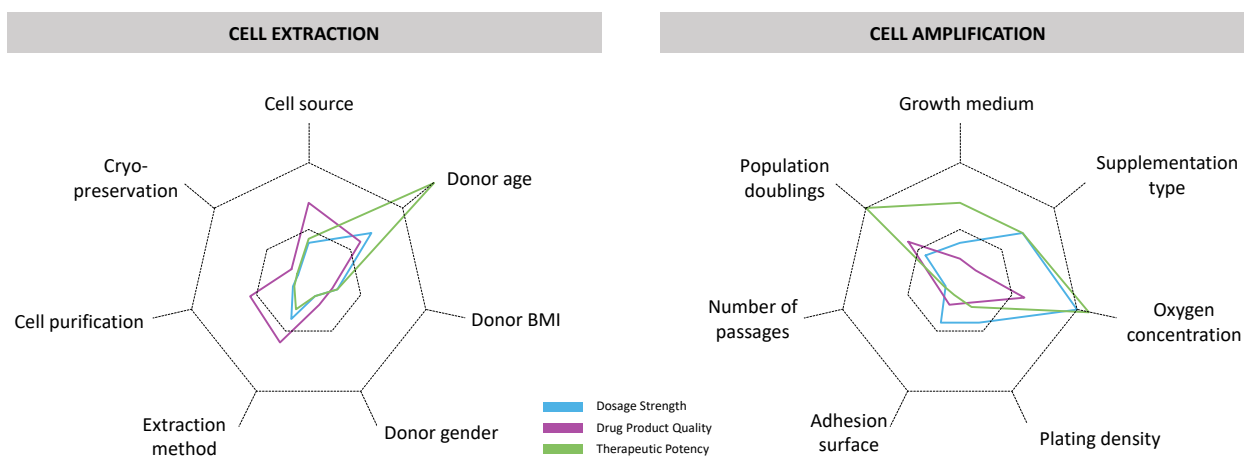
637 C.M. Writing - Original Draft, C.S., D.T., N.I. and E.O. Writing - Review and Editing.

638 **Competing interests**

639 The authors declare no competing interests.

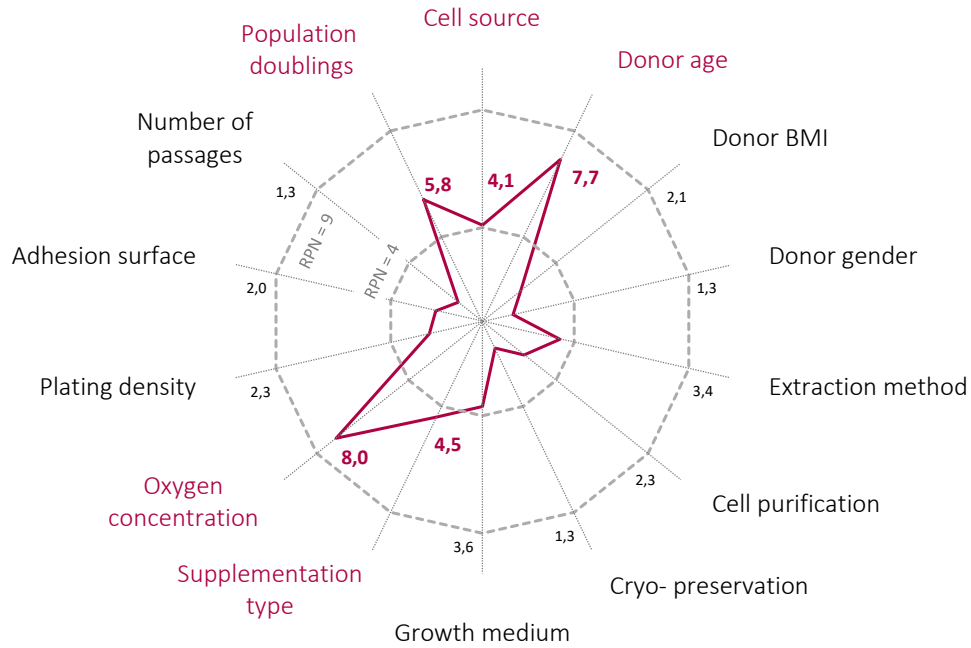


**Figure 1: Overview of the Quality By Design process for Mesenchymal Stem Cell manufacturing.** Drug product characteristics are described in the quality target product profile according to patient requirements in order to identify critical quality attributes which will be sufficient to guarantee product safety and efficacy. The identification of critical process parameters and their impact on the defined CQA's leads to the definition of the design space within which manufacturing processes can be performed without altering product quality. The definition of a control strategy and ongoing process verifications are then required to monitor the process and detect potential deviations during the process.



**Figure 2: Impact of cell extraction and amplification parameters on the Quality Target Product Profile attributes.** All parameters in the inner zone have a Risk Priority Number rating below 4 (possible impact on two Critical quality Attributes). All parameters outside the exterior zone have Risk Priority Number ratings above 9 (proven impact on two Critical Quality Attributes).





**Figure 3: Impact of process parameters throughout MSC manufacturing.** All parameters in the inner zone have a global Risk Priority Number rating below 4 (low criticality). All parameters outside the exterior zone have Risk Priority Number ratings above 9 (high criticality).

**Table 1:** Impact score used for Risk Priority Number calculations.

Impact	Criteria	Score
Low	No discernable effect on CQA, homogeneous results published in literature	1
Low	No results published in literature	1.1
Medium	Unknown effect on CQA due to heterogeneous results published in literature	2
High	Known effect on CQA, homogeneous results published in literature	3

**Table 2:** Generic Quality Target Product Profile for Mesenchymal Stem Cell therapy.

ICH Q8 recommendation	Undifferentiated cell transplantation	In-vitro differentiation
Intended use in clinical setting (e.g., route of administration, dosage form, delivery systems)	Allogenic or autologous MSC transplant at the site of injury	Transplantation of differentiated autologous tissues
Dosage strength(s)	$1-2 \times 10^6$ cells/patient kg	N.A. (as necessary for tissue constructs)
Container closure system	Frozen product for injection	Reconstructed <i>in vitro</i> tissue
Therapeutic potency and attributes affecting pharmacokinetic characteristics	Immunomodulatory properties through secreted biomolecules and/or cell-cell contact after MSC homing mechanisms	Specific and controlled differentiation; functional <i>in vivo</i> accepted tissue graft
Drug product quality criteria (e.g., sterility, purity, stability and drug release)	Fully sterile ; absence of external contaminations ; Frozen stability ; high viability after thawing	Sterility ; <i>In vivo</i> stability ; low tissue immunogenicity

**Table 3:** The definition of Critical Quality Attributes for Mesenchymal Stem Cell therapy based on the defined Quality Target Product Profile.

QTTP	CQA	In-process parameter	Acceptance criteria	Ref.
Dosage Strength	Final Cell Quantity (Qua.)	Individual step yield	(>85 %)	
		Expansion rate	As high as possible	
		Concentration	As high as possible without aggregation or significantly increasing viscosity	Amer et al. [2017]
	Viability (Via.)	Cell viability	>90 %	Lipsitz et al. [2016], Steigman et al. [2008], Gad [2008], Belotti et al. [2015]
Therapeutic Potency	MSC Identity (Id.)	Adherence to plastic	Yes	Dominici et al. [2006]
		Specific positive antigen expression $\geq 95\%$	CD105+, CD73+, CD90+	
		Specific negative antigen expression $\leq 2\%$	CD45-, CD34-, CD14- or CD11b-, CD79 $\alpha$ - or CD19-, HLA-DR-	
	Multipotent Differentiation (Dif.)	Osteoblast, Adipocyte and Chondroblast	Yes ( <i>in-vitro</i> )	Dominici et al. [2006]
<i>In Vivo</i> Effect (Viv.)	To be defined for each application <i>Ex: Inhibition of T-Cell growth in co-cultures</i>	<i>Not Applicable.</i>	Nauta and Fibbe [2007] Gad [2008]	
Product Quality	Genetic stability (Gen.)	Karyotype	23 Pairs of Chromosomes	
		Human Telomerase Reverse Transcriptase activity (hTERT) / Telomere length	High expression of hTERT	Shi et al. [2002]
		<i>in-vivo</i> tumour formation (in mice)	Absent	Gad [2008]
		Morphology	small, spindle-form	Sethe et al. [2006]
	Purity (Pur.)	Sterility testing for each lot	$\leq 10$ CFU/mL for test organisms	Steigman et al. [2008], Gad [2008]
		Mycoplasma assay for each lot	No detectable Mycoplasma (by PCR)	
		Endotoxin assay for each lot	$\leq 5.0$ EU/mL (Limulus amoebocyte lysate LAL)	Gad [2008]
	Cell purity	$\geq 70$ %	Belotti et al. [2015], Lennon et al. [2000]	

**Table 4:** Process parameters during cell extraction and cell expansion and their impact on Critical Quality Attributes. For each parameter, an impact score between 1 and 3 was attributed based on literature and according to Table 1.

Parameter	Range	Strength		Id.	Potency Dif.	Viv.	Quality	
		Qua.	Via.				Gen.	Pur.
Cell Source	BM, UC, AD	3 Kern et al. [2006], De Ugarte et al. [2003]	1 Vishnubalaji et al. [2012]	1 Wang et al. [2004], Kern et al. [2006], Aust et al. [2004], Zaim et al. [2012], Vishnubalaji et al. [2012]	3 Kern et al. [2006], De Ugarte et al. [2003], Zuk et al. [2002], Yang et al. [2014], Bieback et al. [2004], Vishnubalaji et al. [2012]	1.1	2 De Ugarte et al. [2003], Kern et al. [2006]	2 Gronthos [2003], Zuk et al. [2001]
Age	< 10 to > 70	3 Kretlow et al. [2008], Phinney et al. [1999], Dimitriou et al. [2008], Choudhery et al. [2014], Stolzing et al. [2008], Baker et al. [2015], Sethe et al. [2006]	2 Stolzing et al. [2008], Choudhery et al. [2014]	2 Stolzing et al. [2008], Kretlow et al. [2008], Dimitriou et al. [2008], Dimitriou et al. [2008], Baker et al. [2015]	3 Kretlow et al. [2008], Yang et al. [2014], Baxter et al. [2004], Stolzing et al. [2008], Baker et al. [2015], Wagner et al. [2009]	2 Rauscher Frederick M. et al. [2003], Dressler et al. [2005], Bruna et al. [2016] Golpanian et al. [2015]	3 Baxter et al. [2004], Stolzing et al. [2008], Stenderup et al. [2001]	1.1
BMI	< 25, 25-30, > 30	2 Yang et al. [2014], Aust et al. [2004]	1.1	1 Yang et al. [2014]	2 Yang et al. [2014]	1.1	1.1	1.1
Gender	Man, Woman	1 Yang et al. [2014] Phinney et al. [1999]	1.1	1 Yang et al. [2014]	1 Yang et al. [2014]	1.1	1.1	1.1
Extraction	Various protocols	3 Wan et al. [2006], Caterson et al. [2002], Sotiropoulou et al. [2006b], Phinney et al. [1999], Oedayrajsingh-Varma et al. [2006]	1 Wan et al. [2006]	1.1	1 Wan et al. [2006], Caterson et al. [2002]	2 Wan et al. [2006]	1.1	3 Pountos et al. [2007]
Purification	Antigen, MACS, FACS	1.1	1.1	1 Gentry et al. [2007]	1 Simmons and Torok-Storb [1991], Gentry et al. [2007]	1.1	1 Gronthos [2003]	3 Simmons and Torok-Storb [1991], Gronthos [2003], Guo et al. [2006], Gentry et al. [2007]
Cryopreservation	Yes, No	1 Kotobuki et al. [2004], Stolzing et al. [2008]	1 Steigman et al. [2008], Kotobuki et al. [2004], François et al. [2012]	1 Kotobuki et al. [2004]	1 Kotobuki et al. [2004]	1.1	1 François et al. [2012]	1.1
Growth medium	Various	3 Sotiropoulou et al. [2006a], Sotiropoulou et al. [2006b]	1 Pera et al. [2000]	1 Sotiropoulou et al. [2006a]	2 Sotiropoulou et al. [2006a], Pera et al. [2000]	3 Sotiropoulou et al. [2006a], Sotiropoulou et al. [2006b]	1.1	1.1
Supplementation type	FBS, HPL (etc.)	3 Kuznetsov et al. [2000], Zuk et al. [2001], Nasef et al. [2007], Bernardo [2010], Lange et al. [2007], Schallmoser et al. [2007], Kong et al. [2019]	2 Kong et al. [2019]	1 Doucet et al. [2005], Bernardo [2010], Lange et al. [2007], Schallmoser et al. [2007], Kong et al. [2019]	2 Caterson et al. [2002], Zuk et al. [2001], Dimitriou et al. [2008], Doucet et al. [2005], Bernardo [2010], Lange et al. [2007], Schallmoser et al. [2007]	3 Kuznetsov et al. [2000], Nasef et al. [2007], Doucet et al. [2005], Bernardo [2010], Abdelrazik et al. [2011], Schallmoser et al. [2007]	1 Bernardo [2010], Lange et al. [2007], Kong et al. [2019]	1 Bernardo [2010]
Plating density	$10^{-1}$ - $10^5$ MSC's /cm <sup>2</sup>	3 Ikebe and Suzuki [2014], Colter et al. [2000], Sekiya et al. [2002]	1.1	1 Sotiropoulou et al. [2006b]	2 Sekiya et al. [2002], Sotiropoulou et al. [2006b]	1 Sotiropoulou et al. [2006b]	1.1	1 Colter et al. [2000]
Adhesion surface	Varied	3 Sotiropoulou et al. [2006b]	1.1	1 Sotiropoulou et al. [2006b]	1 Sotiropoulou et al. [2006b]	1 Sotiropoulou et al. [2006b]	1.1	1.1
Oxygen	2-21% O <sub>2</sub>	3 Hung et al. [2012], Estrada et al. [2012], Fehrer et al. [2007]	3 Fehrer et al. [2007]	1.1	3 Hung et al. [2012], Fehrer et al. [2007], Fink et al. [2004], Zhou et al. [2005], Csete [2005]	3 Hung et al. [2012], Fehrer et al. [2007]	3 Hung et al. [2012], Estrada et al. [2012], Fehrer et al. [2007], Li and Marbán [2010]	1.1
Passages	1 - 15	1 Zuk et al. [2001]	1.1	1 Schallmoser et al. [2010]	1 Zuk et al. [2001], Conget and Minguell [1999]	1.1	1 Zuk et al. [2001], Conget and Minguell [1999], Schallmoser et al. [2010]	1.1
Population doublings	up to 50 PD	3	1.1	1 Nasef et al. [2007], Bonab et al. [2006], Kim et al. [2009], Bork et al. [2010]	3 Colter et al. [2000], Sekiya et al. [2002], Bonab et al. [2006], Banfi et al. [2000], Bork et al. [2010]	3 Nasef et al. [2007], Kim et al. [2009], Banfi et al. [2000]	3 Bonab et al. [2006], Baxter et al. [2004], Larson et al. [2008], Wagner et al. [2009], Bork et al. [2010]	1.1