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Future Perspectives of Bone Tissue Engineering with Special Emphasis on Extracellular Vesicles

Kaur, Sippy

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Future perspectives

Sippy Kaur[1], Bettina Mannerström[1], Karin Ekström[2] and Riitta Seppänen-Kaijansinkko[1]

1) Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.
2) Department of Biomaterials, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Sippy Kaur, Adjunct Professor, PhD
Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, PO Box 63, 00014 University of Helsinki, Finland
email: sippy.kaur@helsinki.fi

Bettina Mannerström, Adjunct Professor, PhD
Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, PO Box 63, 00014 University of Helsinki, Finland
email: bettina.mannerstrom@helsinki.fi

Riitta Seppänen-Kaijansinkko, Professor, MD, DDS, PhD
Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, PO Box 41, 00014 University of Helsinki, Finland
email: riitta.seppanen-kaijansinkko@helsinki.fi

Karin Ekström, PhD
Department of Biomaterials, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Box 412, 40530 Gothenburg, Sweden
email: karin.ekstrom@biomaterials.gu.se

Tissue engineering therapies to repair or regenerate tissues are based on cells, genes, growth factors, and scaffolds. Currently, bone tissue engineering based on mesenchymal stem cells (MSCs), an osteoinductive scaffold and potentially also a growth factor are applied as a promising strategy for restoring critical-size bone defects and accelerating bone regeneration [1-3]. This cell based approach has several limitations, including time-consuming cell culturing in addition to low homing and survival rates of the transplanted cells[4] Moreover, recent studies have demonstrated that therapeutic effects of MSCs in tissue engineering are facilitated via paracrine mechanisms[5,6] exerted by the cells rather than the cells themselves forming new tissue. Therefore cell-free EV based therapy appears to

be a promising strategy for bone tissue engineering, as EV-based therapy in contrast with stem cell transplantation has several advantages, such as high stability, low immunogenicity[7] and circumventing complications related with cell transplantation, such as embolization. A paradigm shift in the field of bone tissue engineering is beginning to emerge with stem derived EV based therapy.

EVs are diverse nano-sized bilayer membranous vesicles which are mainly responsible for mediating local and systemic responses (Figure 1). They are secreted by most cell types and are found in biological fluids such as saliva, urine, nasal and bronchial lavage fluid, amniotic fluid, breast milk, plasma, serum and seminal fluid[8] (Figure 2). As EVs are known to be involved in both physiological as well as pathological processes, interest in their biological roles and its clinical application is expanding[9] The International Society for Extracellular Vesicles (ISEV), responsible for establishing guidelines for EV research, has suggested the term EV for all the membranous vesicles (exosomes, microvesicles, apoptotic bodies, outer membrane vesicles etc.) released by both prokaryotic and eucaroytic cells. All these vesicles have their own specific biogenesis pathways, size, cargo sorting mechanisms and biological function.[8]

Depending on their cell of origin, EVs contain complex bioactive cargo including proteins, mRNAs, miRNAs, DNAs and lipids. Even though the composition of EV protein is different among cell types, certain proteins, such as the tetraspanins CD9, CD63, and CD81 and proteins from the endosomal compartment, Tsg101 and Alix are enriched in EVs, and these proteins in addition to others are used for EV characterization and identification. Data on the nucleic acid, protein and lipid compositions of EVs from various sources is deposited in the database such as Vesiclepedia (www.microvesicles.org) and EVpedia (http://www.evpedia.info). Stem cell derived EVs are reported to show regenerative effects in different disease models, such as in myocardial ischemia, acute kidney injury, neurological disorders, and wound healing[10] (Table 1). Thus, in-depth

investigation on EV cargo could provide insights into the multiple mechanistic pathways involved in the EV mediated regenerative effects, and future approaches for the treatment of diseases.

MSCs are efficient producers of EVs and by delivery of bioactive molecules the EVs have been shown to mimic the therapeutic effects of MSCs.[11] MSC-EVs have also been shown to play important roles in various physiological activities such as immunomodulation, tumorigenesis, angiogenesis, and wound healing.[12-14] Specifically, MSC-derived EVs inhibit immune reactions in a similar fashion as MSCs, through reduction of inflammatory cytokines and increasing of anti-inflammatory responses[15]. Mechanisms for this mode of action is still not clearly understood. In the study where stem cells and their EVs were studied in parallel, EVs treatment showed similar or even superior therapeutic capacity.[16]

Besides the EVs quality control aspect, another highly important criteria for application of EV therapies is the sufficient amount of EVs. Among different sources of MSCs, adipose derived stem cells are considered abundant and easily accessible source of cells, which can produce scalable amounts of EVs. For tissue engineering applications, EVs from stem cells have shown to have several inherent advantages, such as a) showing expression of several adhesion molecules such as integrins [17]also binding ability to matrix proteins such as type I collagen and fibronectin[18]; b) possessing potent pro-angiogenic capacity both in vitro and vivo, through enrichment in angiogenesis-related growth factors, mRNAs and miRNAs[19,20]; c) ability to induce osteogenesis, by communication between mineralizing osteoblasts and stromal cells in the bone microenvironment; d) ability to be manipulated, to become enriched with eg. mRNAs, miRNAs or other biomolecules that favor tissue regeneration. Current research highlights that MSC-EVs play important roles in angiogenesis, wound healing, regulate osteoblast activity and differentiation and also promote bone regeneration in vivo[22-24]. While the effects of MSC-derived EVs on bone regeneration are not yet clarified, evidence points towards

their promoting effect on osteogenesis being due to the a) protective effect reducing apoptosis in necrotic environment[25]; b) induction of angiogenesis to promote vascularization; c) osteoinductive effect to directly promote the osteogenic differentiation of MSCs[26]. Application of EVs for the treatment of osteochondral and bone-related diseases are shown in table 2. Source of EVs for these diseases were stem cells from various sources such as mesenchymal, synovial, bone marrow and embryonic stem cells.

In a study by Martins et al, concluded that irrespective of their parent cell type or osteogenic induction method,[27,28] EVs in vitro can only promote, rather than complete the process of osteoblastogenesis and also displayed strong proliferative, migrative and chemotactic effects. Additionally, only brief osteoinductive stimulation was needed to produce EVs with osteoinductive capacity which significantly promoted the osteogenesis, which was verified by mineralized nodules, and the gene expression of osteogenic genes such as *RUNX2, ALP*, and *COL1A1*. Since the osteoinductive effect was not increased by extended osteogenic stimulation, the results point towards briefly osteoinduced EVs having capacity to be further developed into clinically applicable alternative tissue engineering to repair bone defects.

An important issue for the use of EVs in the clinic is the mode of administration. Optimal scaffold for EV based bone tissue engineering should be biocompatible, provide temporary physical support, and most importantly, having ability of releasing EVs in a controlled manner to ensure their sufficient duration required to achieve the functional effect. Recently it was demonstrated[29,30] that EVs immobilized on PLGA/pD (polylactic-co-glycolic acid/ polydopamine-coating) scaffolds retained the EVs and enabled their slow and local release in mouse critical-sized calvarial defect. The study indicated that PLGA/pD scaffold is a convenient, viable, and efficient carrier for the release of EVs, resulting in significantly more new bone tissue in vivo.

For successful bone regeneration, vascularization plays a vital role in healing of damaged bone tissue. In a study by Xie et al, scaffolds coated with stem cell EVs were shown to promote bone regeneration in vivo by accelerating vascularization[19], whereas no major effect was seen on proliferation, apoptosis and osteogenesis, indicating that scaffold modification by EVs provides a promising method to promote vascularization, vital for succesful bone tissue engineering. In another study, bone repair and regeneration in rat model of calvarial bone defect was demonstrated when beta tricalcium phosphate scaffold was functionalized with stem cell-derived EVs[31]. The underlying mechanism for the bone regeneration may be the activation of endogenous bone marrow stem cells via EVs present in the scaffold. Polyethyleneimine and poly(lactide) scaffolds complexed with stem cells EVs from human gingiva also contributed to regenerating bone defects induced in rat calvaria.[32] Taken together, all these studies suggests that EVs in combination with scaffolds contribute to osteogenic priming and enhanced vascularization in critical-size bone defects. Therefore, as shown in Figure 3, in the coming years, further research and development of EVs with new or already existing biomaterials will potentially lead to emergence of novel therapeutic alternatives in the field of bone tissue engineering

For the transition of EV-based therapies from animal models to clinical therapy, EV isolation and characterization methods need to be standardized. Currently, there are several methods such ultracentrifugation, density gradient, ultrafiltration, immuno-affinity, precipitation and microfluidics, which are used for EV isolation. Among these, ultracentrifugation is the most widely used method.[33] For EV characterization, combination of different methods such as particle analysis (Nanosight Tracking Analysis, NTA), Advanced Flow Cytometry, dynamic light scattering (DLS), Western blotting and electron microscopy are most commonly used. NTA determines the particle size distribution and number, Western blotting quantifies the protein expression of EVs and electron microscopy is utilized for visualization of EVs. Other ways to establish the EV dose may be by ELISA to measure concentration of EV markers or by using cell equivalents (donor cell number).

ISEV recommends that EVs can be stored in -80C, but the optimal storage conditions of EVs for clinical applications is yet to be determined, as loss of function of EVs after defreezing has been reported[34]. Freeze-drying of EVs could improve their stability at higher temperatures, without significant loss of their biological activities[35]. It is still unclear if all subpopulations of EVs can be freeze-dried by same method and what the maximum shelf-life of the freeze-dried EVs is. For clinical applications, further research is needed to determine the best method for EV isolation, their functional characterization and the optimal storage condition.

EVs have attracted attention in the field of targeted drug delivery and have a demonstrated potential to be used as efficient and functional delivery systems. Compared to synthetic nanoparticles, EVs exhibit important advantages, such as 1) membrane modifications for specific cell targeting, 2) drug loading for targeted therapy, 3) small size enabling them to escape from lung clearance and pass through the blood-brain barrier, 4) potential delivery vehicles as they are easily scaleable to produce large quantities of EVs and would not elicit immune rejection response or adverse effect[36]. Route of EV administration could be intravenous injection, subcutaneous/intravenous, intranasal and in situ injection.

Since the EV cargo contains the information about the pathophysiological conditions of the host cell, they are considered an important tool for biomarker discovery and to monitor the disease status. Therefore genomics, proteomics and lipidomics profiling of EV cargo for the disease in question is fundamental for the detection of new biomarkers. RNAs and proteins in EVs have been found to be useful in monitoring a wide range of diseases. For example, EVs purified from the urine of prostate cancer patients showed elevated levels of prostate cancer antigen-3 (PCA-3), which can be used to monitor the status of the disease[37]. Elevated expression of cancer biomarkers in EVs were also detected in cancers such as gastric cancer, glioblastoma and lung cancer.[38,39]

EVs from stem cells as a therapeutics are already entering clinical trials and several trials are ongoing (Figure 4) where EVs are used as a diagonistic tool or biomarker. Clinical application of EVs is yet to be validated as trials are in very early phases; very few studies are undertaken or are currently ongoing as shown in table 3. Across these studies, a variety of EV isolation and purification methods were applied, which could affect the consistency of the results. In addition to obstacles in EV isolation methods, there remain several other challenges in successful implementation of EVs into clinical use, in particular, detailed characterization of subpopulation EVs, scalable EV production methods to collect uniform EVs and their storage conditions. There are still several unanswered questions before EVs can be exploited fully a novel standardized therapeutic tool.

To receive sufficient amounts of EVs, production scale-up is necessary. This may be performed using bioreactors as well as by stimulation of the donor cells. Cell culturing condition need also to be standardized, since slight changes in the cell culture will affect the release, composition and function of the EVs. Many different EV isolation methods are used, and new novel methods are emerging. They all have their advantages and disadvantages (e.g. high/low yield, high/low purity, large-scale/small-scale, short/long working time) and there is no method that is optimal for all different types of experiments.[40] However, for future isolation of clinical-grade EVs, it is desired to have a reproducible, large-scale method, resulting in high-yield of functional EVs. Furthermore, many parameters including storage temperatures, buffers, repeated freezing-thawing cycles may all affect the EV quality and quantity and thus needs to be evaluated further to find the optimal condition. Furthermore, it is important to note that different EV isolations methods may result in in the isolation of different populations of EVs. It might only be some subtype of EV that is necessary to obtain the desired effect and this will need to be assessed further in specific cases, and methods to isolate specific sup-types of EVs further developed.

The global market for EV diagnostics and therapeutic companies is projected to grow from $16.1 million in 2016 to 111.8 million in 2021[41]. Several companies (Aethlon, Exosomess Sciences, Anosys Inc, Capricor Therapeutics Inc, Caris Life Sciences, Codiak Biosciences, Exosomes Diagnostics, Exovita Biosciences, ReNeuron and Systems Biosciences) are conducting research and development that aim to develop EV based therapeutics or diagnostic biomarkers (Table 4). To date, at least 4 companies (ReNeuron, Capricor and Aegle Therapeutics, Codiak Biosciences) are developing commercial use of EVs. Beneficial applications of EVs in cardiac and muscle disease is undertaken by Capricor Inc. ReNeuron Group PLC are focusing on neurological and ischemic conditions. Application of EV-based vaccines in cancer therapy is developed by Anosys Inc.

From a regulatory and medicinal usage perspective, reasons for using MSC-EVs instead of cells are that they have the beneficial effects as the cells but the easier handling and storage, better stability and possibilities to sterilize by filtration (most EVs are <200 nm). Aspects that require develoment before EVs can be applied in the clinic are related to the controlled manufacturing of sufficient amounts of EVs; which includes the cell culture, EV isolation and storage, quantification, determination of the composition and purity/contamination as well as *in vitro* potency tests [10]. For MSC EVs, a proliferation assay might be suitable since MSC EVs All this will need to be assessed for every batch of EVs that will be later used clinically. EV-based therapeutics will most likely be classified as Biological Medicinal Products and belong to the pharmaceutical class of biologicals (tissue- and cell-based products regulation may be used). Already now, the regulatory framework exist for manufacturing and clinical trials of EVs in Europe, Australia, and United states. A hurdle in EV-based therapeutics may be establishing the Mode of action (MoA), since the EVs consists of a complex composition of molecules, and most likely a combination of molecules is responsible for the MoA.

Concluding remarks: EVs are novel players in cell communication systems and mediators of horizontal transfer of bioactive cargo in gene regulation. Their molecular composition, function, and targeting mechanisms is still a young research field. Therefore, demanding new high resolution technological advances for large scale standardized GMP-grade production, characterization, storage and safety issues needs to be also addressed for subsequent clinical trails. Due to its biological and regulatory complexity, more extensive research on the application of EVs in clinical and therapeutic advancement are warranted.

Table 1. EV based approaches used on animal model for the treatment of the different disease (modified from Willis et al, 2017[10])

Disease Model	Animal model	References
Respiratory		
Bronchopulmonary dysplasia	Mouse	[48]
Pulmonary hypertension	Mouse	[49,50]
Acute lung injury	Mouse	[51]
Silicosis	Mouse	[14]
Pneumonia	Mouse	[52]
Cardiovascular		
Myocardial infarction	Rat	[53-55]
Ischemia/reperfusion	Mouse	[56,57]
Neurological		
Traumatic brain injury	Mouse	[58]
Laser-induced retinal injury	Mouse	[59]
Optical nerve crush	Rat	[60]
Stroke	Rat and Mouse	[61,62]
Musculoskeletal		
Cardiotoxin injury	Mouse	[63]
Hepatic		
Drug-induced liver injury	Mouse	[64]
Liver fibrosis	Mouse	[65]
Gastrointestinal		
Colitis	Rat and Mouse	[66,67]
Dermatological		
Wound healing	Rat and Mouse	[67,68]
Renal		
Ischemia/reperfusion	Rat	[69]
Acute kidney injury	Mouse	[70]

Table 2: EVs used as therapeutic agents in animal models of osteochondral and bone related therapies

Therapeutic agents	Animal model/*in vitro* cell model	References
Osteoarthritis	Rat	[71]

Endochondral ossification in bone fracture	Mouse	[72]
Osteochondral defects	Rat	[31]
protection from cartilage and bone degradation	mouse	[73]
Inflammantion in the synovia	Mouse	[74]
Rheumatoid arthritis	Mouse	[75]
Osteoporosis	Rat	[26]
Femoral head necrosis	Rabbit	[30]
Bone defects	Rat and mouse	[30,31]
Bone regeneration model	Mouse and *in vitro*	[76-78]

Table 3: EV based clinical trials (modified from Willis et al, 2017[10])

Disease	**Phase**	**EV source**	**Status**	**Reference**
Melanoma	I-open label	Autologous monocyte-derived dendritic cells	Complete	[42]
Non-small cell lung cancer	I-open label	Autologous monocyte-derived dendritic cells	Complete	[43]
Colon cancer	I-open label	Autologous ascites	Complete	[44]
Colon cancer	I-open label	Plant based	Ongoing	NCT01294072
Type I diabetes	I-open label	Allogenic umbical cord blood MSC	Ongoing	NCT02138331
Non-small cell lung cancer	II-open label	Tumor cell	Complete	[45]
Wound healing (Ulcer)	I-open label	Autologous plasma	Enrolling	NCT02565264
Cerebrovascular Disorders	I/II	Allogenic Mesenchymal Stem Cell Derived Evs	Not yet recruiting	NCT03384433
Stage IV Pancreatic Cancer	I	Mesenchymal Stromal Cells-derived Evs with KRAS G12D siRNA	Not yet recruiting	NCT03608631
Macular Holes	Early phase I	EVs derived from mesenchymal stem cells	Recruiting	NCT03437759
Polycystic Ovary Syndrome	NA	Plant EVs	Not yet recruiting	NCT03493984
Graft versus host disease	NA	Allogeneic MSC-EVs	Complete	[46]

Table 4: List of companies on EV based products and services (modified from Gimona et al[47])

Name of the company	**Therapeutic target**	**Web address**
Anjarium Biosciences	Broad range of severe diseases	http://anjarium.com/
Aposcience AG	Stroke, spinal cord injury, skin lesions, acute and chronic myocardial infarction	http://www.aposcience.at/the secretome-company/
Capricor Therapeutics	Cardiovascular and non-cardiovascular diseases	http://capricor.com/

Codiak Biosciences	Pancreatic cancer	http://codiakbio.com/
Evox Therapeutics	Inflammatory and neurological diseases	http://www.evoxtherapeutics.com/
ExoCyte Therapeutics	Cancer	http://exocytetherapeutics.com/
Exogenus Therapeutics	Skin lesions	http://www.exogenus-t.com/
Exovita Biosciences	cancers	http://exovitabio.com/
Kimera Labs	Orthopedic, cosmetic and regenerative medicine applications	http://kimeralabs.com/
ReCyte Therapeutics	Vascular disorders	http://recyte.com/
ReNeuron	Neurologic and ophthalmologic disorders	http://reneuron.com/
Stemedica Cell Technologies, Inc.	Cardiovascular diseases, traumatic brain injury and alzheimers disease	http://stemedica.com/
ZenBio	Skin lesions	http://www.zen-bio.com/

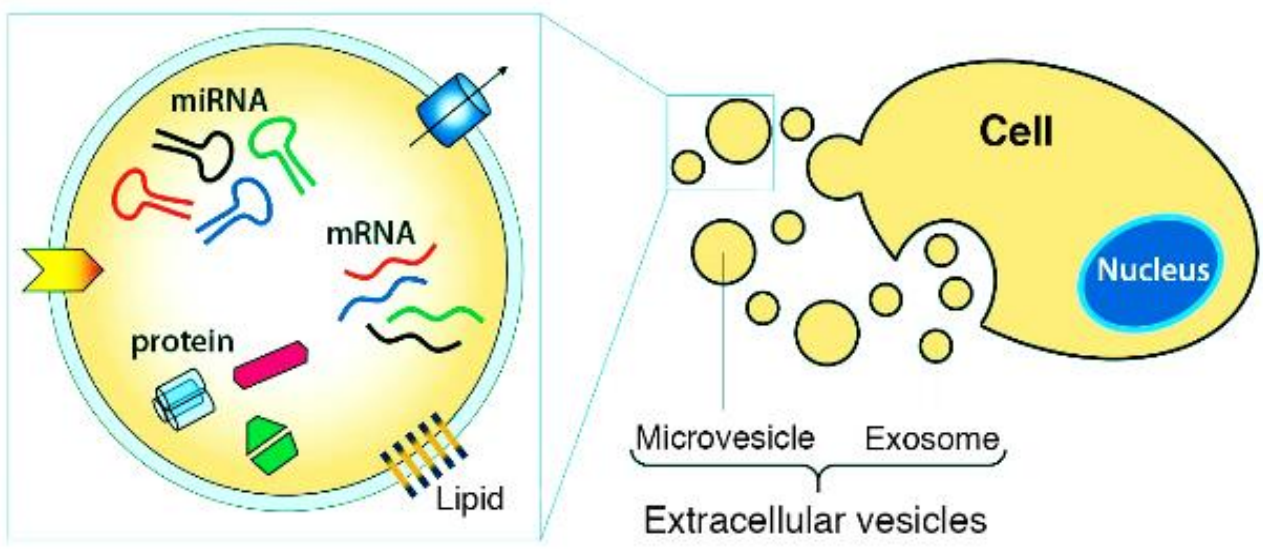

Figure 1: EV anatomy. Nano-sized membrane bound EV released from the parent cell. Cargo of EV includes protein (both cytosolic and membrane bound) , lipids and RNA molecules. Figure modified from (http://www.bioprocessintl.com/manufacturing/cell-therapies/extracellular-vesicles-commercial-potential-as-byproducts-of-cell-manufacturing-for-research-and-therapeutic-use/)

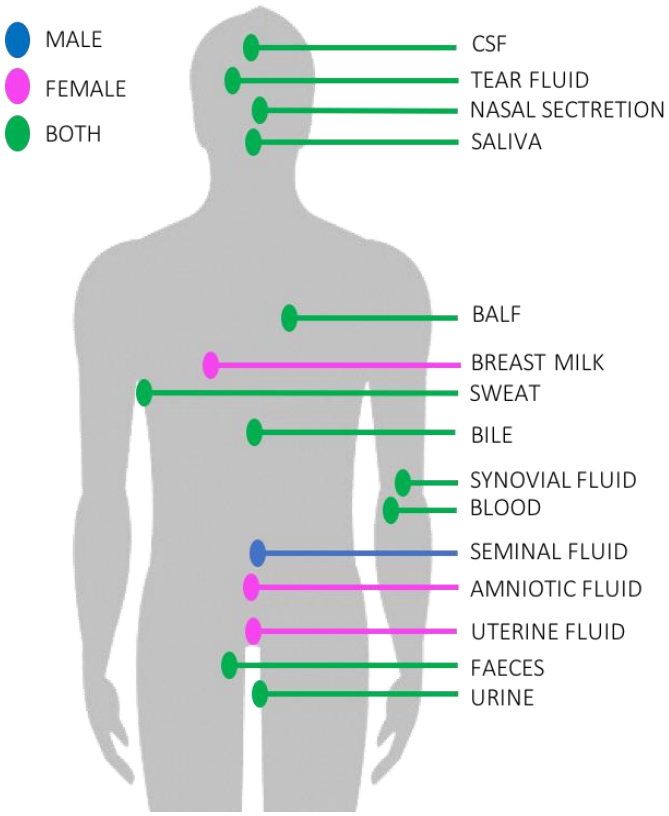

Figure 2. Schematic of in vivo-derived EVs isolated from body fluids. Cells from different human tissues of the body communicate through the secretion of EVs into proximal body fluids. EVs contain proteins, lipids and RNA molecules that may affect the physiology of cells bathed in or lining these body fluids. Highlighted here are the body fluids where EVs have been identified and their possible cellular origin. Pink spots represent body fluids, which are only present in females. Green spots represent body fluids, which are only present in male. Yellow spots represent body fluids present in both female and male. CSF=cerebrospinal fluid; BALF=Broncho alveolar lavage fluid.

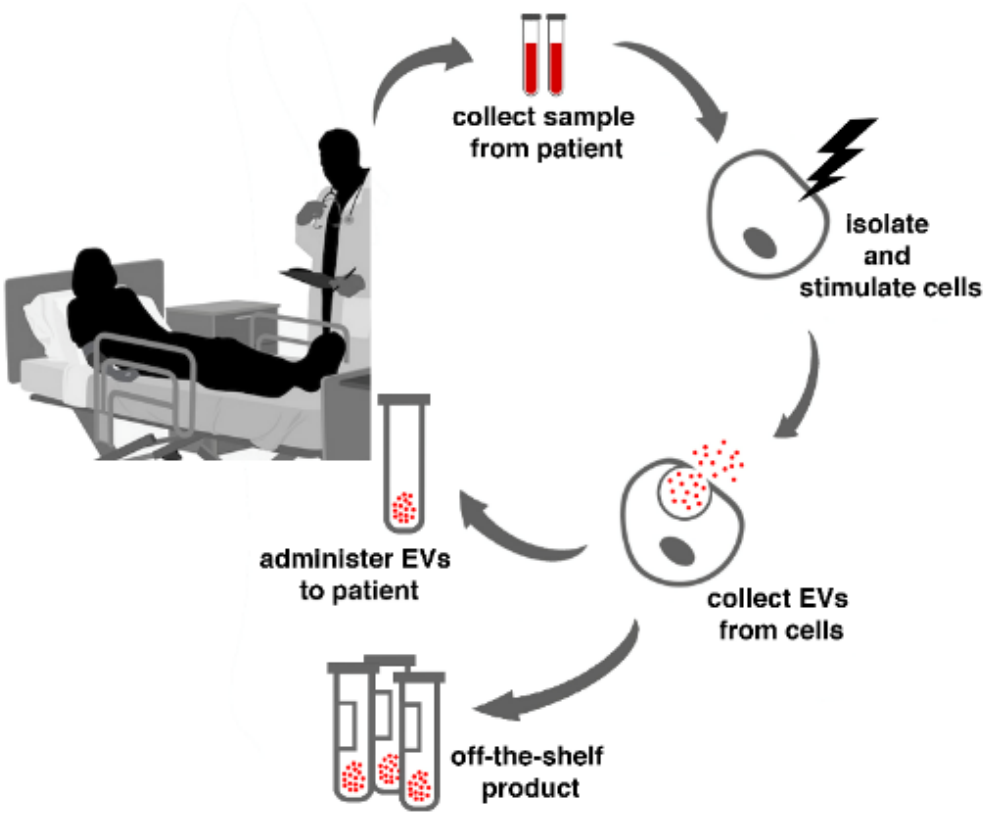

Figure 3: Stem cell EVs for clinical applications. In future, EVs produced under quality controlled regulatory procedures will be stored as off-the shelf product to be used for specific applications. This picture is modified from http://2015.igem.org/Team:NJU-China

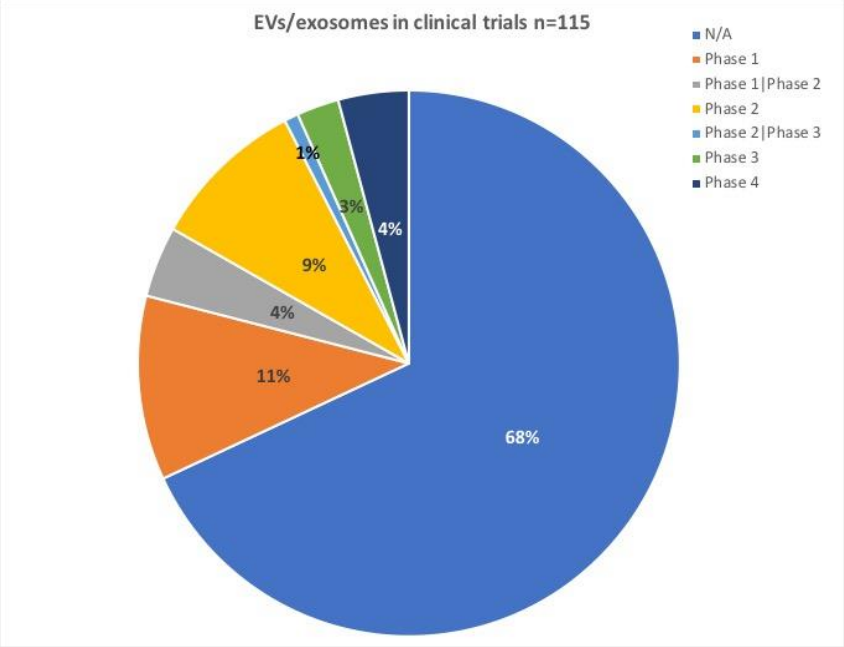

Figure 4: Clinical trials in different phases with administering of EVs/exosomes, according to clinicaltrials.gov 7.8.2018

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