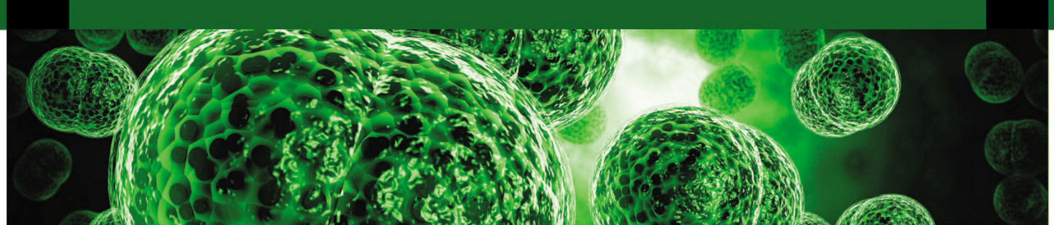

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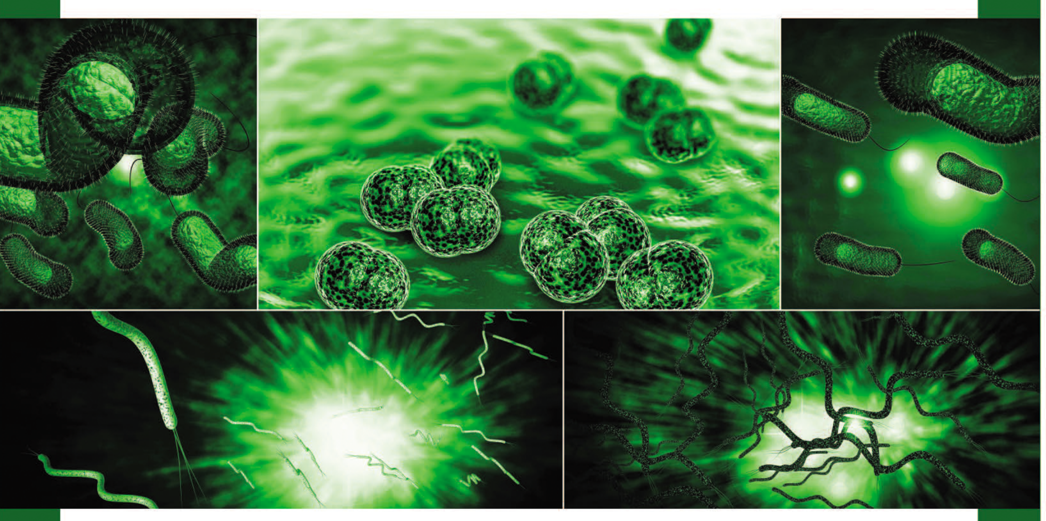

Sheikh Umar Ahmad

Editor

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Dedication

Dedicated to my beloved parents, Wali Mohd Sheikh and Azee Begum,
who against all odds ensured that I completed my education, and
what I am today is because of their selfless and sincere efforts.
I pray to almighty Allah that he accepts their efforts and
keep them steadfast on Iman. May they live a happy,
blessed, and long life, Ameen.

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Abbreviations

3’UTR	3’-untranslated region
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
AA	aplastic anemia
AACR	American Association for Cancer Research
AAT	alpha-1 antitrypsin
AAV	adeno-associated viruses
AB	*Acinetobacter baumannii*
AC	*Acinetobacter calcoaceticu*
ACC	acetyltransferases
ACT	*Actinobacillus*
ACTH	adrenocorticotropic hormone
ACTm	*Actinomyces*
Ad	adenoviruses
AD	Alzheimer’s disease
AER	*Aeromicrobium*
AGO	Argonaute
AHSCT	allogeneic hematopoietic stem cell transplantation
AIET	autologous immune enhancement therapy
AMO	anti-miRNA oligonucleotides
AMP	adenosine monophosphate
AMP	antimicrobial peptides
ANT	aminoglycoside nucleotidyltransferase
APH	aminoglycoside phosphotransferases
APP	amyloid precursor protein
ASC	adult stem cells
AT	agrobacterium tumefacien
ATP	adenosine triphosphates
AuNP	gold nanoparticles
Aβ	β-amyloid
BAC	*Bacillus*
BACT	*Bacteroides*
BC	*Bacillus clausii*

BM	bone marrow
BMI	body mass index
BMSC	bone marrow stem cells
BP	*Bacillus pumilus*
BP	*Burkholderia pseudomallei*
BRM	biological response modifiers
BS	*Bacillus subtilis*
CAMP	childhood asthma management program
CAR	chimeric antigen receptor
CARMEN	combinatorial arrayed reactions for multiplexed evaluation of nucleic acids
CAT	catalases
CAT	chloramphenicol acetyltransferases
CB	*Clostridium butyricum*
CC	*Campylobacter coli*
CD	*Clostridium difficile*
CD	cytosine deaminase
CDI	*Clostridium difficile* infection
CHIP	chromatin immunoprecipitation
CHR	*Chryseobacterium* spp.
CIFN	consensus IFN
CIT	*Citrobacter*
CLGP	cysteinyl leukotriene genetic pathway
CLPP	cysteinyl leukotriene pharmacological pathway
CLR-1	cysteinyl leukotriene receptor 1
ClyA	cytolysin A
CML	chronic myelogenous leukemia
CMV	cytomegalovirus
COR	*Corynebacterium*
CP	*Clostridium perfringens*
CPP	cell-penetrating peptide
CPT	chloramphenicol 3-O-phosphotransferase
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
CRISPR-Cas	clustered regularly interspaced short palindromic sequences–CRISPR-associated protein
CRISPRi	CRISPR interference

crRNA	CRISPR RNA
CSC	cancer stem cell
dCas9	dead Cas9
DHFR	dihydrofolate reductase
DOTAP	1,2-dioleoyloxy-3-trimethylammoniumpropane
DPP	diabetes prevention program
DPP4	dipeptidyl peptidase inhibitors-4
DPSC	dental pulp-derived stem cells
DSB	double-strand break
dsDNA	double-stranded DNA
DSS	dextran sodium sulfate
EA	*Enterobacter aerogene*
EBV	Epstein Barr virus
EC	*Escherichia coli*
EcN	*E. coli* Nissle 1917
ECV	extracellular vesicles
EF	*Enterococcus faecalis*
EGCG	epigallocatechin-3-gallate
ENT	*Enterobacter* spp.
ENT	*Enterococcus*
ENTB	*Enterobacter*
EntC	*Enterobacter cloacae*
ESA	erythropoietic-stimulating agents
ESC	embryonic stem cell
ESC	*Escherichia*
EUB	*Eubacterium*
FDA	Food and Drug Administration
FEV_1	forced expiratory volume in 1 second
FQ	fluorophore quencher
FUS	*Fusobacterium*
GAR	*Gardnerella*
GCK	glucokinase
gFET	graphene-based field-effect transistor
GLP	glucagon-like peptide
GPP	glucocorticoid pharmacological pathway
gRNA	guideRNA
GVHD	graft-versus-host disease
GWAS	genome-wide association studies

H_2O_2	hydrogen peroxide
HA	hemagglutinin
HAE	*Haemophilus*
HBV	hepatitis B infection
HCV	hepatitis C virus
HDR	homology-directed repair
HEPN	higher eukaryotic and prokaryotic nucleotide
HER2	human epidermal growth factor receptor 2
hESC	human embryonic stem cells
HGP	human genome project
HI	*Haemophilus influenza*
HLA	human leucocyte antigens
HMP	human microbiome project
HNF1-α	hepatic nuclear factor 1-α
HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
ICS	inhaled corticosteroids
IFN-α	interferon-α
IKTC	independent Korean trial cohort
ILs	interleukins
IPRs	IP rights
iPSCs	induced pluripotent stem cells
IRIS	immune reconstitution inflammatory syndrome
IST	immunosuppressive therapy
ITRs	inverted terminal repeats
KLE	*Klebsiella*
KLs	*Kluyvera* spp.
KO	knockout
KP	*Klebsiella pneumonia*
LA	*Listonella anguillarum*
LABA	long-acting beta-agonist
LAC	*Lactobacillus*
LAK	lymphokine-activated killer
LFR	lung function response
LL	*Lactococcus lactis*
LM	*Listeria monocytogenes*
LMR	leukotriene modifier response

LNA	locked nucleic acid
LO	lipoxygenase
LOD	limit of detection
LOI	leukotriene inhibitor
LPS	lipopolysaccharide
LR	*Lactobacillus reuteri*
LRA	leukotriene receptor antagonist
LRM	leukotriene receptor modifiers
LTA4H	leukotriene A_4 hydrolase
LTC4S	leukotriene C_4 synthase
MEAC	multiethnic asthma cohorts
MFS transporters	major facilitator superfamily transporters
MHC	histocompatibility complex
MIC	*Micromonospora*
MICC	*Micrococcus*
miRNA	microRNA
MM	*Morganella morganii*
MODY	maturity-onset diabetes of the young
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRP1	multiple drug-resistant proteins 1
MRSA	methicillin-resistant *Staphylococcus aureus*
MSC	mesenchymal stem cells
MT	*Mannheimia taxon* 10
MV	*Mannheimia varigena*
NAE	N-acylethanolamines
NAG	N-any nucleotide
NAPE	N-acylphosphatidylethanolamines
NASBA	nucleic acid sequence-based amplification
NASBACC	nucleic acid sequence-based amplification–CRISPR cleavage
nCas9	nickase Cas9
NCI	US National Cancer Institute
ncRNA	non-coding RNA
NEI	*Neisseria*
NG	*Neisseria gonorrhoeae*
NGS	next-generation sequencing
NHEJ	non-homologous end joining

NM	*Neisseria meningitides*
NPC	neural precursor cells
NSC	neural stem cells
NSP	non-starch polysaccharide
O_2^-	superoxide radical
OH	hydroxyl radical
PA	*Pseudomonas aeruginosa*
PABA	*p*-aminobenzoic acid
PAM	protospacer adjacent motif
PAMAM	poly(amidoamine)
PBPs	penicillin-binding proteins
PD	*photobacterium damselae* subsp. *Piscicida*
PDE	phosphodiesterase inhibitors
PDX-1	pancreatic and duodenal homeobox gene 1
PED	*Pediococcus*
PEFR	peak expiratory flow rate
PEG	polyethylene glycol
pegRNA	prime editing guideRNA
PEI	polyethyleneimine
PEP	*Peptosteptococcus*
PLGA	poly(lactide-co-glycolide)
PM	*Pasteurella multocida*
PM	personalized medicine
PMb	*Proteus mirabilis*
PNA	peptide nucleic acid
POR	*Porphyromonas*
POR	*Proteus*
PP	*Pseudomonas putida*
PRE	*Prevotella*
PSCs	perinatal stem cells
PSEN-1	presenilin-1
QRDR	quinolone resistance determining the region
rAAV	recombinant AAV
RCC	renal cell carcinoma
RES	reticuloendothelial system
RND	resistance nodulation division
RNP	ribonucleoprotein
ROS	reactive oxygen species

RPR	regulatory promoter region
SA	*Staphylococcus aureus*
SABA	short-acting beta-agonist
SAC	*Streptomyces acrimycini*
SAg	*Streptococcus agalactiae*
SAV	self-amplifying vaccines
SCCmec	*Staphylococcal cassette* chromosome mec
SCFA	short-chain fatty acids
SCI	spinal cord injury
SE	*Salmonella enteritidis*
SEL	*Selenomonas*
SF	*Shigella flexneri*
sgRNA	single chimeric guideRNA
SH	*Staphylococcus haemolyticus*
SHERLOCK	specific high sensitivity enzymatic reporter UnLOCKing
SHINE	streamlined highlighting of infections to navigate epidemics
shRNAs	short-hairpin RNAs
SI	*Staphylococcus intermedius*
SLE	systemic lupus erythematosus
SM	*Serratia marcescens*
SNP	short nucleotide polymorphism
SNV	single nucleotide variants
SOD	superoxide dismutase
SP	*Streptococcus pyogenes*
SS	*Streptococcus sius*
SSC	somatic stem cells
ST	*Salmonella typhi*
STA	*Staphylococcus aureus*
STIP1	heat shock organizing protein gene
STM	*Salmonella typhimurium*
StM	*Stenotrophomonas malyophilia*
STP	*Staphylococcus*
STR	*Streptococcus*
STRM	*Streptomyces*
SUR1	sulfonylureas receptor subunit 1
T1D	type 1 diabetes

T2D	type 2 diabetes
TALENs	transcription activator-like effector nucleases
TFFs	trefoil factors
tgRNA	tuned guideRNA
TILs	transfer of tumor-infiltrating T-cells
TPMT	thiopurine methyltransferase
tracrRNA	trans-activating CRISPR RNA
TRE	*Treponema*
TRP	tandem repeat polymorphism
TSPSC	tissue-specific progenitor stem cells
UE	uncultured eubacterium
ULABA	ultra-long-acting beta-agonists
VC	*Vibrio cholerae*
VEI	*Veillonella*
VL	visceral leishmaniasis
WOL	*Wolinella*
ZFNs	zinc-finger nucleases

Preface

Biotechnology, as a fast-developing multidisciplinary modern technology at the interface of biology and engineering and as an advanced science, has already shown its impacts on different aspects of public health, including on many other fields of particular interest to humanity. Biotechnology holds tremendous potential in using fundamental biological knowledge in developing new tools and techniques that can in turn be used in the synthesis and production of novel biological and medicinal entities for the benefit of humankind. This advanced field encompasses a wide variety of highly multidisciplinary subjects and a series of enabling technologies involving the practical application of organisms or their components in manufacturing biological materials, environmental management, and in value addition to food and products of medicinal value. Historically, biotechnology used to be more of an artisan skill rather than a field of science involved in the preparation of wines, beers, and cheese, through traditional approaches and techniques without knowing the actual biological processes and mechanisms involved. As the knowledge and the scientific basis of these traditional biotechnological processes evolved through interdisciplinary research-based approach(es), it has resulted in efficient manufacturing from a commercial point of view and enhanced the rewards of biotechnology viz-a-viz its role in medicine, public health, industry, and agriculture. Interventions in modern biotechnology have led to a range of novel lifesaving biological products and techniques significantly impacting the modern healthcare sector. Modern advanced technology has further evolved in designing molecular tools and techniques allowing desired molecular changes that can be made to living organisms, now heralding into a new field and age of biotechnology called genetic engineering, with a special focus in the areas of environment, medicine, health, and food sustainability. It seems clear that biotechnology will be playing a pivotal role as an enabling technology in the 21st century, having the potential to ensure food and environmental sustainability in addition to its role in revolutionizing the healthcare sector.

Considering its huge impact on the economy, inter- and intra-governmental agencies, including stakeholders from the private sector, have

already taken up numerous measures despite many challenges and are building competent human resources in promoting research and development in this field. However, with the advent of new and enabling modern biotechnological tools, it will be of particular interest to see how advanced biological research using the latest and innovative technologies will revolutionize the public health sector with particular emphasis on disease diagnosis, detection, and treatment.

Keeping these diverse applications of this interdisciplinary field in mind, this book, in a very simple and lucid manner, aims to cover many, if not all, aspects of modern biotechnology and its applications with special attention given to how modern biotechnology will advance the healthcare sector. Reading this book will give readers an idea of the intricate relationship between biotechnology and healthcare and will also explain different biotechnological processes and their applications in medicine.

This volume presents readers with some research trends and areas of improvement to better understand its prospects involving modern biotechnology-based tools and techniques. This volume also sheds light on how biotechnology can be used in disease diagnosis and treatment that will change the perspective of modern biotechnology and its role in healthcare.

This volume:

- Provides an overview of MicroRNAs as next-generation therapeutic and diagnostic agents.
- Discusses the evolving role of CRISPR-Cas-based diagnostics and its reliability.
- Discusses engineered gut microbiome in treating diseases and its applications in modern biotechnology.
- Discusses antibiotics and plant-derived antimicrobials as alternative sources to control infections.
- Sheds light on how understanding the individual pathophysiological intricacies can lead to personalized medicine.
- Discusses stem cell technology (SCT) and regenerative medicine as an emerging therapeutic intervention against a diverse set of diseases.
- Discusses the process methodology and potential role of CRISPR-Cas genome-editing tool as a prospective application to study human diseases.

CHAPTER 1

MicroRNAs: Next-Generation Therapeutic and Diagnostic Agents

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ABSTRACT

microRNAs (miRNAs) are a new class of endogenous regulators for gene expression at the post-transcriptional level whose deregulation is associated with numerous diseases. These small, noncoding molecules have gained a lot of interest in the scientific community across the globe as therapeutic and diagnostic agents. Their prominent presence in biopsies and body fluids makes them highly sensitive and specific circulating biomarkers in nature. Some diagnostic miRNAs are already available to clinicians and sheltered by major insurance companies. On the other hand, many therapeutic miRNAs are in clinical and preclinical trials, but none of them have been used in the market yet. Although there is a large number of obstacles

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to translating the findings of miRNA therapeutics from bench to bedside, but the day is not far when such obstacles will be overcome, and this class of therapeutic drugs will enter into the clinic as next-generation medicine. This chapter summarizes the importance of the chemical modification of oligonucleotides and their delivery systems for the development of therapeutic microRNAs. Further, it also highlights the efforts of various pharmaceutical companies toward the development of therapeutic and diagnostic microRNAs. Lastly, the chapter delivers insights into the major barrier associated with the clinical application of therapeutic miRNAs.

1.1 INTRODUCTION

In the last few decades, exponential progress in the field of biological sciences like metabolomics, systems biology, and proteomics in conjunction with bioinformatics have generated a huge amount of data to address and understand the biological mechanisms associated with diseases and discover therapeutic interventions. It has been well-established that only 1.5% (approximately) of all human genes encode for the proteins that make up our cells and tissues. The rest of the genome is made of noncoding DNA (Li et al., 2001). The noncoding DNA is essential for the regulation and expression of the coding regions (Gloss & Dinger, 2018). microRNAs (miRNAs) are a new class of post-transcriptional regulators of endogenous gene expression. These molecules are short noncoding RNAs (ncRNAs) of ~22 nucleotides that down-regulate expression of their target genes by either mRNA degradation or translational inhibition by guiding Argonaute (AGO) proteins to target sites in the 3'-untranslated region (3'UTR) of messenger RNAs (mRNAs) (Huntzinger & Izaurralde, 2011; Swarts et al., 2014). miRNAs are involved in virtually every cellular process or biological process, including developmental timing, cellular differentiation, proliferation, apoptosis, gene regulation, insulin secretion, cholesterol biosynthesis, and cancer development (Ambros, 2003; Xu et al., 2003). The latest issue of the miRBase database (v22) contains 48,860 different mature microRNA sequences produced from 38,589 hairpin precursor microRNAs entries from 271 organisms. This issue represents 1,917 annotated hairpin precursors and 2,654 mature sequences from the human genome (Kozomara et al., 2019).

The increased research on miRNAs leads to a huge number of studies revealing that miRNA deregulation is associated with numerous diseases

(Esteller, 2011), predominantly cancer (Bracken et al., 2016). These small, noncoding, endogenous molecules have gained a lot of interest in the scientific community across the globe as therapeutic and diagnostic agents. microRNAs are highly sensitive and specific circulating biomarker as these can be easily detected in biopsies and body fluids (De Guire et al., 2013). The potential importance of these small molecules as therapeutics and diagnostics is apparent by the fact that nearly 11,000 studies have been published depicting their huge potentials as future diagnostic agents whereas 3,500 studies reveal their prospective as therapeutic agents (Bonneau et al., 2019).

The entry of Miravirsen (or SPC3649), an antagomiR targeting miR-122 into clinical trial has fueled hope into the scientific community across the globe that these disease modifying agents can be used as future medicines. Further, current advancement in RNA chemistry and technologies to deliver RNA molecules *in vivo* has made it possible for the success of microRNA-based therapeutics (Rupaimoole & Slack, 2017). In the United States, the biotechnology industry revenue was just $20 billion in 1996 and increased up to $70.1 billion in 2008. The major portion of this revenue comes from the pharmaceutical industries. Since 1983, a larger number of pharmaceutical companies have been established which are actively investing in the development of miRNA-based therapeutic molecules (Table 1.1) (Chakraborty et al., 2017).

In this chapter, we will discuss about the chemical modifications of miRNA-based therapeutics, advances in technologies to deliver such therapeutic molecules and microRNAs that are currently in clinical trials. Furthermore, we will discuss about the future prospects of microRNAs as therapeutic and diagnostic agents.

1.2 CHEMICAL MODIFICATIONS OF MIRNA-BASED THERAPEUTICS

Therapeutic microRNAs have emerged as an important biopharmaceutical that are in commercial space as future medicine. The miRNA-based therapeutics approach is based on the fact to restore normal miRNA expression levels either by downregulating high levels of miRNAs using anti-miRs (innovative molecules designed for endogenous miRNA degradation) or by upregulating low levels of miRNAs using mimics (molecules which mimic endogenous miRNAs) in a specific disease stage or pathological

TABLE 1.1 List of Pharmaceutical Companies Involved in miRNA and siRNA Therapeutics

Biopharmaceutical Company	Country	Year of Establishment
Marina Biotech	USA	1983
Quark Pharmaceuticals	USA	1993
Rosetta Genomics	Israel	2000
Alnylam Pharmaceuticals	USA	2002
Santaris Pharma	Denmark	2003
RXi Pharmaceuticals	USA	2003
Asuragen	USA	2006
Mirage Therapeutics	USA	2007
Mirna Therapeutics	USA	2007
Regulus Therapeutics	USA	2007
InteRNA Technologies	Netherlands	2008

condition. MicroRNA mimic is a synthetic RNA duplexes which is identical to the miRNA of interest with chemical changes in order to improve its stability and cellular uptake (Bader et al., 2011; Thorsen et al., 2012) whereas anti-miR is a RNA molecule having a complementary binding site to a particular miRNA or a family of miRNA in order to inhibit their function. It is commonly known as anti-miRNA oligonucleotides (AMOs) (Ebert & Sharp, 2010). The first generation of mimics and AMOs employed were unmodified and they were quickly degraded by ribonucleases present in serum and in the intracellular environment. Scientists quickly realized that chemical modification of these synthetic oligonucleotides is important to prevent their degradation by ribonucleases present in the blood and from phagocytosis carried out by reticuloendothelial system (RES) (Zhang & Farwell, 2008). Current chemical modifications are not carried out just to confer nuclease resistance to synthetic oligonucleotides but are also carried out to increase binding affinity and to increase the cellular uptake of these synthetic oligonucleotides. Other chemical modifications are made to alter the ability to trigger a response by the innate immune system (Lennox & Behlke, 2011).

A range of chemical alterations have been incorporated into these synthetic oligonucleotides particularly the AMOs. These modifications can be carried out in the phosphate moiety, e.g., phosphorothioate-containing oligonucleotides or in sugar group, e.g., 2’-O-methyl ribose containing oligonucleotides. The details of these chemical modifications are described in Table 1.2.

TABLE 1.2 Various Chemical Modifications of Oligonucleotides Improve Stability, Biodistribution, Cellular Uptake, and Delivery Efficiency, and Their Function

Oligonucleotides	Chemical Modification	Structure	References
Phosphorothioate-containing oligonucleotides	• α-oxygen of the phosphate replaced by sulfur • Improves stability		Sharma & Watts (2015)
Methylphosphonate	• α-oxygen of the phosphate replaced by methyl (CH_3) group • Improves stability		
2’-O-methyl-(2’-O-Me)	• 2’-O-methyl group added to 2’-oxygen of the ribose • Improves binding stability and protects from nuclease degradation		Bernardo et al. (2015)
2’-Fluoro oligonucleotides	• Fluoro group added to 2’-oxygen of the ribose • Improves binding stability and protects from nuclease degradation		

TABLE 1.2 *(Continued)*

Oligonucleotides	Chemical Modification	Structure	References
Locked nucleic acid (LNAs)	• 2',4' methylene link in the ribose to form a bicyclic nucleotide • Improves sensitivity and specificity	Base	Chabot et al. (2012)
Terminally modified oligonucleotides	• Contains Cy3- or cholesterol or biotin at the terminal phosphate group • Improves stability and their tracer function in *in-vivo* delivery	Cholestrol Base OH	Krützfeldt et al. (2007)

1.2.1 ADVANCES IN MIRNA DELIVERY SYSTEMS

We all know that the success of microRNA-based therapeutics is directly dependent on the advancement in RNA chemistry and technologies to deliver RNA molecules *in vivo*. Usually, chemically modified oligonucleotides show poor cellular uptake and inadequate tissue-specific delivery when delivered in the absence of a carrier. To overcome such problems, miRNA delivery systems are used, which can be broadly divided into two categories, i.e., viral and nonviral miRNA delivery systems.

1.2.2 VIRAL MIRNA DELIVERY SYSTEMS

The basic idea behind the use of viruses as delivery systems is to remove or replace pathogenic genes with therapeutic gene(s). The Viral based delivery systems are useful for the long-term expression of miRNA (to restore normal miRNA expression) or anti-miRNA (to inhibit upregulated miRNA) into different tissues or organs. The viruses for such oligonucleotide delivery include adenovirus vectors, adeno-associated virus vectors (AAV), retroviral vectors, and lentivirus vectors (Fu et al., 2019).

Although these viral vectors have high delivery efficiency but there are few disadvantages, including low loading capacity, high toxicity, and strong immune response in the host (Buchbinder et al., 2008; McElrath, De Rosa, et al., 2008).

Adenoviruses (Ad) are nonenveloped viruses that contain an icosahedral nucleocapsid and double-stranded DNA (36 kb) with two inverted terminal repeats (ITRs) at its ends (Douglas, 2007). These viruses are highly attractive for scientists as delivery systems because these viruses can infect a broad range of cells without integrating into the host genome. Furthermore, these viruses can be manipulated easily to get the desired transduction efficacy in proliferating as well as quiescent cells (Cao et al., 2004). Currently, researchers use Next generation "gutless" adenoviral vectors with the packaging capacity of ~37 kb compared to the initial adenoviral vectors with a maximum 8 kb. Most of the genes in these Next generation "gutless" adenoviral vectors are completely removed and are left with the genes encoding for packaging and regulatory signals. These manipulations not only increase the loading capacity of Ad but also reduce their immunogenicity (Chen et al., 1997; Amalfitano, 1999). Next-generation "gutless" adenoviral vectors have advantages over initial adenoviral vectors, but these vectors need a helper virus for their propagation (Crettaz et al., 2009).

Despite many advantages as a delivery system, Ad has the disadvantage to induce an immune response (adaptive and innate), resulting in cell toxicity when repeatedly administered (Marshall, 1999).

Adeno-associated viruses (AAV) are nonenveloped viruses having a single-stranded DNA genome (4.7 kb) flanked by ITRs. AAV requires helper virus (adenovirus or herpes simplex virus) for its replication and in the absence of helper virus it releases its genome inside the nucleus where it remains as an episomal form or rarely integrates its DNA into the AAVS1 region of chromosome 19 (Schultz & Chamberlain, 2008). The integration of the AAV genome in the host cell is mainly carried out by the Rep protein. This Rep protein is deleted in recombinant AAV (rAAV), hence making its integration less efficient than wild-type AAV (Herrera-Carrillo et al., 2017). Around 11 nonpathogenic (absence of pathogenicity in humans) serotypes of AAV have been discovered and each of them shows a unique host's cell/tissue tropism with varying immunological properties. This cell/tissue-specific tropism makes AAV a favorite delivery system *in vivo* (Wu et al., 2006). Additionally, AAV shows a long duration of transgene expression

in various organs of mice, including brain, liver, lung, eyes, and muscles (Nakai et al., 1998; Kaplitt et al., 1994; Grant et al., 1997).

Retroviruses are enveloped virion particles with single-stranded RNA (approx. 10 kb) (Pages & Bru, 2004). Once the virus infects the host cell, double-stranded DNA is formed from single-stranded RNA by the activity of reverse transcriptase enzyme. The double-stranded DNA then randomly integrates into one of the host chromosomes, which can allow its sustained expression (Liu & Berkhout, 2011). Although the durable expression of integrated fragment is a favorable characteristic, it can also result in high levels of insertional mutagenesis (Herrera-Carrillo et al., 2017).

Lentiviruses are very similar to Retroviruses as both of them belong to the Retroviridae family. Both of these viruses lead to stable integration of a transgene into the host genome, resulting in sustained gene expression in the host. In contrast to Retroviruses, useful for diving cells, Lentiviruses can transduce both dividing and nondividing cells, which makes Lentiviruses is more preferable than Retroviruses for stable gene expression in the cells (Greber & Fassati, 2003). Finally, Lentiviruses usually integrate within active transcriptional units, which significantly reduces the incidence of insertional oncogenesis (Montini et al., 2009).

1.2.3 NONVIRAL MIRNA DELIVERY SYSTEMS

Nonviral delivery systems also serve as essential platforms for the supply of miRNA-based therapeutics to target sites. These platforms are generally regarded as less immunogenic and toxic in nature. Besides, these delivery strategies do not have any size constraint of the packaged nucleic acid although insert size is an important determinant of transfection efficacy (Yang, 2015). An efficient nonviral delivery system transports exogenous synthetic miRNA or miRNA vectors without their degradation from cellular nucleases (Fu et al., 2019). These platforms encompass physical methods as well as chemical methods. Physical approaches, such as electroporation, gene gun, ultrasound, hydrodynamic-, and laser-dependent energy, apply forces from outside for imparting cell permeability towards the gene therapy (Yang, 2015) and mainly used in *in vitro* culture system and intermittently applied in the miRNA delivery systems (Balacescu et al., 2018). However, these delivery methods occasionally cause damages to cellular integrity. Whereas Chemical methods entail lipid-driven, polymer-driven, and inorganic carriers (Wang et al., 2018).

Lipid-based methodologies exploit the lipid/nucleic acid multiplexes, called lipoplexes or liposome as transporting systems. Cationic, anionic, or neutral liposomes exist, and they are made of a membrane-like exterior, with the miRNA or nucleic acids captured inside (Sforzi et al., 2020). Cationic lipoplexes are frequently employed in nonviral miRNA supplies due to their nonpathogenic and non-immunogenic properties, and high affinity towards cellular membranes. For example, liposome-mediated delivery of miR-300 to the osteoblast cultures resulted in precise overexpression for miR300, followed by a decrease in osteogenic mineralization (Kaur et al., 2020). Several cationic lipoplex (DharmaFECT®, SilentFect™, Lipofectamine® RNAi-MAX, SiPORT™) complexes are commercially available for miRNA delivery (Yoshizuka et al., 2016). However, the most acute difficulty for cationic liposome distribution schemes is their short half-lives in serum. Due to their short half-life and nonspecific interactions with blood proteins, anionic and neutral liposomes are instead utilized for miRNA delivery. For example, the anionic and neutral lipoplexes were effective to the delivery of miR-29b and miR-34a to acute myeloid leukemia and lymphoma, respectively (Garzon et al., 2009). Conjugation of polyethylene glycol (PEG) with the lipids improves steadiness with a longer half-life (up to 72 hours) (Pasut & Veronese, 2009). Pramanik and workers assembled lipid nanoparticles using DOTAP and PEG to deliver miR-143/145 cluster and miR-34a to a xenograft mouse model for a pancreatic cancer study. These assembled particles further established an amplified nanoparticle gathering at tumor sites with reduced tumor size (Kent et al., 2010).

Polymer-dependent methods comprise of poly(lactide-co-glycolide) (PLGA), poly(amidoamine) (PAMAMs) dendrimers, polyethylenimine (PEI) or cell-penetrating peptide (CPP) as delivery transporters. Low molecular weight PEIs display minor cell membrane damage and related cellular toxicity in comparison with high molecular weight PEIs. Treatment of a xenograft mouse model of colon cancer with miR-33a and miR-145 using low molecular weight PEIs diminished tumor development and improved apoptosis (Ibrahim et al., 2011). Nevertheless, the transfection competence is indeed low and associated with limited biodegradability (Yang, 2015).

One crucial Food and Drug Administration (FDA) approved delivery vehicle, namely PLGA, has been used in miRNA-based therapeutic interventions against several critical pathological conditions (Christopher et

al., 2016). However, the hydrophobic trait associated with PLGA reduces its transfection efficiency.

PAMAMs polymers, being positively charged in nature, exhibits high transfection ability compared to other polymers. Anti-miR21-PAMAM dendrimers have been reported to suppress the progression of glioblastoma cells (Játiva & Ceña, 2017). The chief shortcoming of PAMAM dendrimers is the polymer buildup in the liver (Vu et al., 2019). Besides these synthetic polymers, natural polymers viz. CPPs are employed for miRNA delivery. CPP (arginine-rich) from natural protamine efficiently transported miR-29b into osteo-stem cells (Suh et al., 2013). Additionally, Natural polymers CPPs are less toxic but disposed to degradation in serum in comparison to synthetic polymers.

In comparison to lipids and polymers, the importance of inorganic materials as miRNA gene carriers has been reported. Current inorganic miRNA vectors embrace gold nanoparticles (AuNPs), silica nanoparticles and Fe_3O_4-based nanoparticles (Wang et al., 2013). Among these AuNPs are the most habitually used. AuNPs have been stated to deliver miR-130b efficaciously (Bakhshinejad et al., 2014) and anti-miR29b to the surfaces of tumor cells (Roma-Rodrigues et al., 2017). The inorganic delivery platforms exhibit high *in vivo* stability and are devoid of microbial attack. Though, the binding affinity between these carriers and nucleic acids is generally less. To this end, inorganic and organic hybrid materials are innovated and hence employed in molecular delivery systems. AuNP10 conjugated to PEG0.5 delivers miR-1 into cancer cells, depicting higher transfection competence, longer half-lives, and lower toxicity in contrast to lipofection (Yang, 2015).

Viral and nonviral miRNA delivery systems have both advantages and disadvantages. Viral vectors show higher transfection efficiency but result in toxicity with immunogenic response, while nonviral carriers display reduced delivery efficiency but are relatively safe. Consequently, operative and nontoxic miRNA delivery systems are urgent need of the hour. The construct for future delivery systems must combine the benefits from both systems resulting in effective miRNA therapies from bench to clinics.

1.3 THERAPEUTIC MIRNAS IN CLINICAL TRIALS

It is entrenched that miRNAs are restorative for molecular therapy and gene silencing. With the help of emerging pharmaceutical firms, nucleic

acid-based gene therapies are thereby entering the commercial sector. miRNAs are either overexpressed for the therapeutic purpose by miRNA mimics or silenced in the target tissue/cell by AMOs. These oligonucleotides are particularly responsive to nuclease-mediated decay in live cells and chemical changes are normally made prior to their distribution in the body (Lennox et al., 2013). In addition, several biomedical companies, including Rosetta Genomics, Alnylam, and Regulus Therapeutics concentrate on the RNAi driven pharmaceuticals in treatment for myriad of diseases for the societal benefit (Haussecker, 2012). Lately, Onpattro (FDA approved), a medication for the polyneuropathy treatment, has identified the reasons for the drug demand of RNAi technology-driven therapies (Akinc et al., 2019; Kaur et al., 2020). RNA biodrug(s) has been a significant business among numerous pharmaceutical companies from the last 20 years, performing miRNA clinical trials that are functional against several disorders. The majority of pharmaceutical companies are working on the pharmacokinetics and pharmacodynamics for miRNAs in the preclinical and clinical stages of development (Table 1.3).

1.3.1 MIRAVIRSEN

Miravirsen is an antisense phosphorothioate miRNA, modified by LNA (locked nucleic acid) inhibiting miR-122. It has been tagged as the first miRNA that entered phase II clinical trials, funded by Santaris Pharma (Roche Innovation Center), in order to clarify its efficiency and admissibility in patients. Clinical trials for its use against HCV infection have been performed in the US. Besides, phase II clinical trials are currently being conducted in other countries as well (Chakraborty et al., 2020; Gebert et al., 2014).

1.3.2 RG-101 AND RG-012

RG-101, a N-acetylgalactosamine (GalNAc)-conjugated inhibitor that restricts action of liver-specific miRNA-122 for the treatment of HCV, has been developed by Regulus Therapeutics. RG-012, another miRNA silencer targeting miRNA-21, developed by Regulus Therapeutics is currently in a clinical pipeline. It executes an imperative part in fibrogenic ailments associated with distinct organs, specifically the kidneys. Hence

the drug is majorly employed in the prevention of Alport nephropathy (Ali et al., 2016; Chakraborty et al., 2017).

1.3.3 ANTI-MIRS

Unconventional RNA targeting therapy, has been developed by U.S.-based biopharmaceutical corporation miRagen with a special focus on miRNAs for unaddressed human healthcare needs. Several miRNA-dependent therapeutics have been designed from miRagen therapeutics, including but not limited to, MGN-2677, MGN-4220, MGN-1374, MGN-6114, MGN-4893 MGN-8107, and MGN-9103. MGN-9103 was proposed to play a vital role in diabetes and obesity. Besides, miRagen therapeutics is conducting clinical studies on MRG-106 prohibiting miR155. While, for red blood cell expansion, miR-451 is needed and suppression of miR-451 using anti-miR-451 (MGN-4893) in mice showed inhibition for erythrocyte's differentiation. This was proved beneficial against diseases involving anomalous red blood cell count (Chakraborty et al., 2017, 2020).

1.3.4 MRX34

MRX34, double-stranded miRNA mimic supplied in a liposome formulation initially developed by miRNA Therapeutics, which is now under the Synlogic Inc. Preclinical studies demonstrated therapeutic effects of MRX34 on several cancers (renal, hepatocellular, etc.) (Beg et al., 2017).

1.4 DIAGNOSTIC MIRNAS CURRENTLY IN PRECLINICAL AND CLINICAL TRIALS

There is a plethora of studies indicating miRNA as a biomarker in the diagnosis of various disorders (Table 1.4). Rosetta Genomics was the first company that focused on the arena of diagnostic miRNAs. The company launched RossettaGX Reveal (2016), used to distinguish between benign and indeterminate thyroid nucleus, which was authenticated via. multi-center cohort (189) FNA smears (Chakraborty et al., 2020).

Interpace diagnostics (NASDAQ: IDXG), a diagnostic firm that assimilated a combination of miRNA classifier, ThyraMIR®, and oncogene panel ThyGeNEXT®, for the stratification of thyroid cancer. The ThyraMIR® compromises of miR-31-5p, miR-29b-1-5p, miR-146b-5p miR-139-5p, miR-204-5p, miR-155, miR-375, miR-222-3p, miR-138-1-3p, and miR-551b-3p (Partyka et al., 2018).

Further, Hummingbird Diagnostics (Comprehensive Biomarker Center), generated novel miRNA candidates in discovery of human disorders, such as cancer (melanoma, non-small-cell lung carcinoma, breast cancer), neurodegenerative (Alzheimer, multiple sclerosis, Parkinson) and inflammatory disease (Keller et al., 2017). Clinical validation of these miRNA signatures as biomarkers is in progress, supported by three European FP7-funded consortia (BestAgeing, RiskyCAD, and EURenOmics) (Bonneau et al., 2019).

DiamiR has developed the application of miRNAs as biomarkers in various neurodegenerative and neurodevelopmental pathologies. CogniMIR™, panel for early determination of Alzheimer's disease (AD) which formerly is in clinical trials and testified by DiamiR (Chakraborty et al., 2020). Moreover, TAmiRNA developed another miRNA-based detection panel associated with clinical utility for osteoporosis in the form of their first product OsteomiR™ (Ladang et al., 2020). Interestingly, TAmiRNA also offered a panel of 11 miRNAs ThrombomiR™, for evaluating the platelet function and a panel of 19 miRNAs, ToxomiR™ for assessing the toxicity associated with certain tissues (Mussbacher et al., 2020).

Mirnext biomedical firm recognized miR423-5p as a suitable biomarker in heart failure after validation studies in a large number of clinical samples. Additionally, DestiNA developed a patented PCR-free technology for the miRNA discovery and estimation from human samples. The studies demonstrated that the associated assays are capable of detecting miR-122 providing an edge over prevailing protein biomarkers to evaluate liver toxicity (Chakraborty et al., 2020).

1.5 FUTURE PROSPECTS

It has been well established that RNA-based therapeutics are developing rapidly along with enormous potentials for the treatment of various diseases.

TABLE 1.3 Diagnostic miRNAs Currently in the Preclinical and Clinical Trials of Development

SL. No.	Product	miRNA	Companies	Disease	Development Progress
1.	miRview Mets	miRNA library	Rosetta Genomics	Cancer	Available
2.	OsteomiR	Panel of 19 miRNAs	TAmiRNA	Osteoporosis	Available
3.	CogniMIR	Panel (unknown)	DiamiR	Alzheimer	Phase 1
4.	Simoa	miR-122	Quanterix	Liver toxicity	Preclinical
5.	ThyraMIR/ ThyGENX	miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375 and miR-551b-3p	Interpace Diagnostics	Thyroid and pancreatic cancer	Available
6.	Mirnext	Panel with miR 423-5p	Mirnext	Heart failure	Development

Source: Chakraborty et al. (2020); Bonneau et al. (2019); Gebert et al. (2014); Ladang et al. (2020); Mussbacher et al. (2020).

TABLE 1.4 Therapeutic miRNAs Currently in the Preclinical and Clinical Trials of Development

Product	miRNA	Disease	Development Phase	Pharmaceutical Company
RG-101	miR-122	HCV	Phase 2	Regulus Therapeutics
RG-012	miR-21	Alport syndrome	Phase 1	
RG-125	miR-103	NASH	Phase 1	
Mesomir	Mesomir	Mesothelioma	Phase 2	ENGeneIC
MRG-107	miR-155	ALS	Preclinical	MiRagen Therapeutics
MRG-106	miR-155	Lymphoma, leukemia	Phase 1 and Phase 2	
MRG-110	miR-92	Ischemia	Phase 1	
Miravirsen	miR-122	HCV	Phase 2	Roche/Santaris
ABX464	miR-124	IBD	Phase 2	Abivax
MRX34	miR-3	Hepatocellular cancer	Phase I clinical trial	miRNA Therapeutics
–	miR-208	Heart failure	Preclinical	miRagen
–	Let-7	Cancer	Preclinical	miRNA Therapeutics

Source: Chakraborty et al. (2020); Titze-De-Almeida et al. (2020); Nana-Sinkam & Croce (2013).

The discovery of these disease modifying agents is the most exciting drug development, IP rights (IPRs), and the people who are interested in pharmaceutical business. Additionally, the discovery of diagnostic miRNAs is as advanced as the development of therapeutic miRNAs. Several diagnostic miRNAs are already available to clinicians and sheltered by major insurance companies, while the therapeutic miRNAs are yet to enter the market despite their huge potential as therapeutic agents. A major barrier associated with the clinical application of therapeutic miRNAs is to develop a carrier with tissue/cell-specific tropisms to minimize side effects. As this field of therapeutics is new and continues to grow, a better understanding of miRNA biogenesis and function along with the development of efficient vehicles for the targeted delivery to specific cells, tissues, and organs will guide future endeavors in miRNA-based therapeutics. Many obstacles may lie due to safety, target selection, delivery technologies, clinical trial design or commercial considerations. Proper optimization of such factors will minimize the risk of product failure and a high rate of successful drug production. Taken together, miRNA therapeutics is a new and emerging field with large number of obstacles, but the day is not far when such obstacles will be overcome, and this class of therapeutic drugs will enter into the clinic as next-generation medicine.

CONFLICT OF INTEREST

JHS is a scientific co-founder of AAVAA therapeutics and holds equity in the company. Other authors declare no conflict of interest.

KEYWORDS

- **adeno-associated virus vectors**
- **human diseases**
- **microRNAs**
- **molecular diagnostics**
- **next generation therapeutic and diagnostic agents**
- **reticuloendothelial system**

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CHAPTER 2

Evolving CRISPR-Cas-Based Diagnostics and Its Reliability

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ABSTRACT

Detection of genetic material is important for detecting the pathogen or progression of various diseases. The CRISPR-based diagnostic tools are cost-effective in many ways compared to PCR-based DNA detection, as it does not require a thermal cycler machine. Properties of the Cas system, such as detection of the specific protospacer adjacent motif (PAM), specific cleavage of double-strand DNA, and collateral activity on nonspecific sequences, have been utilized for developing diagnostics tools. This chapter explains the types CRISPR-Cas systems, their mechanism, CRISPR-Cas-based diagnostic tools, and their reliability.

2.1 INTRODUCTION

Detection of nucleic acid-based markers is considered highly accurate for determining infectious diseases. Finding nucleic acid-based markers are important for determining the progression of various pathological conditions, including cancer. The recent pandemic of SARS-CoV-2 has increased the need for developing fast nucleic acid detection tools.

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Nucleic acid-based markers are usually detected using PCR-based techniques and often require a thermal cycle machine. The evolution of CRISPR-Cas (clustered regularly interspaced short palindromic sequences–CRISPR-associated protein)-based techniques have changed the face of diagnostic due to their specificity and reliability. CRISPR-based diagnostics has a limit of detection (LOD) in the picomolar range; hence, they can directly detect the target when the sample has a high concentration of DNA or RNA, e.g., Viral infections (Gootenberg et al., 2017; Ramachandran & Santiago, 2021). Ever since the first Cas9-based Zika virus DNA variant detection system was developed, Class 2 CRISPR-Cas systems have been sculptured for multiple diagnostic tools. Diagnostic tools based on the CRISPR-Cas system are extensively reviewed (Kaminski et al., 2021).

The CRISPR-based diagnostic tools are more cost-effective in many cases compared to PCR-based DNA detection, as it does not require thermal cycler machines, and preamplification can be done at isothermal conditions. Properties of Cas enzymes to specifically recognize proto-spacer adjacent motif (PAM), cleave double-strand DNA or single strand (nickase), and initiate collateral cleavage of nucleic acid has been utilized for developing CRISPR-Cas-based diagnostic. NASBACC (nucleic acid sequence-based amplification–CRISPR cleavage), a CRISPR-Cas 9 system in combination with a PCR-based technique, has also been developed to detect Zika virus DNA. A CRISPR Cas12 system-based diagnostic tool, CAT-SMelor, used Cas enzymes as a reporter. CAT-SMelor detects the nanomolar concentration of small molecules such as Uric acid and parahydroxybenzoic acid. The ability of the Cas12 enzyme to specifically cleave double-stranded DNA (dsDNA) and the property of bacterial allosteric transcription factor to detect small molecules were used in combination to develop CAT-SMelor. SHERLOCK one pot Cas 12-based SARS-CoV-2 detecting kit has shown 93.1% sensitivity and 98.5% specificity and could be used widely in low complexity labs (Joung et al., 2020). This chapter explains the types of CRISPR-Cas systems used in diagnostics, their mechanism, and the methods adopted to mold the CRISPR-Cas system to develop various diagnostic tools. Finally, the reliability of diagnostic tools needs to be proved. Various research groups have utilized next-generation sequencing to study the off-target effects of the CRISPR-Cas system. The above-mentioned studies were explored to explain the reliability of CRISPR-Cas-based diagnostic tools.

2.1.1 HISTORY OF CRISPR-CAS SYSTEM

The CRISPR-Cas gene editing technology is an adaption of the bacterial immune system. An unusual genetic structure composed of repeating DNA segments separated by non-repeating sequences was first observed by Japanese scientists (Ishino et al., 1987). It was later discovered that these genomic structures were part of the acquired bacterial immune system (Barrangou et al., 2007). The acquired immunity in bacteria against bacteriophage is achieved through three stages, adaption, expression, and interference (Makarova et al., 2011). In the adaptive stage, upon infection by bacteriophage, Cas enzymes in bacteria cut the phage DNA at a specific site (approximately 30 bp), recognized by PAM. Short segments of bacteriophage genomic DNA (protospacer) were inserted with a duplicated noncontiguous direct repeating segment added adjacent to the inserted segment at the CRISPR locus. The expression stage involves the transcription of cr-RNA, from the CRISPR locus of the bacterial genome. During the expression stage bacterial genome transcribes the spacer-containing region from Pre-crRNA. A Tracer RNA, which is constitutively expressed in a bacterial system, specifically detects and binds to the CRISPR repeat sequence adjacent to protospacers in Pre-crRNA. CRISPR-associated proteins such as RNAase III process the Pre-crRNA:Tracer RNA complex into guideRNA (gRNA). In the interference stage, Cas enzymes get activated upon loading the gRNA and specifically detect and cleaves the target DNA (Makarova et al., 2011). Thus, Cas enzymes loaded with gRNA can specifically damage the bacteriophage DNA and confers resistance.

2.1.2 TYPES OF CRISPR-CAS SYSTEMS

Based on the evolutionary relationship CRISPR Cas system constitute two classes, six types and subtypes (Kick et al., 2017; Xu & Li, 2020). Based on the composition of the ribonucleoprotein (RNP) effector complex (effector nuclease) CRISPR-Cas system is divided into two classes. Class 1 has multiple effector proteins involved in the formation of the effector complex. In the Class 2 systems, effector nuclease involves a single crRNA binding protein. Hence, Class 2 Cas enzymes are widely used in diagnostics as they are easy to reconstitute. Classification of Class 2 enzymes and their requirements for specifically cleaving the target are given in Table 2.1. Mechanisms of Cas enzymes such as Cas9, Cas12, and Cas13 are given in Figure 2.1.

TABLE 2.1 Classification of Class II Cas Enzymes and Requirements for Cleavage of Target

Class	Types	Cas Enzymes	Type of DNA Cleavage	Tracer RNA Requirement	cRNA	Collateral Activity	References
Class II	Type II	Cas9	Blunt cut, double-strand break	Required	Required	No	Jinek et al. (2012); Makarova et al. (2020)
	Type V	Cpf I (Cas12a) or C2c1 (Cas12b)	Generates 5’ overhang, double-strand break	No for Cas12h and Cas12i; Yes for Cas12b and Cas12g.	Required	Cas12a, Cas12b-collateral ssDNA degradation	Chen et al. (2018); Rusk (2019)
	Type VI	C2c2 (Cas 13a), C2c6 (Cas 13b)	Targets single-strand RNA	No	Required	Cas13-collateral RNA degradation	Abudayyeh et al. (2016)

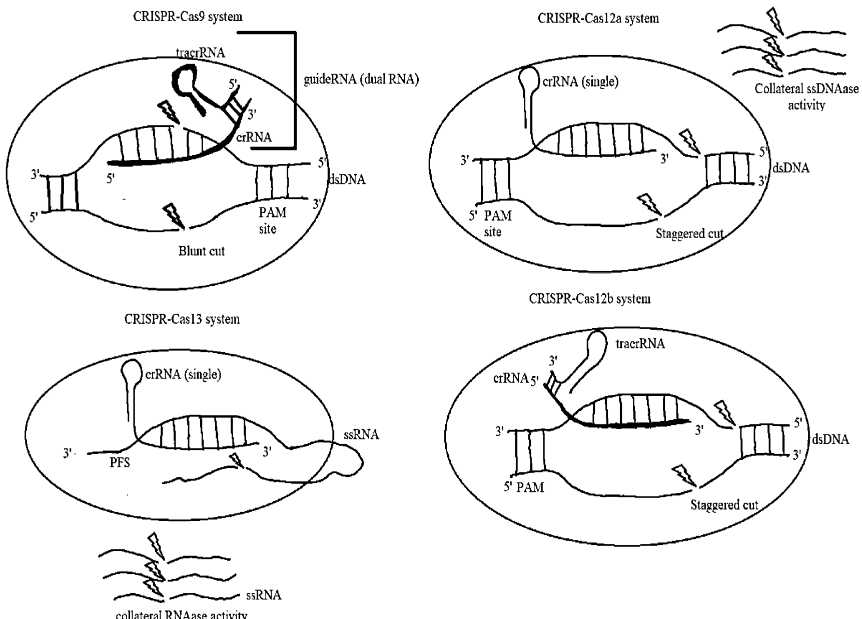

FIGURE 2.1 Mechanism of Cas9, Cas12, and Cas13 enzymes binding to target sequence.

2.2 CRISPR-CAS 9 SYSTEM AND ITS MODIFICATION FOR DIAGNOSTICS

Cas9 enzymes are best characterized Class II enzymes and have been widely used and modified for diagnostic purposes. The enzyme requires Tracer RNA for generating gRNA. The effector nuclease forms double-teamed blunt cuts on target DNA. Cas9 enzyme require PAM motif, NGG, and very rarely NAG (N-any nucleotide) to bind to the DNA. PAM motifs are located downstream to the binding site of Cas9. Cas9 enzymes cleave the DNA using two separate catalytic domains, a RuvC-like domain and C-terminal HNH-like domain. RuvC domain cleaves the noncomplementary strand and HNH domain cleaves the complementary strand binding to cRNA-gRNA (Jinek et al., 2012). Each catalytic domain cleaves one strand of DNA resulting in double-stranded blunt-end cleavage of DNA or with short 3 bp overhang (Vriend et al., 2014). Cas9 enzymes can be made catalytically dead by mutating both catalytic domains, but the mutated Cas9 can still bind to DNA at specific sites. This property of Cas9 has been put to use in visualizing genomic loci in living cells (Chen et al., 2013).

2.2.1 CAS9-NASBA-BASED DETECTION OF TARGET GENE

Cas9 system in combination with nucleic acid sequence-based amplification (NASBA) technique was used to differentiate between African and American Zika virus strains. After preamplification of virus RNA by NASBA technique, the sample will be then applied to a toehold RNA-regulated LacZ mRNA along with guideRNA and Cas9 enzyme. Cas9 enzyme specifically detects strain-specific PAM motif and cleaves the DNA. If strain Specific PAM motif is not present on target RNA, it will activate toehold RNA upstream to LacZ gene. Activation of LacZ gene leads to color production in the presence of substrate (Pardee et al., 2016). Figure 2.2 shows the NASBA-Cas9-based method of detection and differentiation of viral strains.

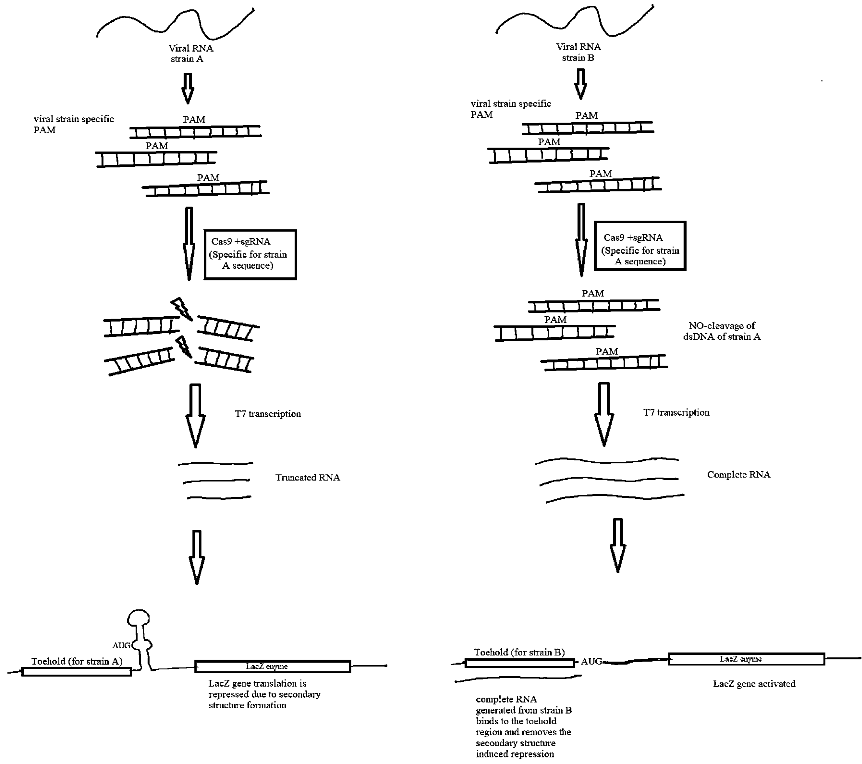

FIGURE 2.2 NASBA-Cas9-based method of detection and differentiation of viral strains.
Source: Pardee (2016).

2.2.2 CRISPR-CAS9 CHIP FOR DETECTION OF TARGET GENE

Catalytically deactivated Cas9 system directly immobilized on a graphene sheet to develop CRISPR-enhanced graphene-based field-effect transistor (gFET or CRISPR-chip). CRISPR-Chip is the best example for using the Cas9 system for preamplification-free detection of nucleic acid biomarkers. Catalytically deactivated Cas9 scan the whole genome sample binds to the target sequence. Hybridization of target DNA to CRISPR-gRNA complex will lead to change in electric characteristics of CRISPR-Chip (Hajian et al., 2019). Figure 2.3 shows the diagram of CRISPR-Cas9-based chip for detecting target DNA sequences.

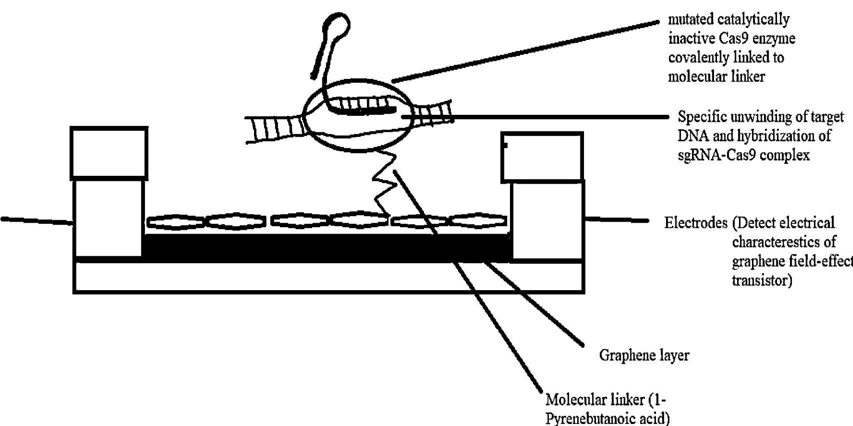

FIGURE 2.3 CRISPR-Cas9-based chip for detecting target DNA sequence.
Source: Hajian (2019).

2.2.3 CRISDA TECHNIQUE FOR ISOTHERMAL AMPLIFICATION OF TARGET GENE

Using PCR machine to amplify DNA is a major hurdle in onsite diagnostics for detecting nucleic acid biomarkers. CRISPR–Cas9-triggered nicking endonuclease-mediated Strand displacement amplification method (CRISDA) technique using Cas9H840A system could isothermally amplify and detect the target DNA. Cas9 enzymes with H840A mutation is a nickase, which cleaves only one of the strands of dsDNA (Nickases) while exposing the other strand. CRISDA method of isothermal amplification require Cas9H840A enzyme, Specific guideRNA, forward and

reverse primer with 5' Nb.BbvCI endonuclease nicking site for initiating the amplification. Specific gRNA is designed to target the DNA at two sites (~186 bp). Upon reconstituting the enzyme and gRNAs, the enzyme will nick the DNA from both sides exposing the complementary strand for primer binding. Upon reconstituting the klenow DNA polymerase (exo-), DNA polymerization will be initiated from both ends leading to amplification of the target DNA. The targetDNA has active Nb.BbvCI nickase site at the 5' end. Nb.BbvCI nickase enzyme cleaves the DNA, Klenow polymerase (exo-) binds to the nicked site and add new nucleotide and displaces the other strand. Thus, a continuous cycle of nicking activity and strand displacement by klenow polymerase (exo-) leads to amplification of target DNA at isothermal conditions. A specific peptide nucleic acid (PNA) probes detects the amplified target DNA (Zhou et al., 2018). Figure 2.4 shows the diagrammatic view of CRISDA technique.

2.2.4 CAS-EXPAR-BASED ISOTHERMAL AMPLIFICATION OF TARGET GENE

Isothermal amplification of target gene was possible using Cas9 system. Cas9 enzymes cleave single-stranded DNA and single-stranded RNA when Cas9-sgRNA system is treated with separate PAM containing oligonucleotide nucleotide (PAMmer) (O'Connell et al., 2014). This property of Cas9 system was made used to achieve isothermal amplification of the target gene in CRISPR/Cas9 triggered exponential amplification method (CAS-EXPAR). Cas-Expar technique requires an exogenous single-stranded Expar template oligonucleotide containing Nickase enzyme recognition site (Nt.BstNBI) flanking two sgRNA target sequences. After specific cleavage of single-stranded DNA by Cas9-sgRNA-Pammer complex, the specifically cleaved single-stranded DNA will bind to the Expar template oligonucleotide. DNA polymerase enzymes further add nucleotide, and a complete dsDNA is generated. The generated new dsDNA has a nickase enzyme recognition site in the center, Nt.BstNBI creates a nick and vent (exo-) DNA polymerase binds to the nicked region and adds nucleotides, and displaces the complementary single-strand DNA. This released single-stranded DNA serves as a primer for amplifying the target DNA. Thus, cycle of Cas9 cleavage, binding of Expar template oligonucleotide, nicking by Nt.BstNBI, vent (exo-) DNA polymerase activity to release

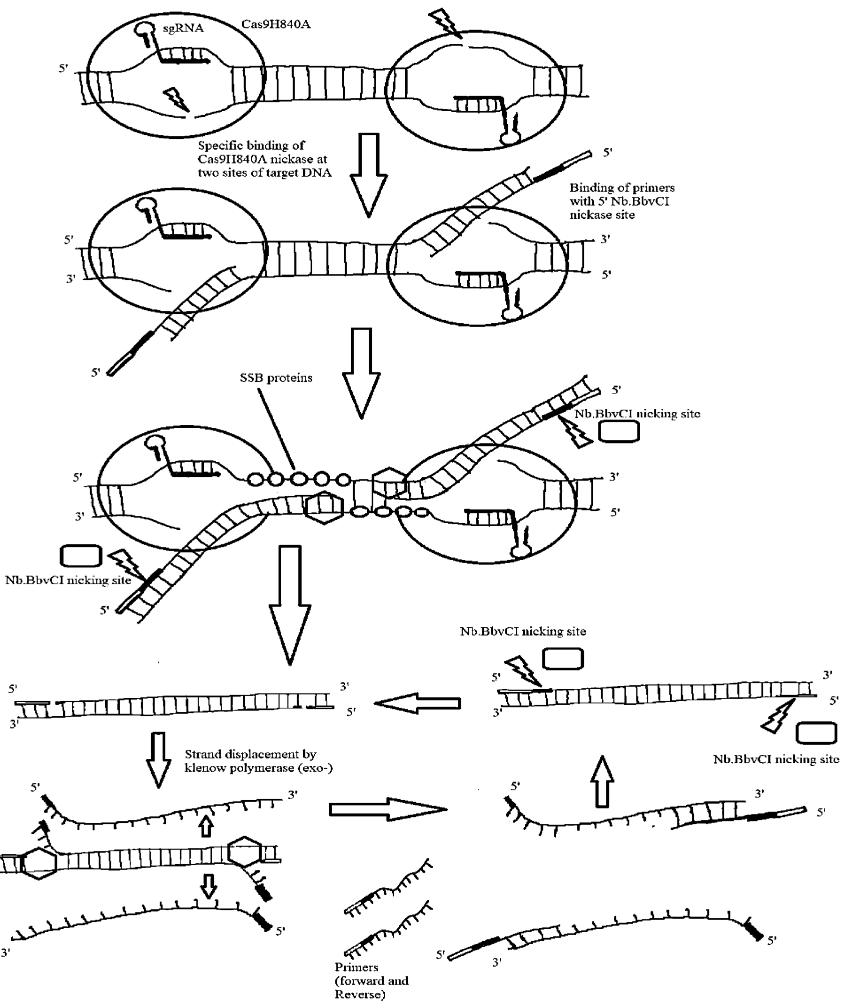

FIGURE 2.4 Diagrammatic view of CRISDA technique.
Source: Zhou et al. (2018).

a single strand leads to amplification of the target gene (Huang et al., 2018). Figure 2.5 shows the diagrammatic view of the Expar technique of isothermal amplification using CRISPR-Cas9 system.

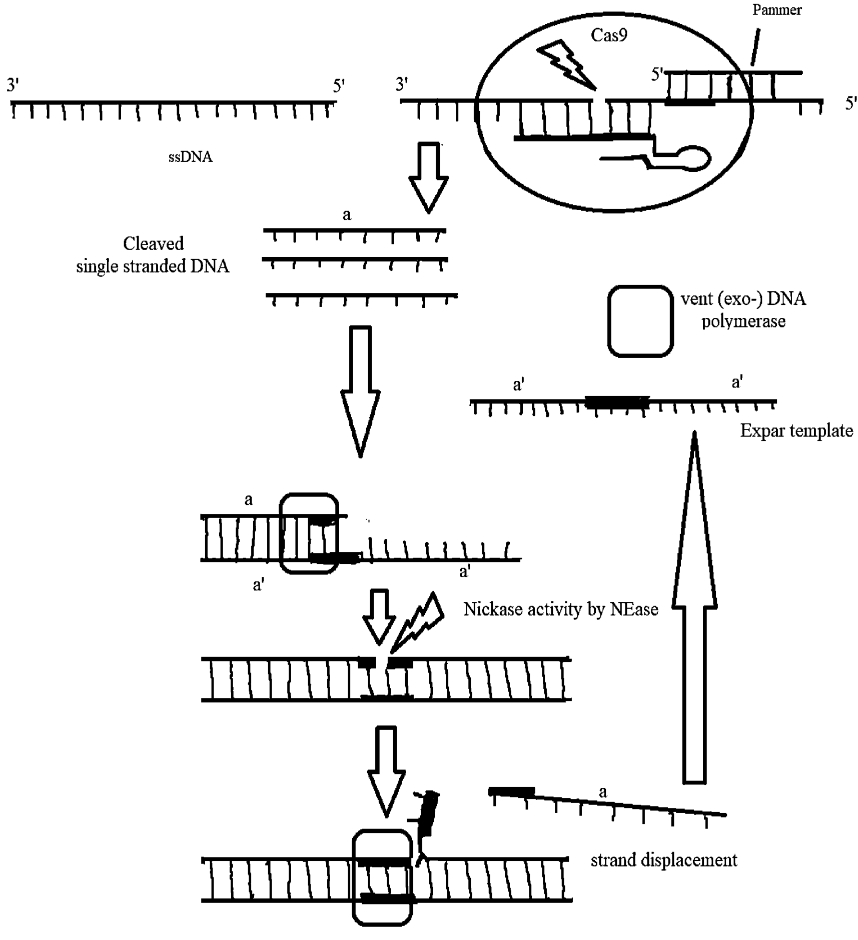

FIGURE 2.5 Cas-Expar technique of isothermal amplification using CRISPR-Cas9 system.

Source: Huang (2018).

2.2.5 RNA-GUIDED CAS9 SYSTEM FOR ISOTHERMAL AMPLIFICATION

Isothermal amplification was achieved using RNA-guided Cas9 system and (exo-) klenow polymerase. The target dsDNA is specifically nicked using nCas9 (D10A) and sgRNA. (exo-)klenow polymerase adds nucleotides and

displaces the opposite single-stranded DNA. Specific primers containing protospacer and PAM on 5' end will bind to the released single-stranded Target DNA. The (exo-)klenow polymerase adds nucleotides and forms dsDNA. The dsDNA formed has protospacer and PAM motif at 5' end, nCas9-sgRNA complex target this region and again insert a nick. The (exo-)klenow polymerase binds to the nicked DNA region and add nucleotide and displaces the complementary single stranded DNA. The second set of primers containing protospacer and PAM on 5' end will bind to the released single-stranded Target DNA. Thus, the cycle of specific primers containing protospacer and PAM motif at 5' end specific nCas9 nicking leads to amplification of target at isothermal condition (Wang et al., 2019).

2.3 CRISPR/CAS12 SYSTEM AND DIAGNOSTICS

Cas12 are class II type V enzyme. It requires gRNA for specific target DNA detection and induces specific cleavage of target DNA. Cas12 enzymes, Cas12a requires single crRNA for activation, but Cas12b requires dual RNA, both tracer and gRNA for activation (Murugan et al., 2017). Cas12a enzyme process its own crRNA (Swarts et al., 2017; van der Oost et al., 2017). Cas12 enzyme specifically recognizes a T rich PAM. Cas12a enzyme specifically targets dsDNA and form a staggered cut. Cas12a also degrade nonspecific single-stranded DNA along with specific cleavage of dsDNA target. This property of the Cas12a enzyme is made used in CRISPR-Cas12-based diagnostics to detect viral genomes (Chen et al., 2018). Cas12a enzyme cleaves the specific double-stranded target DNA by RuvC catalytic domain (Stella et al., 2017). Nonspecific dsDNA degradation (collateral activity) is proposed to be happening in RuvC domain itself (Li et al., 2018).

2.3.1 CAS12-BASED DETECTION OF RNA-DETECTR

DETECTR is a Cas12-based diagnostic method to detect human papilloma virus directly from anal swabs. Crude DNA extracted from the anal swabs were treated with either Cas12a-crRNA specific to Human papillomavirus 16 or 18 (hypervariable loop region of HPV16 or 18). crRNA specifically detected the target and induced a double-stranded cleavage of target DNA. Collateral ssDNAse activity initiated immediately after cleavage of specific target DNA leads to the cleavage of the fluorescent quenched

reporter (ssDNA). Fluorescent signal released indicates the presence of HPV16 or HPV18 (Chen et al., 2018).

2.3.2 CAS12-BASED DNA DETECTION-HOLMES

A Cas12a-based diagnostic HOLMES (a one-HOur Low-cost Multipurpose highly Efficient System) combines Rapid PCR-based amplification of target gene and then detection by Cas12a-crRNA complex. Similar to DETECTR technique This method also made use of the collateral ssDNA degradation activity of Cas12a enzyme to cleave the quenched fluorescent ssDNA reporter following binding to the target DNA. Amplification of the target DNA using PCR increased the specificity of detection by HOLMES. HOLMES could rapidly detect a gout risk-related short nucleotide polymorphism (SNP), rs101429 and was able to discriminate between PRV Ra classical strain and Bartha-K61 vaccine strain (Li et al., 2018).

2.3.3 CAS12B-BASED DNA DETECTION (CD DETECTION)

Compared to Cas12a enzyme Cas12b require both tracerRNA and gRNA for detection of specific target DNA. In the CD detection technique, tuned guideRNA (tgRNA)-Cas12b system was able to detect the dsDNA target specifically. CD detection could directly detect the human papilloma dsDNA concentration at attomolar level. CD detection with fine-tuned specific sgRNA could discriminate the human blood groups with high specificity. The tgRNA-Cas12b system also detected cancer-based SNPs such as *TP53* 856G>A, *BRCA1* gene (3232A>G and 3537A>G). Cas12b enzyme had preference for non-targeted ssDNA cleavage. Cas12b-sgRNA complex preferred to cleave poly-thyamine, poly-adenine, or poly-cytosine-based fluorophore quencher (FQ)-labeled homopolymer reporters (Teng et al., 2019).

2.4 CRISPR-CAS13 SYSTEM AND ITS MODIFICATION FOR DIAGNOSTICS

Cas13 enzymes are class II type VI enzyme. The Cas13 enzyme has RNAase activity and gRNA processing activity. The enzyme has both

RNAse and gRNA maturation activity. The RNAse activity is through higher eukaryotic and prokaryotic nucleotide (HEPN) binding domain and gRNA maturation activity is mediated by both HEPN2 and helical-1 domain (O'Connell, 2019). One of the important properties of Cas9 enzyme is its collateral activity, once the HEPN domain of Cas13 enzyme is activated by binding to specific gRNA it cleaves specific target RNA and in addition to the target it will also degrade the neighboring nonspecific RNA (Abudayyeh et al., 2016). This collateral degradation activity of Cas13 enzyme is put to use in CRISPR/Cas13-based diagnostics to amplify the signal after specific cleavage.

2.4.1 CAS13-BASED DIAGNOSTIC SPECIFIC HIGH SENSITIVITY ENZYMATIC REPORTER UNLOCKING (SHERLOCK)

Cas13-based diagnostic SHERLOCK could detect Zika virus and Dengue virus at attomolar sensitivity. In this method, the recombinase polymerase amplification (isothermal preamplification) technique was coupled with T7 Transcription to convert target DNA into RNA. Cas13 with sgRNA specifically detect the target DNA and induce cleavage of specific viral RNA. Collateral RNA degradation initiated by Cas13 after detecting specific RNA target degrades quenched RNA probes releasing the fluorescence. Appearance of fluorescence indicates the presence of viral RNA. SHERLOCK technique was also able to differentiate between related Zika virus and Dengue virus (Gootenberg et al., 2017). SHERLOCK one pot testing kit for detecting SARS-CoV-2 is a Cas13-based diagnostic tool, it showed 98.5% specificity and 93.1% sensitivity (Joung et al., 2020).

2.4.2 CAS13-BASED DIAGNOSTIC STREAMLINED HIGHLIGHTING OF INFECTIONS TO NAVIGATE EPIDEMICS (SHINE)

SARS-CoV-2 detection was done using Cas13-based diagnostic, SHINE. SHINE technique was able to detect 10 copies of viral RNA per microliter. SHINE is a modification of SHERLOCK method. This method combined various steps – reverse transcription of RNA to DNA, recombinase-based polymerization (isothermal amplification of target), T7 transcription and Cas13-based detection of target RNA into a single step. Upon detection of

viral RNA, Cas13-gRNA cleaves the quenched fluorescent reporter tagged to ssDNA (Arizti-Sanz et al., 2020).

2.4.3 CAS13-BASED DETECTION OF VIRUS (CARMEN)

Combinatorial arrayed reactions for multiplexed evaluation of nucleic acids (CARMEN) is a Cas13-based platform for multiplexed pathogen detection (Ackerman et al., 2020). In this method, input samples are initially amplified by RPA method and then undergone T7 transcription, and then the samples are emulsified using fluorous oil to form emulsion droplets of 1 nanoliter size. Each sample for analysis is color coded for identification. Similarly, emulsion droplets containing Specific crRNA for target recognition, Cas13 enzyme and fluorescent quenched ssRNA reporters were prepared. All color-coded emulsions were mixed and loaded onto a microwell array chip, each well in microarray can hold two nanoemulsion droplets in random. Nanodroplets will be merged after application of electric field. Upon merging of nanodroplet, specific cRNA-Cas13 system will bind to the target and fluorescent reporter will be cleaved. Each droplet is identified and monitored by fluorescent microscopy. Figure 2.6 shows the diagrammatic view of the microwell array chip containing nanodroplet.

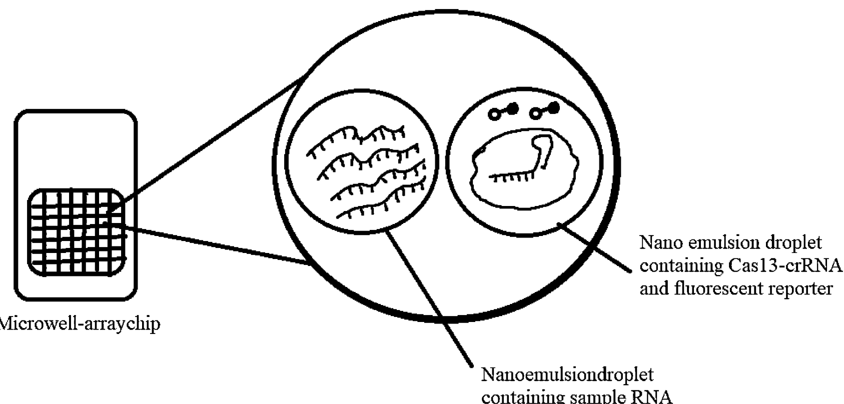

FIGURE 2.6 Diagrammatic view of microwell-array chip used in Cas13-based CARMEN.

Source: Ackerman (2020).

2.5 CRISPR-CAS SYSTEM AS REPORTER

Collateral activity of CRISPR-Cas system can be used as a method to amplify signals. Cat-SMelor technique combines the ability of a transcription factor to bind to specific sequence and the ability of CRISPR-Cas system to specifically bind to the target DNA (Liang et al., 2019). Cat-SMelor-based techniques were used to detect uric acid (25–500 nM) and p-hydrobenzoic acid (9–180 nM) in serum. In this method target DNA for both transcription factor and CRISPR-Cas system are same therefore, they compete for the target DNA sequence. In CaT-SMelor technique a bacterial transcription factor HucR fused to cellulose binding domain was immobilized on microcrystalline cellulose. The HucR-based CaT-SMelor technique was able to detect the levels of uric acid in serum. The bacterial transcription factor HucR constantly bind to the target DNA containing HucR binding motif and PAM sequence. HucR is a uric acid responsive protein, upon binding of uric acid HucR factors dissociate from target DNA, allowing the specific CRISPR-Cas12a system to bind and cleave the target dsDNA. Collateral nuclease activity initiated cleaves the fluorescently quenched ssDNA probes. The rate of cleavage of quenched fluorescent probe was depended on the concentration of uric acid. Figure 2.7 shows the diagrammatic view of CAT-SMelor technique.

2.6 CRISPR-BASED DIAGNOSTICS AND RELIABILITY

Recent whole genome sequencing studies following chromatin immunoprecipitation (CHIP) of a catalytically inactive Cas9 enzyme showed that only 1 to 5 bp of the immunoprecipitated DNA matched to the complimentary region in gRNA. These regions of gRNA are called seed sequence. This indicates that 1 to 5 bp adjacent to the PAM sequence or seed sequence on gRNA determines the specificity of Cas9 binding (Wu et al., 2014). The sequencing studies following CHIP only gave information of DNA bound to Cas9, but not the cleavage event (Duan et al., 2014; Zhang et al., 2015). Therefore, it is not sure if all these Cas9 bound sites are cleaved. Frequency of the seed sequence in the genome determines the specificity of binding. Therefore, CRISPR-Cas9-based diagnostics, which does not involve cleavage events such as direct observation of genomic loci could be interfered by off-target effects and therefore require optimization of guideRNA. The PAM sequence also affects the specificity

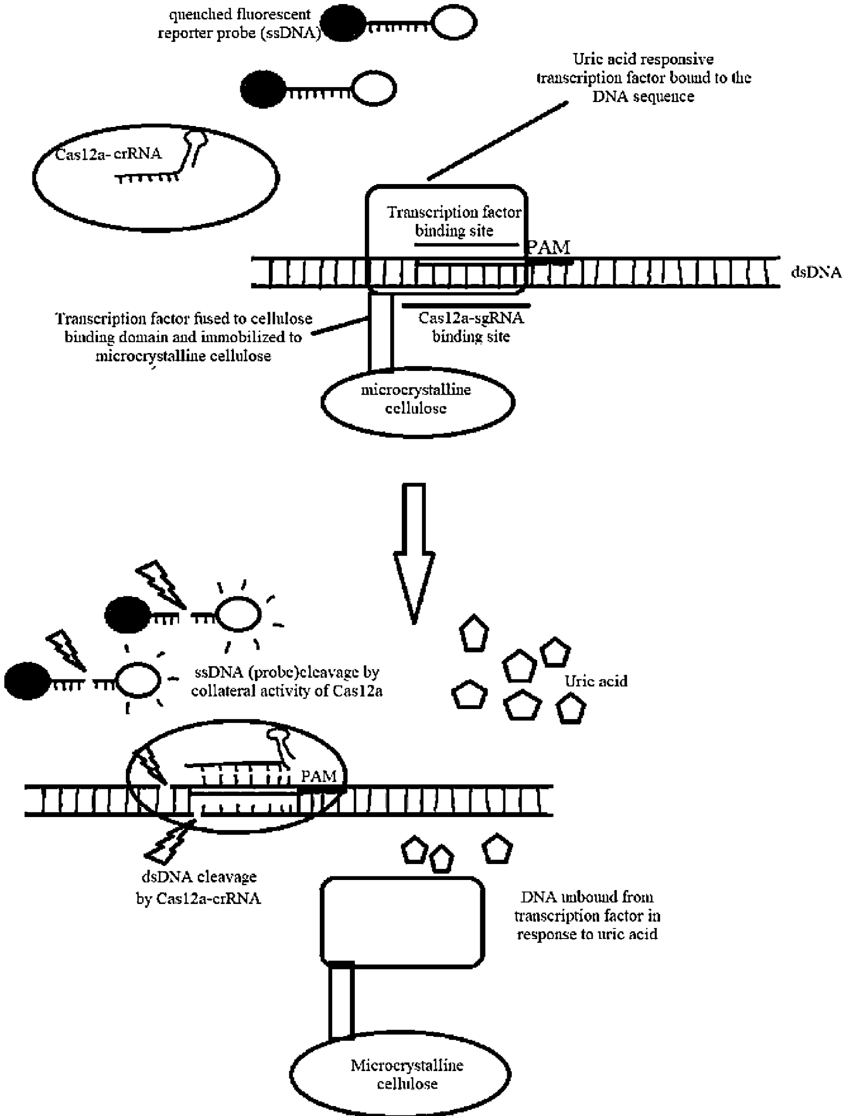

FIGURE 2.7 Diagrammatic view of CAT-SMelor technique.
Source: Liang (2019).

of binding, it was shown that Cas9 system uses the PAM sequence NGG (N for A, T, G or C) but Cas9 system also has less frequent preference for PAM sequence NRG (R for G or A) therefore diagnostics tools designed based on the Cas9 system should carefully consider for the presence of other less frequent PAM sequences in the system (Zhang et al., 2015). For improving the specificity of the CRISPR-Cas system, potential off target regions should be predicted and avoided. Target sequence with global similarity should be avoided (Figure 2.8).

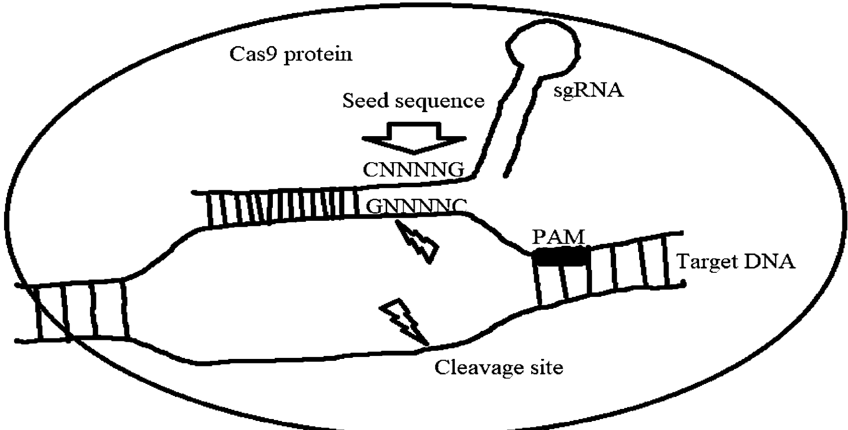

FIGURE 2.8 Seed sequence determines the specificity of Cas9 binding to target DNA.
Source: Zhang (2015).

2.7 CONCLUSION

Detecting genetic material of pathogens is the major requirement to confirm a disease condition. Detecting the pathogens at very low concentration could help in finding the disease at very early stages. Therefore, specificity and sensitivity of diagnostic tools need to be improved. In addition, diagnostics should be able to be performed without heavy machinery. The current pandemic has shown the requirement of portable diagnostic tools. CRISPR-Cas-based diagnostics tools are solution for all these problems. CRISPR diagnostic tools help in detecting DNA-based or RNA-based target DNA directly without machinery, such as thermal cycler. Different

isothermal DNA amplification techniques have helped to improve the sensitivity of the CRISPR-Cas-based diagnostic tool. The recent development of various CRISPR-Cas-based diagnostic tools showed that they are very reliable. Collateral activity observed in Cas12 and Cas13 system largely helped in amplifying the signals in CRISPR-Cas-based diagnostics. Various studies show that CRISPR-Cas system-based diagnosis has very high specificity and sensitivity. SHERLOCK-CRISPR Cas13-based technique could detect SARS-CoV-2 RNA with 100% specificity and 100% sensitivity in fluorescent read-out method (Patchsung et al., 2020). Cas12b-based diagnostic tool could detect SARS-CoV-2 mRNA with 98.5% specificity and 93.1% sensitivity. The CRISPR-Cas system was modified to use as reporter, in CaT-SMelor. In CAT-Smelor it is used to detect the concentration of small molecules in serum (Liang et al., 2019). The CaT-SMelor technique was able to detect the concentration of small molecules in the serum. This study showed that collateral activity of Cas enzymes can be quantified by comparing the rate of cleavage of fluorescent probes.

KEYWORDS

- **CRISPR-Cas**
- **deoxyribonucleic acid**
- **disease diagnostics**
- **peptide nucleic acid**
- **protospacer adjacent motif**
- **ribonucleic acid**
- **short nucleotide polymorphism**

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CHAPTER 3

Engineered Gut Microbiome in Treating Diseases and Its Applications in Modern Biotechnology

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ABSTRACT

Microbiomes are composed of trillions of diverse microbial communities that evolved with us and existed in all ecosystems. Any hindrance in the balance of the microbial communities leads to chronic diseases and creates an imbalance in the ecosystem. Gut microbes play a crucial role in health and disease and in maintaining the ecosystem. Manipulation of the commensal bacterial metabolites can be applied in treating diseases such as IBD, cancer, diabetes, metabolic diseases, and many more. Microbiome engineering thus holds a promising future in increasing agricultural sustainability and balance in the ecology. This chapter mainly focuses on the engineered bacterial strain to produce metabolites which then incorporate into the host microbiota for therapeutic treatment, and the current applications of microbiome engineering, mainly in humans, plants, animals, and soil reviewed in improving human health and agricultural productivity.

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3.1 INTRODUCTION

Adaptation to microorganisms to form ecological communities (microbiomes) on earth occurred over millions of years of co-evolution before any existence of humans, which shows that humans have learned and co-evolved with them (Grice & Segre, 2012; de Vos & de Vos, 2012). These microbial communities exist in living hosts such as humans, plants, animals, oceans, soil, and air. They interact with their hosts and form synergistic relationships. These interactions in human determine the composition of the gut microbiome, which is associated with the physiology and physiological health of the host (Grice & Segre, 2012), and the microbiome composition of the soil influence the productivity of crops and ecosystem (Chaparro et al., 2012).

With the increase in recent advances in metagenomic analyzes, it has provided us with many information about the composition of the gut microbiota and their differences in healthy and diseased individuals. More than 25 diseases are linked to the alteration of the intestinal microbiota (de Vos & de Vos, 2012). The most studied diseases that are linked to the altered gut microbiota are metabolic syndrome, obesity, type II diabetes, inflammatory bowel diseases (IBDs), Crohn's disease, colorectal cancer, Alzheimer's disease (AD), autoimmune disease, and Parkinson's disease (Backhed et al., 2004). Studies showed that in few cases metabolites released from the gut microbiota directly affect the host (Feng et al., 2018; Bilotta & Cong, 2019). Such studies suggest manipulation of the gut microbial metabolites in improving host health and treating diseases. Approaches for alteration of gut microbial metabolites include use of medication, manipulation of diet, manipulation of gut microbiota by probiotics (Mukherjee et al., 2018; Choi & Cho, 2016). This chapter focuses on the genetically engineered specific bacterial species to produce beneficial metabolites which then incorporate in the host microbiota of target clinical condition and the current applications of microbiome engineering in improving human health and agricultural productivity.

Engineered bacteria offer more therapeutic benefit than localized bacteria because they can be manipulated to have additional features and also to produce higher yields of bacterial products (Pinero-Lambea et al., 2015). Till now, engineered products produced by therapeutic bacteria include interleukins (ILs), enzymes, vaccine antigens, and antibodies. Bacteria chosen for modification must be localized to the appropriate target

region since the composition of the gut microbiota varies longitudinally and latitudinally (Sekirov et al., 2010). The commonly used genetically engineered bacterial strains are *Escherichia coli* and lactic acid bacteria as it can culture in large-scale, easy genetic manipulation and has low toxicity (Behnsen et al., 2013).

3.2 ENGINEERED LACTIC ACID BACTERIA IN INFLAMMATORY BOWEL DISEASE (IBD)

Lactic acid bacteria are gram-positive, non-pathogenic, non-spore forming, facultative anaerobes that are used in food fermentation. Studies showed that lactic acid bacteria like *Lactococcus lactis* is not a part of the human gut flora but can survive in the human gut environment (Klijn et al., 1995). In IBD patients, it has been observed that there is an overall decrease in microbial diversity and stability in the intestinal microbiota (Scaldaferri et al., 2013). The current therapies to treat IBD are based on anti-inflammatory drugs integrated with immunosuppressives (Kuhbacher & Folsch, 2007). Recombinant lactic acid bacteria such as *L. lactis* strain was used to treat and prevent colitis by producing and delivering an anti-inflammatory cytokine IL-10 in different mouse models *in situ* (Steidler et al., 2000). Colitis in mice induced by administration of dextran sodium sulfate (DSS) showed a decrease in 50% after daily mucosal administration of recombinant *L. lactis* secreting IL-10 (Dumot et al., 2001).

Lactic acid Bacteria producing trefoil factors (TFFs) to treat IBD has also been studied. TFFs are non-mitogenic peptides which plays an important role in repairing and protection of intestinal mucosa after damage (Playford et al., 1995), which is why it has become an interesting molecule to treat IBD. These molecules when administered by an oral route, adheres to the gut and then absorbed at the intestinal level. Interestingly, when recombinant *L. lactis* secreting TFF were administered intragastric, it expressed active peptides in the colon, which prevents and repairs the damage caused by acute colitis induced by DSS (Vandenbroucke et al., 2004). Another study showed that use of recombinant *L. lactis* secretes LcrV antigen, an inflammatory protein produced by *Yersinia pseudotuberculosis* is also used to treat colitis. Two colitis murine models, i.e., DSS and trinitrobenzene sulfonic acid was used to evaluate the therapeutic and protective potential of this strain (Foligne et al., 2007).

Another strategy to treat IBD is oral administration of antioxidant enzymes. Previous studies have shown that the inpouring of macrophages and neutrophils with the production of inflammatory mediators like cytokines, proteases, and reactive oxygen species (ROS) leads to inflammation in the gastrointestinal tract (Segui et al., 2005). ROS includes hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (·OH). Reactivity of these ROS towards DNA, proteins, and lipids causes mutagenic cellular damage and cytotoxicity (Grisham et al., 1990). To detoxify ROS, antioxidant enzymes like catalases (CAT) and superoxide dismutase (SOD) degrade H_2O_2 and O_2, respectively—thus preventing the formation of ·OH (Wardman, 2007). Many studies with this context have shown that use of recombinant strain of *Lactobacillus* spp. expressing either CAT or SOD can reduce GIT inflammation in mouse models (Rochat et al., 2007; Watterlot et al., 2010). A natural protease inhibitor elafin which is expressed in healthy intestinal mucosa is diminished in IBD patients, therefore, use of lactic acid bacteria secreting elafin in acute and chronic colitis showed protection against inflamed epithelium from increased intestinal permeability and from release of chemokines and cytokines (Motta et al., 2012).

It has been well established that recombinant lactic acid bacteria producing *L. lactis* strain is widely used in the production of heterologous protein and is considered as live delivery vector model and lactic acid bacteria model (Bermudez-Humaran, 2009). However, this bacterium has a shorter life span of about 24 hours in the human GIT thus leading to a reduced time of action. A study carried out by Hamady et al. chose *Bacteroides ovatus* bacterium for *in vivo* delivery of proteins due to its ability to utilize properties of xylan and colonize the colon (Hamady, 2013; Hamady et al., 2008). They developed a xylan-regulated delivery of human tumor growth factor TGF-b_1 in mice to treat colitis (Hamady et al., 2011) and human keratinocyte growth factor-2 to treat inflamed colon (Hamady et al., 2010). These promising developments confirm the potentiality for *in vivo* delivery of recombinant commensal.

3.3 ENGINEERED *E. COLI* IN OBESITY, DIABETES, CARDIOMETABOLIC DISEASE, AND CANCER

E. coli are gram-negative facultative anaerobe reside in the normal gut of mammals. Several *E. coli* are pathogenic which causes infectious

diseases, but the majority belongs to commensals (Huang et al., 2012). Some common lab strains of *E. coli* used for engineering are BL21DE3 and MG1655 but *E. coli* Nissle 1917 (EcN), a probiotic strain that highly colonize, was used previously by Germany for treating intestinal infections, chronic constipation and ulcerative colitis (Behnsen et al., 2013). *E. coli* has been used as a model organism for several synthetic biology studies because of its rapid growth, high yield production, ease of genetic manipulation, biochemistry, and well-characterized genetics (Huang et al., 2012).

A recent study revealed that incorporation of EcN in gut microbiota can inhibit the development of obesity and can treat related metabolic disorders. N-acylphosphatidylethanolamines (NAPE) and N-acylethanolamines (NAE) produce bioactive lipids which regulates food intake but production of these is downregulated in fat diet. NAPE is a biosynthetic precursor of NAE and several studies are focusing on increasing its bacterial production (Gillum et al., 2008). Reduced inflammation, reduced pain and increased satiety are some of the beneficial effects that shown on increasing NAEs (Piomelli, 2013). In a study, C57BL/6J mice were maintained on a high-fat diet and administered with NAPE expressing EcN in the drinking water, showed an inhibition of the development of obesity. Due to colonization in the intestine, this anti-obesity effect persisted for 4 weeks after ending the treatment (Chen et al., 2014).

Engineered EcN have also been developed for diabetes treatment. Engineered EcN has revealed that it produces insulin topic protein GLP-1 or pancreatic and duodenal homeobox gene 1 (PDX-1) (Duan et al., 2008) which stimulate the intestinal epithelial cells to synthesize insulin. *In vitro* studies of GLP-1 (1–37) secreting bacteria are recently exploring whether by oral administration of these bacteria in rat models can enhance hyperglycemia in diabetes (Duan et al., 2015).

Increased risk of cardiometabolic diseases which includes diabetes, non-alcoholic fatty liver and cardiovascular diseases are associated with obesity. NAPE expressing EcN significantly reduced early signs of fibrosis, serum cholesterol, body weight, liver fat as well as inflammation, and some extent of necrosis in a mouse model of fatty liver disease and atherosclerosis (May-Zhang et al., 2019).

The use of engineered *E. coli* in treating cancer is similar to *Salmonella* as it efficiently targets the necrotic region of the solid tumors (Kocijancic, 2016; Weibel et al., 2008). Engineered *E. coli* expressing cytosine

deaminase (CD) ameliorate conversion of 5-Fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU) at the site of tumor (Lehouritis et al., 2013). For the treatment of tumors, several novel deliveries in *E. coli* have been engineered. CTNNB1 (catenin β-1), a gene mutated or over-expressed in colorectal cancer can be suppressed by engineered *E. coli* BL21DE3 through production of short-hairpin RNAs (shRNAs) against CTNNB1 (catenin β-1). Xiang et al. when administered these bacteria to nude mice xenografted with human colon cancer cells, showed significant suppression of CTNNB1 expression in those cancer cells (Xiang et al., 2006). Cytolysin A (ClyA) is a hemolytic bacterial protein that forms pore and has the ability to kill mammalian cells is expressed by engineered *E. coli* MG1655. Single-dose administration of ClyA slows down the rate of tumor growth in BALB/c mice implanted with CT26 colon cancer cells subcutaneously. Treatment with ClyA suppresses the lung metastases thus extending survival in mice injected with CT26 cancer cells intravenously (Jiang et al., 2010).

3.4 ENGINEERED *BIFIDOBACTERIUM* SPP., *BACILLUS* SPP., AND *CLOSTRIDIUM* SPP. IN VARIOUS DISEASES

Bifidobacterium spp. are non-pathogenic, non-spore forming gram-positive anaerobes present in mammals in the lower gastrointestinal tract. *Bifidobacterium* spp. provide many benefits such as preventing carcinogenesis and protection against viral infections (Hidaka et al., 2007). Engineered *Bifidobacterium* are used as a therapeutic for treating IBD. *Bifidobacterium longum* was engineered to produce α-melanocyte-stimulating hormone that has anti-inflammatory properties. This engineered bacterium inhibits DSS-induced ulcerative colitis by colonizing the intestine in mice (Wei et al., 2016). For the treatment of type 2 diabetes (T2D), Wei et al. engineered *Bifidobacterium longum* to express bioactive penetratin – GLP-1 fusion protein (Wei et al., 2015). Another therapeutic strategy with the concept of delivering engineered *B. longum* secreting human manganese superoxide dismutase (rhMnSOD) through transporter peptide PEP-1 penetratin fusion protein in the colon has also been studied (Liu et al., 2018). Oral administration of *Bifidobacterium* is more stable in the intestine and increases its efficiency and retention time of target drugs. *Bifidobacterium* are used as a model organism for treating cancers

because similar to *E. coli* and *Salmonella*, *Bifidobacterium* grow in the hypoxic region of tumors. Engineered *Bifidobacterium* strains delivers cytosine deaminase enzyme to hypoxic region of solid tumors that inhibit tumor growth. Engineered *B. adolescentis* secreting endostatin inhibits angiogenesis therefore suppressing the growth of Heps liver cancer cells in induced BALB/c mice (Li et al., 2003).

Bacillus subtilis are gram-positive endospore forming aerobic bacterium (Buescher et al., 2012; Koo et al., 2017). It is found in the gastrointestinal tract of humans and in soil. *Bacillus subtilis* used as a probiotic for both humans and animals due to its GRAS status (Hong et al., 2005). *B. subtilis* production and secretion ability is high (van Dijl & Hecker, 2013) which makes it suitable to use it as a model organism for various genetics and metabolism studies (Buescher et al., 2012; Koo et al., 2017). *B. subtilis* produces vitamins, enzymes, sugars, and engineered *B. subtilis* developed biofilms known as smart living glues (Zhang et al., 2019) Killed *B. subtilis* spores express streptavidin which enable the targeting colon cancer cells (Nguyen et al., 2013). In 2017, *B. subtilis* with 168 strains were constructed with a reduction of genome by 36% (Reuß et al., 2017). In genetically engineered *B. subtilis*, insertion of two chimeric genes in two thymidylate synthase genes, i.e., *thy*A and *thy*B makes the spores more dependent on thymine or thymidine, thus unable to survive in thymine or thymidine deficient environment (Hosseini et al., 2018; Duan et al., 2015).

Clostridium spp. are gram-positive anaerobic bacterium, and it can grow in necrotic or hypoxic regions of solid tumors (Lehouritis et al., 2013). *C. histolyticum* was the first strain to induce lysis and tumor regression in mice. *Clostridia* are injected as spores since it is a spore forming bacteria that travel to the site of the tumor and proliferate only in anoxic areas (Parker et al., 1947). *Clostridium* are pathogenic strains that lead to intestinal infections (Lehouritis et al., 2013). One example of pathogenic strain is *C. tetani* that has found to grow in and shrunk the tumors which results in rapid death of those animals due to elevated toxicity (Malmgren & Flanigan, 1955). Therefore, it is clear that for safe use of treatments, *Clostridium* strains should be non-pathogenic. One such strain is *Clostridium novyi* which was genetically engineered to become non-pathogenic by removing the gene encoding for lethal α-toxin NT. This engineered strain then gets its ability to destroy tumors (Dang et al., 2001) and it also secretes liposomase which inflate the delivery of liposome encapsulated drugs within the tumors (Cheong et al., 2006).

Radio-induced promoters control the expression of gene temporally and spatially, therefore fusion of this promoter improves the gene specificity targeting to hypoxic tumors. Administration of cytosine deaminase expressing *C. sporogenes* incorporated with radio-induced promoters increases the specificity and anti-tumor response of rats induced with rhabdomyosarcoma (Nuyts et al., 2001). Cytosine deaminase expressing *Clostridium sporogenesis* delivers the enzyme to tumor cells in mice which results in anti-tumor effect through intravenous administration of 5-FC (Liu et al., 2002). Engineered *Clostridia* induced with radiation-inducible recA promoter secretes TNFα that directly gets delivered in tumor, thus amplifying the therapeutic use for cancer treatment (Nuyts et al., 2001). Therefore, incorporation of radiotherapy with *Clostridium* expressing protein delivery proves to be a new possibility for cancer therapy.

3.5 APPLICATIONS OF MICROBIOME ENGINEERING

With the advances in meta-omics tools, certain culture independent analysis of microbiome has been made possible to do, which showed a clear picture of how the composition of the microbiome can impact the ecosystem (Chaparro et al., 2012; Mueller & Sachs, 2015). As it becomes more clearer, there is an increasing demand in engineering the microbiomes for shaping the microbiota to change the ecosystem. Microbial communities as well as microorganisms and host within themselves have a complex network of interconnection between them which portray the idiosyncratic characteristics of each ecosystem (Waldor et al., 2015). Any perturbation to the balanced microbiomes leads to dyshomeostasis, which then cause detrimental effect to both host fitness, productivity, and soil fertility (Round & Mazmanian, 2009; Navarrete et al., 2015). Therefore, microbial compositions can be manipulated by microbiome engineering to improve health and ecosystem.

3.5.1 HUMAN MICROBIOME ENGINEERING

Microbiome engineering is mostly applied to human microbiome due to the possibilities of manipulating the gut microbiota for the treatment of various diseases (Grice & Segre, 2012). The largest and most diverse population of microbes inhabit in the gastrointestinal tract of human

(Zhao, 2013). The introduction of human microbiome project (HMP) led to characterize and identify the particular microbiota in human body such as skin, urogenital tract, oral, gut, nasal, and can differentiate the microbiota with healthy and diseased ones (Group et al., 2009). It is well established that any disturbance to the microbiome can lead to gastrointestinal diseases like IBD, ulcerative colitis, *Clostridium difficile* infection (CDI), also diabetes, metabolic diseases, obesity, and many more (Gough et al., 2011).

Fecal microbiota transplantation is a method of collecting fecal material from healthy donors and transplanting it to the gastrointestinal tract of the patients suffering from any disease (Jung Lee et al., 2015). Studies have shown that FMT holds a promising therapy in treating CDI. Previously, CDI was treated traditionally using antibiotics vancomycin and metronidazole to remove *Clostridium difficile,* but more use of antibiotic makes the pathogens resistance to antibiotic which leads to washing out of the beneficial bacterial population from the gut, thus causing the patients to get ill again. Through FMT, it restores beneficial bacteria like *Bacteroidetes* and *Firmicutes*, thus reviving a healthy microbiome (Russell et al., 2014). FMT has also shown its potential in treating IBD, diabetes, multiple sclerosis, rheumatoid arthritis, and autism (Rossen et al., 2015).

Other strategies besides FMT are the antimicrobial peptides (AMPs), i.e., thuricin CD (Rea et al., 2011), pyocin S5 (Saeidi et al., 2011) which by targeting the pathogens can alter the gut microbiota explored in animal model studies. Another strategy is using of prebiotics signaling inhibitor LED209 which prevents autophosphorylation of membrane which in turn activate the virulent factors that eliminate pathogenic strains (Rasko et al., 2008). Other prebiotics, for example, galactooligosaccharides increase beneficial *Bifidobacterium* and decrease pathogenic *Clostridium histolyticum,* which improves health (Costabile et al., 2016). All these studies show the potentiality of microbiome engineering in therapeutic applications performed in mice models, but to understand the efficacy of these strategies, clinical studies need to be performed in humans. Treatment of skin diseases such as psoriasis, atopic dermatitis is treated using probiotic *Lactobacillus* which enhance the growth of *Staphylococcus epidermidis* and hinder the growth of pathogenic bacteria in skin (Grice, 2014). Using AMP C16G2, oral microbiome has been engineered to eliminate *Streptococcus mutans* which causes tooth decay (Guo et al., 2015).

3.5.2 ANIMAL MICROBIOME ENGINEERING

Animals, like humans, also inhabit diverse and large population of microbial communities. Rats and mice were extensively studied but as an animal model for engineering human microbiome. More studies should focus on understanding the animal microbiome with respect to animals such as cows, rats, mice, broiler for improving the health and agricultural productivity. Prebiotics, probiotics, and feed enzymes usage can alter the microbial composition of livestock animals.

Studies shows that most dominant prebiotics such as fructooligosaccharides, xylooligosaccharides, inulin have been used to alter the microbial composition in animals. Administration of prebiotics leads to the production of: (i) antimicrobial factors; (ii) short chain fatty acids (SCFAs) that provides energy, regulate immune system and metabolism; (iii) prebiotic derivatives (Pourabedin & Zhao, 2015). Prebiotics benefits have also been noticed in broiler chicken (De Maesschalck et al., 2015). Delivery of prebiotic inulin in swine gut has shown a rise in probiotic and decline of pathogenic microorganisms (Samanta et al., 2015).

For engineering animal microbiomes, the administration of probiotic has proven to be efficacious. Administration of probiotic has been used to treat infectious diseases and to increase the growth performance as seen in *Bacillus subtilis* CH16. On administration of this probiotic in broiler chicken, gaining weight and reduction in food conversion rate have been observed. The most used dominant probiotic strains *Bacillus*, *Saccharomyces boulardii*, *Lactobacillus*, *Enterococcus*, and *Bifidobacteria* (Nguyen et al., 2015). Amalgamate of probiotics with prebiotics has been shown to increase the probiotic bacterial strain in the gastrointestinal tract (Tanner et al., 2015).

Feed enzymes improve gut health in swine by increasing prebiotics production from non-starch polysaccharide (NSP)-degrading enzymes and enhancing digestion of substrate. Feed enzymes include NSP-degrading enzymes, amylase, lysozyme, phytase, and proteases. So far feed enzymes have shown positive effects in inhibiting pathogens, reducing infectious diseases like salmonellosis, dysentery in piglets and in aiding animal growth (Kiarie et al., 2013).

3.5.3 SOIL MICROBIOME ENGINEERING

Soil microbiome engineering has led to create more microbial and diverse communities which improves soil health and plant fertility.

Soil microbiome is attained by implementing agricultural practices like organic farming (Chaparro et al., 2012). By practicing organic farming, it magnifies biological cycles, microbial diversity of soil, and biological activity with the slightest usage of pesticides and herbicides. Long-term use of organic fertilizers improves soil health by changing the structure of soil microbiome, reduces scattering and increases richness of soil (Hartmann et al., 2015). Other strategies for soil microbiome engineering, which can alter the structure and function of soil microbiome is to change of land utilization that has been observed in tropical forests in South-East Asia where the forests were converted into oil palms plantation (Tripathi et al., 2016). Besides, change of vegetation, other agricultural practices include cropping systems (Xiong et al., 2015), logging (Hartmann et al., 2014) and tillage (Souza et al., 2016) have shown to be effective in shifting the structure and function of soil microbiome that led to alter the microbial diversity and functions of soil. For decades, agricultural practices have been applied to improve crops.

3.5.4 PLANT MICROBIOME ENGINEERING

Microbial communities of plant are diverse, including fungal and bacterial taxa which reside in the root and rhizosphere hence defines as root associated microbiome. The structures of communities get affected due to microbe-microbe interaction, abiotic environment, and host genotypes (Agler et al., 2016). Therefore, by engineering the root associated microbiome it can alter the microbial composition of the plant and can improve the plant fertility and growth. It has been noted that, for engineering plant microbiome, synthetic microbiomes, microbiome transfer have been engaged. A recent approach has been adapted for plant disease management is to merge disease conducive soils and disease suppressive soils. This strategy has been applied to inhibit tobacco black root rot, potato common swab and sugar beet infection (Gopal et al., 2013). Another approach is the use of a plant model (*Arabidopsis thaliana*) for transferring microbiome from soil to plant to increase biomass and avoid drought condition (Zolla et al., 2013).

Soil that manufactures phenotypes in previous generation's rhizosphere microbiomes is engineered by generating duplication of *Arabidopsis*

thaliana in the soil microbiome to alter the biomass and detoxification of the toxic compounds. Hence, usage of this host mediated artificial selection can alter ecology and develop plant microbiomes (Swenson et al., 2000; Edwards et al., 2015). Chemical molecules such as salicylic acid from *Arabidopsis thaliana* plant which is the regulator of plant immune system increases the microbial composition in root. Hence, this approach can be taken into consideration for improving microbial diversity, its productivity and increasing resistance to environmental stress (Lebeis et al., 2015). *Arabidopsis thaliana* is used extensively as a plant model for studying plant microbiome, this studies can then be extrapolated to study other plants. Extensive crop microbiome engineering studies are required for improving productivity, sustainability, drought, and disease resistance.

3.6 CONCLUSION AND FUTURE DIRECTIONS

It is not too distant in our future, when we will be able to manipulate our gut with genetically engineered bacteria that will detect and eliminate chronic diseases at its earlier stage. Intestinal microbiota is an integral part of the human body, and with its interaction, it can lead to long lasting hereditary programming. Engineered bacteria expressing therapeutic compounds which when administered, colonize the gut, shows a remarkable potentiality in treating various chronic diseases such as cancer, gastrointestinal diseases, metabolic diseases, and many more. The futile hunt for diseases like Crohn's disease has set off a new theory that all these lifestyle diseases may have been caused by the intestinal microbiota. Therefore, the approach of modifying or engineering the gut by administration of probiotics, prebiotics or FMT can prevent and treat chronic diseases.

Expression of various proteins by engineered bacteria seems to be a new tide of medicine. Despite all the success in animal study experiments, there is a lack of clinical trials in human which will prove the efficacy of therapeutic prospects of engineered bacteria. The evident roadblock for conducting human trials is the unsettlement of whether the pre-emptive measures are enough to ensure that the individual consented to treatment are exposed to the engineered bacteria. Therefore, to obtain the effectiveness of the therapeutic bacteria, public confidence and support, more rigorous

and well controlled studies are needed urgently. Another roadblock for both conducting of human trials and acceptance of therapeutic engineered bacteria is the absence of antibiotic selection for production of large stocks of the recombinant therapeutic protein. Large scale fermentation approach without antibiotic selection for engineered bacteria needs to be developed fully, since large-scale fermentation strategy is well-rooted for unaltered probiotic strains.

Microbiome engineering in the field of synthetic biology holds great potential in improving agricultural productivity and sustainability of the ecosystem because of the advances in high throughput multi omics tools. With the help of synthetic biology smart microbes that have beneficial effects can be engineered. This strategy can be applied to eliminate the pathogenic strains from the microbial community for microbiome engineering. Despite development of omics tools in determining microbial composition and attaining knowledge of the microbiomes, kinetics of the microbiome as well as how it interacts to regulate the composition of microbiome still needs to be investigated. More research needs to be focused on other microbiomes located at other places of a body, such as oral cavity, lungs, urogenital tract, and skin because peripheral effects of microbiome far off from the site need to expand to understand the relevancy of therapeutic microbiome engineering. Advance studies of microbiomes for clinical trial are obligatory to understand the potential of microbiome engineering.

KEYWORDS

- **dextran sodium sulfate**
- **inflammatory bowel disease**
- **microbiome engineering**
- **microbiota**
- **N-acylethanolamines**
- **reactive oxygen species**
- **superoxide dismutase**

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CHAPTER 4

Antibiotics and Plant-Derived Antimicrobials as an Alternative Source to Control Infections

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ABSTRACT

The discovery of penicillin started the era of antibiotics and was used for the treatment of infectious diseases to ensure the elimination of the major cause of human morbidity. However, microbial antibiotic resistance was soon observed after the introduction of antibiotics. Diverse mechanisms of antibiotic resistance have been revealed, including molecular mechanisms. Intrinsic mechanisms are attributed to a mutation in the host's chromosomal gene, whereas mutations in antibiotic-targeted genes and the transfer of resistance traits found on extrachromosomal elements like

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plasmids, bacteriophages transposons, and integrons are responsible for bacterial antibiotic resistance. The development of drug resistance results in clinical failures in the treatment of infections. Hence, new antibiotics are required to combat pathogens. Additionally, the development pace of new antimicrobial drugs is not enough to counteract the fast emergence of resistant strains. Natural small molecules could be used as an alternative to antibiotics.

4.1 INTRODUCTION

The invention of antibiotics has been considered as one of the greatest achievements of biomedicine. Antibiotics are compounds that inhibit or kill microorganisms (Pathogens). The use of antibiotics has decreased mortality rate and morbidity rate of humans. Penicillin was the first antibiotic discovered by Fleming (1929). Nowadays, numerous different classes of antibiotics are known, and they are classified based on their mode of action. Mode of action of antibiotics includes inhibition of DNA replication, transcription, translation, inhibition of cell wall synthesis, and inhibition of energy metabolism (Walsh, 2000).

Broad and uncontrolled use of antibiotics leads to resistance development in pathogens. Resistance in bacteria is not new. It was found in antimicrobial compound-producing microorganisms. Other peripheral organisms also became resistance due to selective pressure exerted by the compound to which they are exposed (Walsh, 2000; Barbosa & Levy, 2007). Over the past 70 years, bacteria have been exposed to different antibiotics used to treat human diseases and adapted genetic traits to nullify the effect of the antibiotic (Walsh, 2000). This chapter will discuss the antibiotic mode of action and both intrinsic as well as acquired resistance mechanisms.

4.2 MOLECULAR MECHANISMS OF RESISTANCE

In response to antibiotics, bacteria adapt genetically and physiologically to combat the action of antibiotics (Barbosa & Levy, 2007). The mechanisms involved in resistance emergence are enzymatic inactivation of antibiotics, reduced cell permeability, efflux of the antibiotics by specific or non-specific pumps and modification of the antibiotic targets (Figure 4.1). There are two mechanisms through which bacteria develop resistance

against antibiotics; one, intrinsic mechanism and second, acquired mechanism (Barbosa & Levy, 2007; Wright & Sutherland, 2007; Alekshun & Levy, 2007).

The intrinsic mechanism includes a mutation in the host's chromosomal gene that encodes antibiotic targets and overexpression of efflux systems. In acquired mechanisms, bacteria become resistant by mutations in antibiotic-targeted genes and the transfer of resistance traits found on plasmids (accomplished by conjugation), bacteriophages (accomplished by transduction), transposons, and integrons (Barbosa & Levy, 2007; Wright & Sutherland, 2007; Alekshun & Levy, 2007). For several classes of antibiotics, development of intrinsic and acquired resistance is described in Figure 4.1 along with its mode of action.

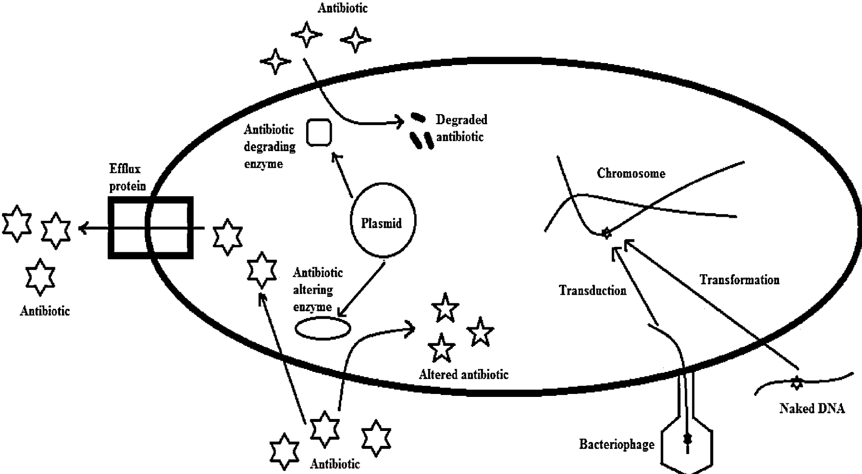


FIGURE 4.1 The antibiotic resistance mechanism of bacteria. Red star represents mutation.

4.2.1 AMINOGLYCOSIDES

4.2.1.1 HISTORY AND MODE OF ACTION

In 1943, the first aminoglycoside discovered was streptomycin (Figure 4.2), isolated from *Streptomyces griseus*. Streptomyces was clinically used to treat tuberculosis. Several years later, new aminoglycosides, such as neomycin (1949); gentamycin (1963); tobramycin (1967); and sisomycin

(1970), were developed to overcome the development of resistance against streptomycin. They show bactericidal activity against selective gram-positive bacteria as well as gram-negative bacteria. There is the rapid emergence of resistance against first-generation aminoglycosides, like dibekacin (1971); amikacin (1972); arbekacin (1973); isepamicin (1975); and netilmicin (1976) (Becker & Cooper, 2013).

FIGURE 4.2 Aminoglycoside antibiotic structure: (A) streptomycin; (B) gentamycin; (C) neomycin; and (D) tobramycin.

Source: All chemical structures are drawn with Chem Draw Software, Perkin Elmer Informatics.

Aminoglycoside show dose dependent bactericidal activity and inhibit protein synthesis by specifically binding to A site of 16S ribosomal (rRNA). Thus, the presence of aminoglycoside in cytosol generally hampers the peptide elongation by inhibiting of translocation of ribosomes (Becker & Cooper, 2013; Kaul et al., 2004; Ramirez & Tolmasky, 2010).

4.2.1.2 RESISTANCE MECHANISMS

Intrinsic resistance mechanism against aminoglycoside includes efflux pumps, mutations in 16S rRNA and 16S rRNA methylases mediated resistance. Mutation in A1408 leads rearrangement of the molecular structure of 16S rRNA. This rearrangement causes resistance to neomycin and gentamycin (De et al., 1989). Ribosomal protein S12 (RpsL) is also the main target of streptomycin-mediated translational inhibition. Mutation in *the rpsL* gene affects binding of streptomycin, thereby leading to resistance (Springer et al., 2001).

16S rRNA methylase mediated methylation of specific nucleotides within the A-site of 16S RNA prevents binding of aminoglycosides to the 30S ribosomal subunits, initiating emergence of resistance against aminoglycoside such as amikacin, tobramycin, and gentamycin (Doi & Arakawa, 2007). It has been reported that self-transmissible plasmid *pIP1204* confers 16S rRNA methylase in *Klebsiella pneumonia*, isolated from urinary tract infection and became resistance to aminoglycoside (Galimand et al., 2003).

Resistance to aminoglycoside in *P. aeruginosa* strains was due to changes to the outer membrane lipopolysaccharide (LPS). In limited Mg^{2+} concentrations, the PhoP-PhoQ two-component regulatory systems induce the changes of outer membrane LPS level in the presence of polyamines. Ultimately, organisms reduce the net negative charge of the outer membrane (Macfarlane et al., 2000; Kwon & Lu, 2006; Wang & Quinn, 2010).

The efflux pumps of the family of resistance nodulation division (RND), which is mainly present in gram-negative bacteria, are able to transport aminoglycoside antibiotics. The RND family efflux pump-mediated resistance has been reported in *Pseudomonas*, *Acinetobacter*, *Brucella*, *Bukholderia*, *Enterobacter*, *Escherichia*, *Helicobacter*, and *Stenotrophomonas* species (Li & Nikaido, 2009).

Acquired resistance mechanism involves modification of aminoglycoside by enzymes. These enzymes can be classified into three families based

on the type of modification: aminoglycoside acetyltransferases (ACCs), aminoglycoside phosphotransferases (APHs) and aminoglycoside nucleotidyltransferase (ANTs). Aminoglycoside acetyltransferase transfers acetyl group to amino group of aminoglycosides. Aminoglycoside phosphotransferase catalyzes phosphorylation reaction to the hydroxyl group of aminoglycosides while ANTs transfers adenosine monophosphate (AMP) group from adenosine triphosphate (ATP) to a hydroxyl group of the aminoglycoside. There are four acetyltransferases: AAC(1), AAC(2'), AAC(3), and AAC(6'); seven phosphotransferases: APH(2"), APH(3'), APH(4), APH(6), APH(7"), and APH(9) and five nucleotidyltransferases: ANT(2"), ANT(3"), ANT(4"), ANT(6), and ANT(9) have been reported till date (Table 4.1) (Becker & Cooper, 2013; Ramirez & Tolmasky, 2010).

TABLE 4.1 Aminoglycoside Modifying Enzymes and Their Antibiotic Substrates and Sources (Ramirez & Tolmasky, 2010)

Enzyme	Classification	Type	Antibiotic Substrate	Bacteria Source
N-Acetyltransferases	AAC(1)	–	Apramycin Butirosin Lividomycin Paromomycin	*Escherichia coli*, *Campylobacter* spp.
–	–	–	Butirosin Lividomycin Paromomycin	*Actinomycete*
–	AAC(3)	I	Gentamicin Sisomicin Fortimicin	Gram-negative *Enterobacteriaceae*
		Ia		*Serratia marscens*
–	–	Ie	–	*Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica*
–	–	IIa	Gentamicin Netilmicin Tobramycin Sisomicin	*Acinetobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., *Pseudomonas* spp., *Salmonella* spp.
–	–	IIb	–	–
–	–	IIc	–	–
		III	–	*Pseudomonas* spp., *Klebsiella* spp., *Pneumoniae* spp.

TABLE 4.1 *(Continued)*

Enzyme	Classification	Type	Antibiotic Substrate	Bacteria Source
		IV	–	*Escherichia coli*, *Campylobacter jejuni*, *Pseudomonas stutzeri*
		Via	–	*Escherichia coli*, other *Enterobacteriaceae*, *Salmonella enterica*
		VII VIII IX X	Kanamycin Dibekacin Arbekacin Amikacin	*Actinomycetes*
	AAC(2)	Ia	Gentamicin	*Providencia stuartii*
		Ib	Tobramycin Dibekacin	*Mycobacterium fortuitum*, *Acinetobacter baumannii*
		Ic	Kanamycin Netilmicin	*Mycobacterium tuberculosis*, *Mycobacterium bovis*
		Id		*Mycobacterium smegmatis*
		Ie		*Mycobacterium leprae*
–	AAC(6')	I	Amikacin Gentamicin C1a, C2	Gram-positive, gram-negative
		II	Gentamicin C1, C1a, C2	
O-Nucleotidyltransferase	ANT(6)	I	Streptothricin	*Enterococcus faecalis*, *Campylobacter jejuni*, *Staphylococci* spp., *Enterococci* spp., *Bacillus subtilis*, *Campylobacter fetus* subsp. *fetus*
	ANT(9)	Ia	Streptomycin	*Enterococcus avium*, *Enterococcus faecalis*, *Enterococcus faecium*
		Ib		*Enterococcus faecalis*
	ANT(4')	Ia	Dibekacin Tobramycin Amikacin Isepamicin	*Staphylococci* spp., *Enterococci* spp., *Bacillus* spp.
		IIa	Tobramycin Amikacin	*Pseudomonas* spp., *Enterobacteriaceae*
		IIb	Isepamicin	*Pseudomonas aeruginosa*

TABLE 4.1 *(Continued)*

Enzyme	Classification	Type	Antibiotic Substrate	Bacteria Source
	ANT(2”)	Ia	Gentamicin Tobramycin Dibekacin Sisomicin Kanamycin	*Enterobacteriaceae* Gram-negative
–	ANT(3”)	Ia	Spectinomycin Streptomycin	*Pseudomonas aeruginosa*
O-Phosphotransferase	APH(4)	Ia	Hygromycin	–
	APH(6)	Ia	Streptomycin	*Streptomyces griseus*
		Ib		*Streptomyces glaucescens*
		Ic		Gram-negative
		Id		Gram-negative Gram-positive
	APH(9)	Ia	Spectinomycin	*Legionella pneumophila*
		Ib		*Streptomyces flavopersicus*, *Streptomyces spectabilis*
	APH(3’)	I	Kanamycin Neomycin Paromomycin Ribostamycin Lividomycin	*Corynebacterium* Gram-negative
		IIa IIb IIc	Kanamycin Neomycin Paromomycin Ribostamycin Butirosin	*Pseudomonas aeruginosa* *Salmonella maltophilia*
–	–	IIIa	Kanamycin Neomycin Lividomycin Paromomycin Livostamycin Butirosin Amikacin Isepamicin	Gram-positive
		Iva	Neomycin Paromomycin Ribostamycin	*Bacillus circulans*

TABLE 4.1 *(Continued)*

Enzyme	Classification	Type	Antibiotic Substrate	Bacteria Source
		Va	Neomycin	*Actinomycetes*
		Via	Paromomycin	*Acinetobacter baumannii*
		VIb	Butirosin Amikacin Isepamicin Kanamycin Ribostomycin	*Klebsiella pneumonia*, *Serratia marcesans*
		VIIa	Kanamycin Neomycin	*Campylobacter jejuni*
	APH(2")	I	Gentamicin	Gram-positive
		II		–
	APH(3")	Ia	Streptomycin	*Streptomyces griseus*, *Mycobacterium fortuitum*
		Ib		*Vibrio cholarae*
		Ic		*Mycobacterium fortuitum*
–	APH(7")	Ia	Hygromycin	*Streptomyces hygroscopicus*

There are two different nomenclature systems in use for the aminoglycosides modifying enzymes. One is from an enzymatic perspective and consists of a three-letter code to identify the activity (APH, ANT, and AAC), followed by a number in parentheses that identifies the site of modification, then a Roman numeral that describes a particular resistance profile that is emerged in the organism (Subclass), and lastly a lower case letter as an individual identifier. The parentheses and the subclass are separated by a hyphen, AAC(3)-IIa, for example. The other nomenclature system has a genetic perspective, with three lower case letters in italics for the type of activity (*aph, aac*, and *aad*), a capital letter for the site of modification and a number as a unique identifier of the individual genes (Becker & Cooper, 2013; Ramirez & Tolmasky, 2010).

4.2.2 B-LACTAM ANTIBIOTICS

4.2.2.1 HISTORY AND MODE OF ACTION

The β-lactam antibiotics (Figure 4.3), one of the largest classes of antibiotics, contain a β-lactam nucleus in their molecular structure. Penicillin

was the first β-lactam antibiotic discovered in 1929 by Alexander Fleming (1929). The β-lactam antibiotic family includes penicillin and its derivatives, cephalosporins, carbapenems, and monobactams. Early *Penicillins* were less antibacterial against gram-negative pathogens. Aminopenicillin was developed later that showed antibacterial activity against *E. coli*, *Shigella*, and *Salmonella* but not against *P. aeruginosa* and *Klebsiella* species. Carboxypenicillin was generated by replacing an amino group of aminopenicillin with a carboxy group. Carboxypenicillin was active against *P. aeruginosa* (Bodey, 1990).

FIGURE 4.3 β-lactam antibiotic structure: (A) benzylpenicillin; (B) cefotoxamine; (C) imipenam; and (D) aztreonam.

In the late 1940s, Cephalosporin was developed to treat β-lactamase producing staphylococcal infections. According to their spectrum of activity and time of introduction, cephalosporins can be categorized into first, second, third, and fourth generations. The first generation shows good gram-positive activity and modest activity against gram-negative bacteria; second generation cephalosporin has increased gram-negative activity and less gram-positive activity while the third generation shows

improved gram-negative and variable gram-positive activity. The fourth generation has broad spectrum activity against both gram-negative as well as gram-positive bacteria (Harrison & Bratcher, 2008). Imipenem, first carbapenems introduced in 1985, shows broad spectrum activity as they diffuse easily in the bacteria. The discovery of monobactams provides therapeutic options for bacterial infection. Aztreonam is one of the monobactam introduced in 1986 (Bodey, 1990).

The β-lactams antibiotics inhibit the cell wall synthesis by binding to penicillin binding proteins (PBPs) in bacteria, which catalyzes the cross-linking of peptidoglycan. Inhibition of transpeptidation reaction results in less integrity of cell wall and finally leads to cytolysis due to osmotic pressure (Bodey, 1990; Tipper & Strominger, 1965).

4.2.2.2 RESISTANCE MECHANISMS

The β-lactam antibiotic resistance is increased at a significant rate in bacteria. Hence it constitutes one of the most common problems for public health care. Resistance to β-lactam antibiotics occurs through two mechanisms: (i) the production of β-lactamase, which hydrolyzes β-lactam antibiotics; and (ii) production of altered PBPs with lower affinity for most β-lactam antibiotics.

By late 2009, more than 890 β-lactamases were reported (Bush et al., 1995). Most of the β-lactamases are encoded by genes located on plasmids and transposons, whereas others are chromosomally encoded. β-Lactamases can be classified on the basis of either the functional or molecular characteristics of the enzymes. Based on the molecular characteristic, β-lactamases can be divided into four classes. This classification is also known as "Ambler" classification. Classes A, C, and D β-lactamases contains serine in their active sites and catalyzes the reaction by forming an acyl enzyme whereas class B β-lactamases are metalloenzymes who require zinc as a metal cofactor for their catalytic activity (Bush, 1989). Based on the functional characteristic, β-lactamases can also be classified into 1 to 4 groups, also called Bush Jacoby-Medeiros groups (Figure 4.4). AmpC is the first β-lactamases encoded from the chromosome. AmpC found in many species of *Enterobacteriaceae* and *P. aeruginosa*, is a member of class C β-lactamases. In 2005, it has been reported that plasmid-borne *ampC* genes encode AmpC protein. Class D enzymes, also

called oxacillinases, have been found in *P. aeruginosa*, *Acinetobacter* ssp., and *Aeromonas* spp. (Drawz & Bonomo, 2010).

Based on their potent activity to hydrolyze a small number or a variety of β-lactam, the enzymes can be further subdivided into narrow, moderate, broad, and extended-spectrum β-lactamases. The broad spectrum β-lactamases can hydrolyze penicillins and cephalosporins, but they are susceptible to β-lactamase inhibitors such as clavulanic acid and tazobactam. The extended-spectrum β-lactamases enable to provide resistance to the penicillins in the first, second, and third generation cephalosporins and aztreonam, while they are susceptible to carbapenems and β-lactamase inhibitors (Bradford, 2001).

Another mechanism of β-lactam resistance is altered PBPs. The first methicillin resistance *Staphylococcus aureus* was identified in 1961, soon after two years of the introduction of methicillin. The Staphylococci emerged as methicillin resistant by acquiring gene encoding for PBPs. The altered PBPs of methicillin resistant *S. aureus* are conferred by *mecA*. The *mecA* gene is regulated by *mecR1-mecI*. The *mecA* is transformed as mobile genetic elements, called staphylococcal cassette chromosome *mec* (SCC*mec*). The *mecA* operon encodes the site-specific recombinases responsible for transformation (Fuda et al., 2005).

During treatment of cystic fibrosis, *Pseudomonas aeruginosa* developed resistance to β-lactam antibiotics by altered PBPs, which have a low affinity for penicillin G (Godfrey et al., 1981). Clinically isolated *S. pneumonia* from the United States showed a decrease in the affinity of PBP group 1 and 2 for Penicillin G. The most resistant strain showed a decrease in the amount of PBP2b (Hakenbeck et al., 1980).

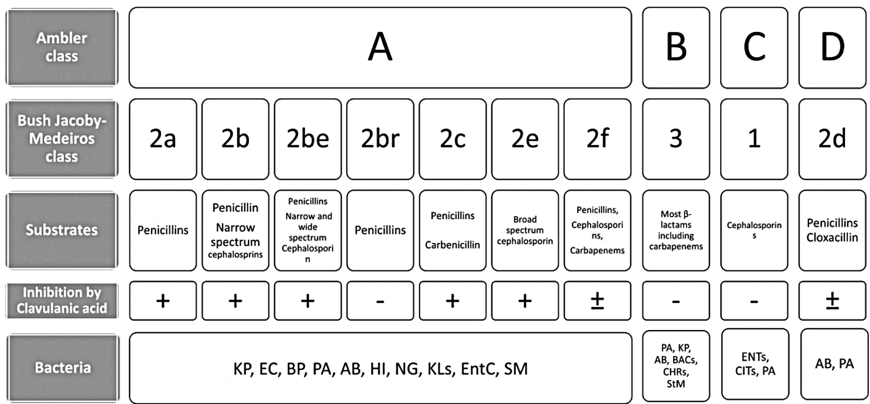
4.2.3 CHLORAMPHENICOL

4.2.3.1 HISTORY AND MODE OF ACTION

Chloramphenicol (Figure 4.5), initially known as chloromycetin, was isolated from *Streptomyces venezuelae*. Production of chloramphenicol was originally observed by Ehrlich in 1947 (Mclean et al., 1949; Izard & Jacqualine, 2000; Schwarz et al., 2004). Three derivatives of chloramphenicol have been developed by substitution of functional groups. Azidamfenicol (Figure 4.5) is developed by replacing two chlorine atoms

(Cl_2) with an azide group. In thiamphenicol (Figure 4.4), the nitro group ($–NO_2$) is substituted by a methyl sulfonyl residue ($–SO_2CH_3$) while florfenicol (Figure 4.4) is developed by substituting the nitro group (NO_2) and hydroxyl group (–OH) with a methyl sulfonyl group and fluorine (–F), respectively (Schwarz et al., 2004).

Chloramphenicol inhibits protein synthesis by preventing peptide chain elongation. It binds specifically to the peptidyl transferase center at the 50S ribosomal subunit of 70S ribosomes. It has broad spectrum activity as it is active against gram-positive, gram-negative, aerobic, and anaerobic bacteria (Izard & Jacqualine, 2000; Schwarz et al., 2004).



Ambler class	A							B	C	D
Bush Jacoby-Medeiros class	2a	2b	2be	2br	2c	2e	2f	3	1	2d
Substrates	Penicillins	Penicillin Narrow spectrum cephalosprins	Penicillins Narrow and wide spectrum Cephalosporin	Penicillins	Penicillins Carbenicillin	Broad spectrum cephalosporin	Penicillins, Cephalosporins, Carbapenems	Most β-lactams including carbapenems	Cephalosporins	Penicillins Cloxacillin
Inhibition by Clavulanic acid	+	+	+	-	+	+	±	-	-	±
Bacteria	KP, EC, BP, PA, AB, HI, NG, KLs, EntC, SM							PA, KP, AB, BACs, CHRs, StM	ENTs, CITs, PA	AB, PA

FIGURE 4.4 β-lactamase classification schemes (Drawz & Bonomo, 2010).

Abbreviations: AB: *Acinetobacter baumannii*; BACs: *Bacillus* spp.; BP: *Burkholderia pseudomallei*; CHRs: *Chryseobacterium* spp.; CITs: *Citrobacter* spp.; EC: *Escherichia coli*; EntC: *Enterobacter cloacae*; ENTs: *Enterobacter* spp.; HI: *Haemophilus influenza*; KLs: *Kluyvera* spp.; KP: *Klebsiella pneumonia*; NG: *Neisseria gonorrhoeae*; PA: *Pseudomonas aeruginosa*; SM: *Serratia marcescens*; StM: *Stenotrophomonas malyophilia.*

FIGURE 4.5 The structure of chloramphenicol and related antibiotics.

Name of Antibiotic	R_1	R_2	R_3
Chloramphenicol	$-NO_2$	–OH	$=Cl_2$
Azidamfenicol	$-NO_2$	–OH	Azide group
Thiamphenicol	$-SO_2CH_3$	–OH	$=Cl_2$
Florfenicol	$-SO_2CH_3$	–F	$=Cl_2$

4.2.3.2 RESISTANCE MECHANISMS

The mechanisms of bacterial resistance to chloramphenicol involved enzymatic inactivation, efflux system, mutation, and permeability of barriers. Chloramphenicol acetyltransferases (CATs) modifies the molecular structure of chloramphenicol and azidamfenicol by replacing hydroxyl group (-OH) with an acetyl group. However, the hydroxyl group is absent in florfenicol and hence, it is resistant to CATs inactivation. Two types of genes encoding CATs, which distinctly differ in their structure, have been reported: the classical CATs and the Xenobiotic CATs (Figure 4.6) (Schwarz et al., 2004). All CATs are heterotrimer of 24 to 26 kDa (Murray & Shaw, 1997).

Chloramphenicol 3-O-phosphotransferase (CPT) has a polypeptide chain of 19 kDa that inactivates the chloramphenicol by phosphorylation. The CPT is active as a homodimer. CPT-mediated phosphorylation requires ATP as phosphoryl donor to transfer the γ-phosphate to the (C-3) hydroxyl of chloramphenicol (Izard & Jacqualine, 2000).

Specific exporters play a key role in chloramphenicol resistance (Schwarz et al., 2004). The first non-enzymatic chloramphenicol resistance was observed in *P. aeruginosa* having Inc P plasmid R26 and transposon Tn1696 on the Inc P plasmid R1033. Both contain the same gene (*cmlA1*) that encodes transmembrane transport proteins of the major facilitator superfamily (George & Hall, 2002). Currently, eight different groups of specific exporters are known (Table 4.2).

4.2.4 GLYCOPEPTIDE

4.2.4.1 HISTORY AND MODE OF ACTION

Vancomycin, the first glycopeptide, was isolated as secondary metabolites from a soil bacterium; *Streptomyces orientalis* (now renamed *Amycolatopsis orientalis*). Teicoplanin is the second glycopeptide, used against gram-positive bacterial infections (Pootoolal et al., 2002).

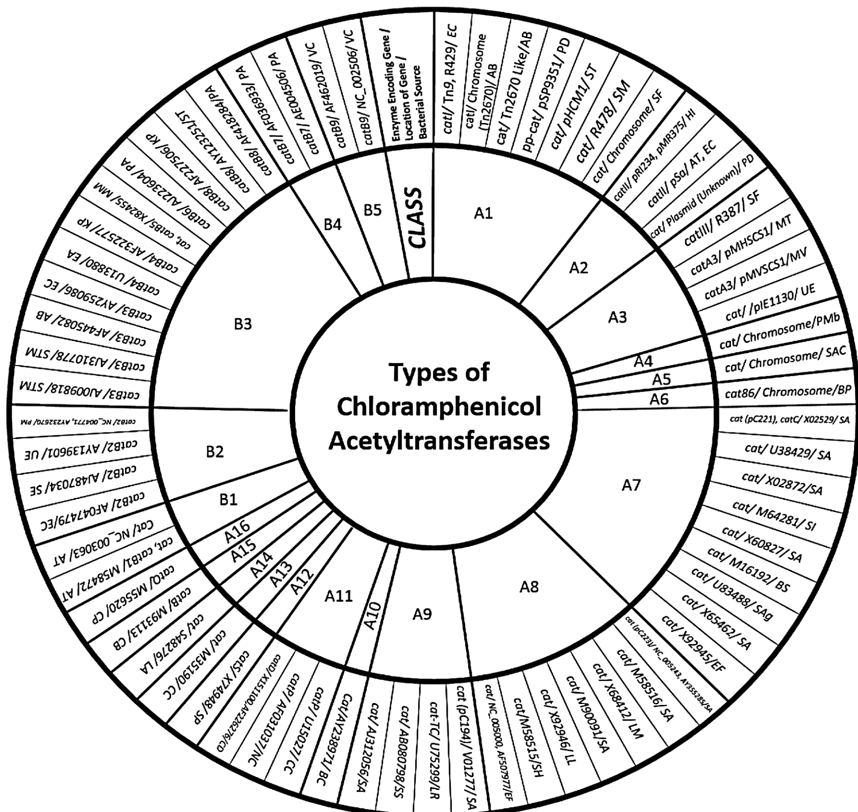


FIGURE 4.6 Types of chloramphenicol acetyltransferases (Schwarz et al., 2004).

Note: Pattern of reading in outermost concentric circle: Name of enzyme encoding gene/ location of gene/bacterial source.

Abbreviations: AB: *Acinetobacter baumannii*; AC: *Acinetobacter calcoaceticus*; AT: *Agrobacterium tumefaciens*; BC: *Bacillus clausii*; BP: *Bacillus pumilus*; BS: *Bacillus subtilis*; CB: *Clostridium butyricum*; CC: *Campylobacter coli*; CD: *Clostridium difficile*; CP: *Clostridium perfringens*; EA: *Enterobacter aerogenes*; EC: *Escherichia coli*; EF: *Enterococcus faecalis*; HI: *Haemophilus influenza*; KP: *Klebsiella pneumonia*; LA: *Listonella anguillarum*; LL: *Lactococcus lactis*; LM: *Listeria monocytogenes*; LR: *Lactobacillus reuteri*; MM: *Morganella morganii*; MT: *Mannheimia taxon* 10; MV: *Mannheimia varigena*; NM: *Neisseria meningitides*; PA: *Pseudomonas aeruginosa*; PD: *Photobacterium damselae* subsp. *Piscicida*; PM: *Pasteurella multocida*; PMb: *Proteus mirabilis*; PP: *Pseudomonas putida*; SA: *Staphylococcus aureus*; SAC: *Streptomyces acrimycini*; SAg: *Streptococcus agalactiae*; SE: *Salmonella enteritidis*; SF: *Shigella flexneri*; SH: *Staphylococcus haemolyticus*; SI: *Staphylococcus intermedius*; SM: *Serratia marcescens*; SP: *Streptococcus pyogenes*; SS: *Streptococcus sius*; ST: *Salmonella typhi*; STM: *Salmonella typhimurium*; UE: Uncultured eubacterium; VC: *Vibrio cholera.*

TABLE 4.2 The Specific Exporters Involved in Chloramphenicol or Chloramphenicol/Florfenicol Resistance (Schwarz et al., 2004)

Group	Gene Designation	Plasmid/ Transposon/ Chromosome	Database Accession No.	Bacterial Source
E1	*cmlB, cmlA2*	pIP833	AF034958	*Enterobacter aerogenes*
	cmlA	Plasmid	AJ487033	*Salmonella typhimurium*
	cmlA5	R751 (Tn2000)	AF205943	*Escherichia coli*
	cmlA1	pILT-3	AF458080	*Klebsiella pneumoniae*
	cmlA1	RPL11 (Tn1403)	AF313472	*Pseudomonas aeruginosa*
	cmlA4	pTK1	AF156486	*Klebsiella pneumoniae*
	cmlA5	pSp1	AY115475	*Uncultured bacterium*
	cmlA6	Plasmid	AF294653	*Pseudomonas aeruginosa*
	cmlA7	Chromosome	AJ511268	*Pseudomonas aeruginosa*
	cmlA, cmlA1	pR1033:Tn1696	U12338 M64556 AF078527	*Pseudomonas aeruginosa*
E2	*Cml*	R26	M22614	*Escherichia coli*
E3	*cmlA-like*	Chromosome	AF071555	*Salmonella typhimurium DT104*
	floR	Chromosome	AF118107	*Salmonella typhimurium DT104*
	Flo	Chromosome	AJ251806	*Salmonella typhimurium DT104*
	floR	Chromosome	AF261825	*Salmonella typhimurium DT104*
	floR	Chromosome	AY339985	*Salmonella typhimurium DT104*
	Flo	Plasmid	AF252855	*Escherichia coli*
	floR	Plasmid	AF231986	*Escherichia coli*
	floR	pMBSF1	AJ518835	*Escherichia coli*
	floR	R55	AF332662	*Klebsiella pneumoniae*
	floR	Chromosome (SXT element)	AY034138	*Vibrio cholerae*
	floR	Chromosome (SXT element)	AY055428	*Vibrio cholerae*
	pp-flo	pSP92088	D37826	*Photobacterium damselae* subsp. *piscicida*

TABLE 4.2 *(Continued)*

Group	Gene Designation	Plasmid/ Transposon/ Chromosome	Database Accession No.	Bacterial Source
E4	*fexA*	pSCFS2	AJ549214	*Staphylococcus lentus*
E5	*Cml*	Chromosome	X59968	*Streptomyces lividans*
E6	*Cmlv*	Chromosome	U09991	*Streptomyces venezuelae*
E7	*cmrA*	Tn5561	AF015087	*Rhodococcus rhodochrous*
	Cmr	pRF2	Z12001	*Rhodococcus fascians*
E8	*Cmr*	pXZ10145	U85507	*Corynebacterium glutamicum*
	Cmx	pTP10:Tn5564	AF024666	*Corynebacterium striatum*

Haemophilus influenza, which lacks CAT activity, developed chloramphenicol resistance due to the loss of an outer membrane protein (Burns et al., 1985). The same mechanism is also observed in *P. cepacia* from a patient with cystic fibrosis (Burns et al., 1989). The clinically isolated *Salmonella typhi* developed as it lacks porin (OmpF) (Toro et al., 1990). *E. coli* and *B. subtilis* have developed chloramphenicol due to a mutation in the major ribosomal protein gene cluster (Baugman & Fahnestock, 1979; Anderson et al., 1984). Mutation in 23S rRNA in *E. coli* is also responsible for chloramphenicol resistance emergence (Ettayebi et al., 1985).

Glycopeptide inhibits peptidoglycan synthesis. It inhibits transpeptidase and transcarboxylase activity by specific interaction with D-Ala-D-Ala terminus of the cell wall peptidoglycan precursors (Pootoolal et al., 2002).

4.2.4.2 RESISTANCE MECHANISM

Six different phenotypes (VanA, VanB, VanC, VanD VanE and VanG) are responsible for the emergence of glycopeptide resistance in enterococci. *Enterococcus faecium* acquired Tn1546 contains three genes of seven genes which are responsible for Vancomycin resistance: *vanH*, *vanA*, and *vanX*. The *vanH*, *vanA*, and *vanX* conferred α-keto acid reductase, D-Ala-D-Ala ligase, and a Zn binding metallodipeptidase, respectively. The α-keto acid reductase generates the D-isomer of lactate. Dipeptidase cleaves D-Ala-D-Ala, whereas D-Ala-D-Ala ligase ligates the D-Ala-D-Lact (Figure 4.7). Thus, cells having transposon become resistant to a change in the antibiotic target structure (Pootoolal et al., 2002).

The cells having VanB and VanD phenotype produce D-Ala-D-Lact terminating peptidoglycan. The production of D-Ala-D-Lact terminating peptidoglycan requires VanHB, VanAB, and VanXB enzymes, having similar role enzyme produced in cells having VanA phenotype.

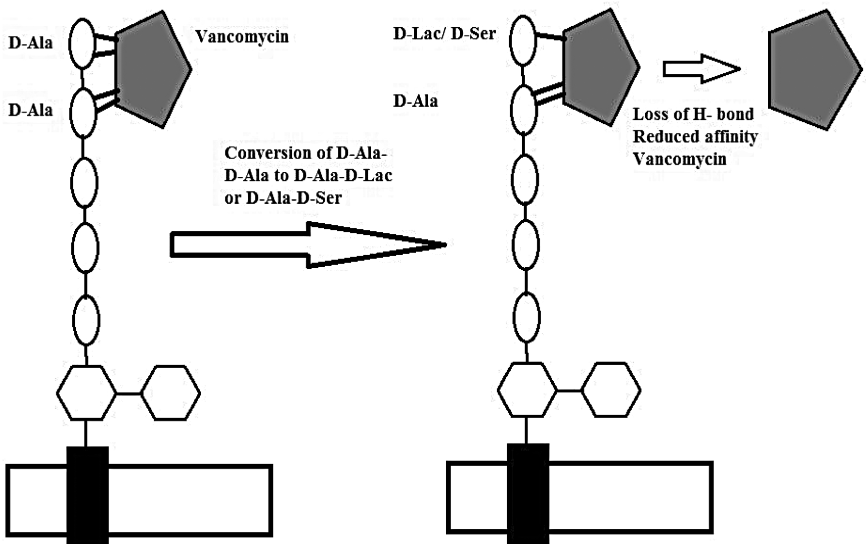


FIGURE 4.7 Vancomycin resistance. Conversion of D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser results in loss of one hydrogen bond, which reduces vancomycin affinity towards peptidoglycan.

VanD phenotype is encoded by chromosome and responsible for vancomycin and teicoplanin resistance. The VanC, VanE, and VanG phenotypes produce D-Ala-D-Ser terminating peptidoglycan that lowers the affinity for glycopeptides (Figure 4.7) (Pootoolal et al., 2002).

Staphylococcus aureus developed vancomycin resistance by thickening the cell wall, increased peptidoglycan synthesis and altered PBPs. Mutation in VncS, a sensor His kinase of a two-component regulatory system, leads autolysis in response to vancomycin. Phosphorylation of VncR represses autolysin gene expression under normal conditions. VncS has both kinase and phosphatase activity. In the presence of vancomycin, VncR dephosphorylates VncR, which leads to autolysin production and cause cell lysis. Inactivation of VncS inactivates the phosphatase activity (Pootoolal et al., 2002).

4.2.5 MACROLIDE-LINCOSMAIDE-STREPTOGRAMIN B (MLS ANTIBIOTICS)

4.2.5.1 HISTORY AND MODE OF ACTION

Structurally, macrolides contain a large lactone ring to which amino acids or neutral sugars are attached by glycosidic bonds. The first macrolide, erythromycin, was discovered in 1952, isolated from *Saccharopolyspora erythraea*. The new 14, 15, and 16-membered ring macrolides, such as clarithromycin and azithromycin, have been developed to mimic the chemical instability, poor absorbance, and bitter taste of erythromycin (Roberts et al., 1999). Although lincosamide, streptogramin, and macrolide are structurally unrelated to each other, they have a similar mode of action. However, many macrolides resistance genes code for the resistance of two or all three of the macrolide-lincosamide-streptogramin B (MLSB) (Roberts, 2002).

MLSB binds to the 50S ribosomal subunit and stimulates dissociation of the peptidyl-tRNA from the ribosome during elongation, and thus inhibiting the protein synthesis (Roberts et al., 1999; Roberts, 2002; Weisblum, 1998).

4.2.5.2 RESISTANCE MECHANISMS

MLS resistance mechanisms involve enzymatic modification, mutation of ribosomal RNA (rRNA) target site and efflux system. The most common mechanism of MLS resistance is methylation of 23S rRNA by rRNA methylases, which is encoded by the *erm* gene (*erm* tends erythromycin–ribosome methylation) (Figure 4.8). The rRNA methylases methylate at the site of the peptidyl transferase circle of the 23S rRNA domain. It has been observed that A2058 of 23S rRNA methylates post-transcriptionally by 23S rRNA methylase (Roberts et al., 1999; Roberts, 2002).

Two esterases, two hydrolases, seven transferases and three phosphorylases have been reported to inactivate the MLS antibiotics (Figure 4.8). Mutation in 23S rRNA domain V and domain II results in MLS resistance. Mutational alteration of 23S rRNA at A2058 was first reported in clinically isolated *Mycobacterium intracellulare*. Mutation in domain II of the 23S rRNA contains a Shine Dalgarno sequence (GGAGG), which destabilizes the secondary structure of Domain II.

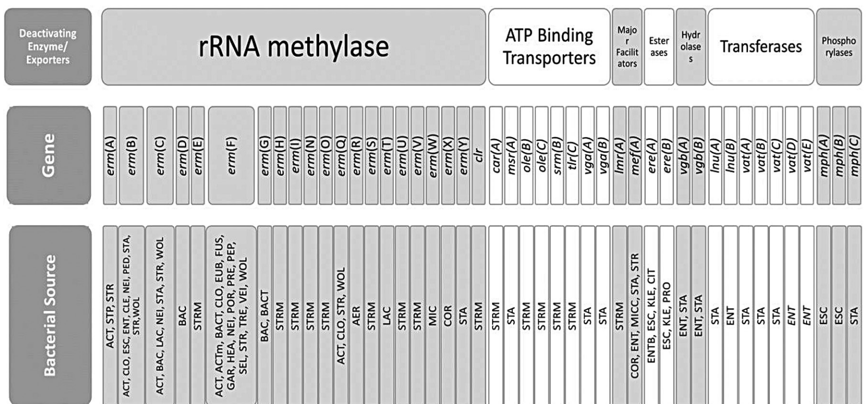


FIGURE 4.8 Inactivating enzymes and exporters genes, which confer resistance to MLS antibiotics.

Abbreviations: ACT: *Actinobacillus*; ACTm: *Actinomyces*; AER: *Aeromicrobium*; BAC: *Bacillus*; BACT: *Bacteroides*; CIT: *Citrobacter*; CLO, CLE: *Clostridium*; COR: *Corynebacterium*; ENT: *Enterococcus*; ENTB: *Enterobacter*; ESC: *Escherichia*; EUB: *Eubacterium*; FUS: *Fusobacterium*; GAR: Gardnerella; HAE: *Haemophilus*; KLE: *Klebsiella*; LAC: *Lactobacillus*; MIC: Micromonospora; MICC: *Micrococcus*; NEI: *Neisseria*; PED: *Pediococcus*; PEP: *Peptosteptococcus*; POR: *Porphyromonas*; PRE: *Prevotella*; POR: *Proteus*; SEL: *Selenomonas*; STA: *Staphylococcus aureus*; STP: *Staphylococcus*; STR: *Streptococcus*; STRM: *Streptomyces*; TRE: *Treponema*; VEI: *Veillonella*; WOL: *Wolinella.*

Erythromycin resistance is regulated by either transcriptional or translational attenuation (Weisblum, 1998). Instead of efflux of the drug out of the cell or the cellular membrane, the efflux protein pumps keep drug intracellular concentration lower so that the ribosome can function normally. ATP transporters and major facilitator super family transporters (MFS transporters) play a crucial role in MLS resistance emergence. Eight different ATP transporter genes and two different MFS transporters have been reported in gram-positive and gram-negative bacteria (Figure 4.8). The *msr* and *vga* genes are found in *Enterococcus* and *Staphylococcus* ssp. The *lmr* and *mef* genes encode MFS transporter proteins and confer lincosamide resistance. The *lme* gene is reported as present in *Streptomyces*, whereas the *mef* gene is present in a gram-positive and gram-negative bacterium (Roberts et al., 1999; Roberts, 2002; Weisblum, 1998).

4.2.6 QUINOLONE

4.2.6.1 HISTORY AND MODE OF ACTION

Nalidixic acid, a quinolone derivative was first introduced in clinical care in 1962, as it has antibacterial activity against the gram-negative bacteria. Nalidixic acid was produced during the synthesis of chloroquine (Lesher et al., 1962). Later, fluoroquinolone was generated by adding fluoride atom at the 6th position of quinolone molecule. The addition of fluoride atom to quinolone enhances its antibacterial activity. The second and third generations of quinolone were introduced in health care in the 1980s and 1990s, respectively. The second generation of quinolone includes ciprofloxacin, norfloxacin, and ofloxacin, while the third generation comprises levofloxacin and sparfloxacin. These generations of quinolone show wide spectrum antibacterial activity against gram-positive as well as gram-negative (Hooper, 2000; King et al., 2000).

They kill the bacteria directly by inhibiting DNA replication (Hooper, 2001). They trap the DNA-DNA gyrase or topoisomerase IV complex and forms barrier to DNA replication and transcription (Hiasa et al., 1996; Willmott et al., 1994; Shea & Hiasa, 1999). Both are type 2 topoisomerase, having tetramer subunits (GyrA and GyrB subunits of DNA gyrase, ParC, and ParE subunits of topoisomerase IV).

4.2.6.2 RESISTANCE MECHANISM

Two main mechanisms of quinolone resistance have been reported: alterations in the targets of quinolones and decreased intracellular concentration due to impermeability of the membrane and/or over-expression of efflux pump systems. Both of the mechanisms are intrinsic. Furthermore, the *qnr* genes are found on a plasmid, which confers quinolone resistance (Ruiz, 2003).

Quinolone inhibits the action of the type II topoisomerases, DNA gyrase and topoisomerase IV. Mutation in *gyrA*/*gyrB* and *parC*/*parE* leads in an amino acid substitution that has been reported in *Escherichia coli*. Mutations in *gyrA* are predominantly found in the quinolone resistance determining the region (QRDR). Mutation at the 67th and 106th positions in the QRDR results in the substitution of serine in place of alanine and

arginine/histidine in place of glutamine. Additionally, mutation is found also in the *parC* gene (Ruiz, 2003; Yoshida et al., 1990).

Decreased intracellular concentration of quinolone may be due to an increase in the bacterial impermeability or the overexpression of efflux pumps. Hydrophobic quinolones are reported to diffuse through the porins. Thus, alteration in the composition of LPS and expression of porins may lead to the creation of quinolone resistance. Decreased expression of OmpF increases the resistance level in *E. coli* to quinolone. Expression of OmpF is regulated by chromosome loci such as MarRAB and SoxRS. The MarRAB contains three genes: *marR* that encodes repressor protein, *marA* that encodes activator protein and *marB* that encodes an unknown protein. SoxRS operon contains two genes, *soxR* encoding regulatory protein and *soxS* encoding activator protein (Alekshun and Levy., 1997; Oethinger et al., 1998).

In *E. coli*, the MarRAB and SoxRS operons also reported regulating the expression level of efflux pump AcrAB. The disruption or inhibition of AcrAB expression increase susceptibility to ciprofloxacin, enrofloxacin, and marbofloxacin in *S. typhimurium*. Three different efflux systems such as MexAB-OprM, MexCD-OprJ or MexEF-OprN have been reported in *P. aeruginosa*. The fourth Mex XY efflux system has also been described, but no downstream open reading frame for an outer membrane protein has been found. In *S. aureus*, NorA, an ATP dependent efflux pump, is reported to pump out hydrophilic quinolones like enoxacin or norfloxacin, but not sparfloxacin (Hydrophobic quinolone). The over-expression of Bmr and Blt, the NorA related efflux pumps in *B. subtilis,* provide quinolone resistance (Hiroshi, 1996).

Plasmid contains a *qnr* gene that encodes a protein of 218 amino acids, belonging to the pentapeptide repeat family. This protein protects the DNA gyrase from quinolone inhibition, although its effect on topoisomerase IV is unknown. In 1998, plasmid-mediated quinolone resistance was first reported in *Klebsiella pneumoniae* (Strahilevitz et al., 2009; Tran & Jacoby, 2002).

4.2.7 SULFONAMIDES

4.2.7.1 HISTORY AND MODE OF ACTION

Sulfonamides were the first synthetic drugs with a selective effect on bacteria. They were first used to treat *Streptococcus pyogenes* infection

in mice by Domagk in 1932 (Skold, 2000). To combat the rapid development of sulfonamide resistance after their introduction, sulfonamide was combined with trimethoprim due to the synergistic bactericidal effect resulted from the combination of both drugs (Huovinen et al., 1995).

Sulfonamide, structural analog to *p*-aminobenzoic acid (PABA), completely inhibits the dihydropteroate synthase (DHPS). DHPS catalyzes the condensation of PABA and 7,8-dihydro-6-hydroxymethylpterin pyrophosphate to form dihydropteroic acid, which is the final step in the folate biosynthetic pathway. Folate biosynthesis is required for thymine production and bacterial cell growth (Skold, 2000).

4.2.7.2 RESISTANCE MECHANISM

Sulfonamide resistant *Escherichia coli* have been isolated under selective pressure of sulfonamide. The mutant *E. coli* produces altered DHPS that shows lower affinity to sulfonamide, compared to the parent strain of *E. coli* (Pato & Brown, 1963). The sequencing of a *folP* gene that encodes DHPS revealed that the mutation results in the substitution of leucine codon instead of phenylalanine codon.

Plasmid borne sulfonamide resistance has been observed in *E. coli*. They produce altered DHPS enzymes (Skold, 1976; Wise & Abou-Donia, 1975). The plasmid mediated genes are characterized as *sul1* and *sul2*. The *sul1* gene is normally found on the *Tn21* type integrin, while *sul2* is usually located on *IncQ* family plasmid and *pBP1* (Swedberg & Skold, 1983).

4.2.8 TETRACYCLINE

4.2.8.1 HISTORY AND MODE OF ACTION

The first members of the tetracycline group were characterized in the late 1940s as Chlortetracycline and Oxytetracycline. These molecules were produced by *Streptomyces aureofaciens* and *Streptomyces rimosus*, respectively. Later, other tetracyclines were discovered either as naturally occurring molecules mostly in *Streptomyces* spp. or as semi-synthetic products such as methacycline, doxycycline, and minocycline (Swedberg & Skold, 1991).

Tetracycline enters in bacterial cells through porin channels and prevents the interaction of aminoacyl tRNA with the bacterial ribosome. They mainly bind to the 30S subunit of the ribosome in the protein synthesis machinery (Swedberg & Skold, 1991).

4.2.8.2 RESISTANCE MECHANISMS

The bacteria use three strategies to become resistant to tetracycline: efflux systems, ribosomal protection proteins and enzymatic inactivation. Acquisition of tetracycline resistance (*tet*) and oxytetracylcine (*otr*) gene is responsible for the development of tetracycline resistance in most bacteria. Approximately 38 different *tet* and *otr* genes have been reported so far. Out of the 38 different *tet* and *otr* genes, 23 genes encode energy dependent efflux proteins, 11 genes encode for ribosome protection proteins and 3 genes encode an inactivating enzyme (Table 4.3) (Roberts, 2005).

TABLE 4.3 Distribution of *tet* Genes in Bacteria (Roberts, 2005)

Gene	Mechanism	Bacterial Source
tet(A)	Efflux	*Acinetobacter*, *Haemophilus*, *Veillonella*
tet(B)	Efflux	*Acinetobacter*, *Brevundimonsa*, *Neisseria*, *Photobacterium*, *Pseudomonas*
tet(C)	Efflux	*Aeromonas*, *Chlamydia*
tet(D)	Efflux	*Alteromonas*
tet(E)	Efflux	–
tet(G)	Efflux	*Escherichia*, *Providencia*
tet(H)	Efflux	*Actinobacillus*, *Acinetobacter*, *Moraxella*, *Pasteurella*
tet(J)	Efflux	–
tet(K)	Efflux	*Lactobacillus*, *Norcardia*, *Streptomyces*
tet(L)	Efflux	*Actinobacillus*, *Morganella*, *Norcardia*, *Salmonella*, *Veillonella*
tet A(P)	Efflux	–
tet(V)	Efflux	–
tet(Y)	Efflux	–
tet(Z)	Efflux	–
tet(30)	Efflux	–
tet(31)	Efflux	–
tet(33)	Efflux	*Corynebacterium*
tet(35)	Efflux	*Stenotrophomonas*, *Vibrio*

TABLE 4.3 *(Continued)*

Gene	Mechanism	Bacterial Source
tet(38)	Efflux	*Staphylococcus*
tet(39)	Efflux	*Acinetobacter*
tcr3	Efflux	–
Otr(B)	Efflux	–
Otr(C)	Efflux	–
tet(M)	Ribosomal protection	*Acinetobacter*, *Afipia*, *Enterobacter*, *Erysipelothrix*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Microbacterium*, *Mitsuokella*, *Mycobacterium*, *Neisseria*, *Prevotella*, *Porphyromonas*, *Ralstonia*, *Photobacterium*, *Pseudomonas*, *Selenomonas*, *Streptomyces*, *Vibrio*
tet(O)	Ribosomal protection	*Megasphaera*, *Neisseria*
tet(S)	Ribosomal protection	*Lactococcus*, *Lactobacillus*, *Veillonella*
tet(T)	Ribosomal protection	–
tetB (P)	Ribosomal protection	–
tet(Q)	Ribosomal protection	*Neisseria*
tet(W)	Ribosomal protection	*Actinomyces*, *Arcanobacterium*, *Bacillus*, *Butyrivibrio*
–	–	*Clostridium*, *Lactobacillus*, *Mitsuokella*, *Megasphaera*, *Neisseria*, *Prevotella*, *Porphyromonas*, *Roseburia*, *Selenomonas*, *Staphylococcus*, *Streptococcus*, *Streptomyces*, *Veillonella*
tet(32)	Ribosomal protection	*Clostridium*
tet(36)	Ribosomal protection	*Bacteroides*, *Clostridium*, *Lactobacillus*
Tet	Ribosomal protection	–
Otr(A)	Ribosomal protection	–
tet(X)	Enzymatic inactivation	–
tet(34)	Enzymatic inactivation	*Aeromonas*, *Pseudomonas*, *Serratia*, *Vibrio*
tet(X37)	Enzymatic inactivation	*Unknown*
tet(U)	Unknown	*Staphylococcus*

Energy-dependent and membrane-associated proteins, encoded by *tet* and *otr* genes, decrease intracellular concentration of tetracycline. Thereby it protects the bacterial ribosomes *in vivo* (Roberts, 2005). Upon binding of tetracycline, a conformational change occurs in the ribosome, which disrupts the elongation cycle and protein synthesis stops. The ribosomal protection proteins interact with the ribosome and cause an allosteric change of tetracycline binding site, which results in the release of tetracycline molecule from the ribosome. Thus, ribosome regains its active conformation and starts protein synthesis. The ribosomal protection protein requires GTP to be functional. Tet(O) and Tet(M) are extensively studied ribosomal protection proteins (Connell et al., 2003).

An NADPH-dependent oxidoreductase, encoded by *tet(X)*, inactivates tetracycline in the presence of oxygen and NADPH. The *tet(X)* gene is found only in strict anaerobe, bacteroides (Chopra & Roberts, 2001). The *tet(34)* in *Vibreo* spp. encodes xanthine-guanine phosphoribosyl transferases, which inactivates tetracycline (Nonaka & Suzuki, 2002).

4.2.9 TRIMETHOPRIM

4.2.9.1 HISTORY AND MODE OF ACTION

Trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxy-benzyl)-pyrimidine was synthesized in 1962 and being explored for its antibacterial activity (Barbara et al., 1962). All the later agents have been developed from older antibiotics, which belong to families of agents. Like sulfonamides, trimethoprim is also a synthetic antibiotic that belongs to the diaminopyrimidine group of compounds (Skold, 2001).

Trimethoprim binds to dihydrofolate reductase (DHFR) and inhibits its activity. The DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate. Thus, it is also known as antifolate (Skold, 2001).

4.2.9.2 RESISTANCE MECHANISMS

There are three types of intrinsic trimethoprim resistance: one, chromosomally located in Tn7 transposon, second, thymine auxotrophic mutation in bacteria and third, mutational changes in the chromosomally encoded DHFR. It has been reported that Tn7 transposon is responsible for

trimethoprim resistance. The Tn7 specifically and efficiently transposes into the *Escherichia coli* chromosome (Lichtenstein & Brenner, 1981). Under selective pressure of trimethoprim, the mutation occurs in the gene coding for thymidilate synthetase. The *thy*-mutants require thymine exogenously to synthesize DNA and cause trimethoprim resistance (Maskell et al., 1978). The mutation in *dhfr* gene leads to the overproduction of altered enzyme and trimethoprim resistance (de Groot et al., 1988; Powell et al., 1991). Similarly, several 100-fold overproductions of the chromosomally encoded DHFR are reported in highly trimethoprim resistant *E. coli*. The enzyme overproduction is due to a mutational substitution of a glycine for a tryptophan at position 30 (Flensburg & Skold, 1987).

In 1972, plasmid mediated trimethoprim resistance was first reported in *E. coli* and *K. aerogenes*, isolated from infected urines (Fleming et al., 1972). During investigations on the R-plasmids mediated trimethoprim resistant *E. coli* and *Citrobacter*, it has been found that two types of R-plasmids DHFRs were identified. Both were different from the chromosomally encoded enzyme in terms of binding of dihydrofolate, NADPH, folate, and 2,4 diamino pyrimidine (Pattishall et al., 1977). Based on phylogenetic analysis, plasmid borne DHFR can be classified into two families. The family 1DHFR includes enzyme type I, V, VI, VII, and Ib. They are 64 to 88% identical. They are a homodimer of pentapeptide containing 157 amino acids. They confer a very high level of trimethoprim resistance. The family 2 DHFRs includes type IIa, IIb, and IIc.

They are homotetramer proteins of 78 amino acids (Huovinen et al., 1995). The Tn5086 transposon, 15.3 kb trimethoprim resistant, was found in two distinct plasmids. They contain the *dhfrVII* gene that encodes DHFR which has got glutamate instead of aspartic acid residue at the 27th position of the chromosomally encoded enzyme from *E. coli* (Sunstrom et al., 1993). In trimethoprim resistant *E. coli* and *Shigella* spp., *dhfrXII* gene has been found in the Tn21 like element (Heikkila et al., 1993).

4.3 NATURAL SMALL MOLECULES AS A SOURCE OF BIOACTIVE AND THERAPEUTIC AGENT

Antibiotics derived from natural sources, semi-synthetic processes, and synthetic processes, are commonly used for the treatment of ailments. Researchers believed that the infectious diseases would be soon eradicated

from this world. However, nature has given strength to every living organism to fight for survival under adverse conditions. With the introduction of novel antibiotics in clinical usage, resistance to them also evolved in microorganisms. Over the past 70 years, bacteria have been exposed to different antibiotics during treatment of human diseases, and many adapted genetic traits to nullify the effect of antibiotics. Since past two decades, several reports indicating the development of resistance in bacteria not only to one antibiotic but multiple antibiotics have been reported (Walsh, 2000), which disturbs the scientist and doctors alike.

Spreading of resistance to antibiotics has fascinated the researcher to develop new antibiotics. However, it is predicted from history that new antimicrobials will have a short life. Thus, resistance to new drugs leads shortage of antimicrobials as the discovery of new antimicrobial drug is not enough to cope with the fast emergence of resistant strains (Levy & Marshall, 2004). This requires new potent antimicrobial compounds as an alternative to existing antibiotics (Cowan, 1999). Natural molecules are a more promising source of drugs. Natural molecules have diverse chemical structure and biochemical specificity. Biologically active natural molecules usually have low molecular weight; they are easily absorbed and metabolized in the body. Major resources of natural molecules include plants, marine organisms, and microbes (Harvey, 2008). The plant produces innumerable secondary metabolites with several biological activities. Hence, it is worthwhile to explore the potential of plants for the development of new therapeutic agents (Lahlou, 2013).

4.3.1 PHYTOCHEMICAL AS AN ANTIMICROBIAL AGENTS

The plant produces secondary metabolites which help in plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some metabolites are responsible for odor and pigments production. These metabolites are known as phytochemicals. Depending on species and climate, they have a wide range of biological activity (Cowan, 1999). Phytochemicals can be separated into several categories: terpenes, phenolics, and nitrogen containing compounds (Chinou, 2008). Each plant species, genus, and family produce a mixture of these metabolites.

4.3.1.1 TERPENES

Terpene is a simple hydrocarbon molecule which contains isoprene units. Terpenes are the most structurally diverse plant molecule. The isoprene unit contains five carbon molecules. Therefore, isoprene is the most basic class of terpenes, which is known as hemiterpenes (C5). Monoterpene (C10) contains two isoprene units. Diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) have two, three, and four terpene units, respectively. Sesquiterpenes (C15) contain three isoprene units. When oxygen is added to terpenes, they termed as terpenoids (Cowan, 1999; Taiz & Zeiger, 2010). Sterols are triterpenes which contain an alcoholic group. Sterol acts as a precursor for the synthesis of cholesterol (Taiz & Zeiger, 2010).

The Essential oils commonly comprise monoterpenes and sesquiterpenes. They have pharmaceutical properties like antifungal, antimalarial, etc. The isolated monoterpenes, diterpenoids, sesquiterpenes, and triterpenoids have been reviewed recently for their antibacterial activity (Kurek et al., 2011). Sesquiterpenes (Diterpenoids) isolated from diverse medicinal plants showed antibacterial activity against gram-positive bacteria and also have growth inhibition activity against *Mycobacterium tuberculosis* (Kurek et al., 2011; Garcia et al., 2012). Terpenoids are effective against bacteria and fungi. However, the mode of action of terpenoids is not identified yet. It is assumed that they interact with a lipophilic compound of the membrane and disrupt the membrane. It has been reported that essential oil of plant origin showed inhibitory effect on *Listeria monocytogenes* (Aureli et al., 1992). Essential oil of tea tree having a composition of terpinene 4-ol, α-pinene, linalool, and α-terpineol have antibacterial activity and reported to disrupt the cell membrane of bacteria (Gustafson et al., 1998; Carson et al., 2002).

4.3.1.2 PHENOLICS

Plants produce a large number of secondary metabolites that have phenolic compounds. Many phenolic compounds protect plants from herbivores and plant pathogens. Some compounds act as an attractant of pollinator. Plants secrete many phenolic compounds to inhibit the growth of other plants near its periphery. Others act as a shield to protect from ultraviolet radiation damage (Taiz & Zeiger, 2010).

Polyphenolic compounds have potential antioxidant activity and antimicrobial activity. Simple phenolic acids, ferulic acid and gallic acids are effective against bacteria by rupturing or making pores in the cell membrane (Borges et al., 2013). Catechol and pyrogallol are the allelochemicals which are produced by plants. It is believed that they are bio-synthesized by plants to control plant interaction with pathogens. Both compounds are toxic to bacteria (Kocacaliskan et al., 2006). Quinones are also classified as a phenolic compound having potential antimicrobial properties. They make a stable complex with amino acids in microbial proteins and leads to the loss of functions (Cowan, 1999; Saleem et al., 2010).

Flavonoids are phenolic compounds, and most of them have flavone nucleus with two side aromatic rings. These compounds play a key role in the protection of plant from pathogens as well as predators and also play a major role in physiological functions (Cowan, 1999; Taiz & Zeiger, 2010). Antibacterial activity of flavonoids has been extensively studied. Olive leaves showed antibacterial activity and found to have luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside and luteolin 4'-O-glucoside (Pereira et al., 2007). Seven pure flavonoids were isolated and evaluated for their antibacterial activity. Isolated flavonoids show more activity on gram-negative bacteria (Basile et al., 1999). Epigallocatechin-3-gallate (EGCG) is a flavonoid compound found in high concentrations in green (unfermented) leaves of *Camellia sinensis* (tea). It has been found that consumption of green tea has a variety of health benefits. It also showed anticancer and antimicrobial properties. Gram-positive, gram-negative, and fungal pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and MDR *Acinetobacter baumannii,* were sensitive to EGCG (Taylor et al., 2005).

Tannin is high molecular weight phenolic phytochemical which is produced by diverse medicinal plants. Tannins can be categorized into two categories: hydrolysable and condensed tannins. Condensed tannins are a polymer of flavonoid units. They can be easily converted into anthocyanidins in the presence of strong acids. Hydrolyzed tannins are a polymer of phenolic acids like gallic acids and simple sugars (Cowan, 1999; Taiz & Zeiger, 2010). Like other phenolic compounds, tannins also protect from herbivores and plant pathogens. They act as a repellent and force animals away from the plant (Taiz & Zeiger, 2010). However, it has been reported that the ingestion of beverages having a substantial amount of tannin, especially green teas, and red wines, can provide immunity against

ailments in humans (Cowan, 1999; Taiz & Zeiger, 2010). The duramen has a higher concentration of tannin that inhibits decaying activity initiated by bacterial as well as fungal growth (Taiz & Zeiger, 2010). Thus, it also has antimicrobial activity. Gallotannins were isolated from Mango (*Mangifera indica* L.) Kernels and showed gram-positive bacterial growth inhibition activity. However, bacterial growth was regained by the addition of iron to the medium. Thus, it has been proved that the inhibitory effects are due to their iron-binding properties (Engels et al., 2009).

4.3.1.3 NITROGEN-CONTAINING COMPOUNDS

Structurally, alkaloids are a vastly distinct group of compounds that comprise an aromatic ring structure and a nitrogen atom. Alkaloids are produced by a wide variety of medicinal plants. Alkaloids have the excellent property to act as either hydrogen acceptor or hydrogen-donor for hydrogen bonding which plays a crucial role in the interaction of the molecule with enzymes, proteins, and receptors (Kittakoop et al., 2014). Berberine is isoquinoline alkaloids. It usually occurs in the root, rhizome, and stem bark of different group of plants like *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (Barberry), *Berberis aristata* (Tree Turmeric), etc. Berberine showed antibacterial activity against enterovirulent multidrug resistance *Escherichia coli* (Bandyopadhyay et al., 2013). Two new benzophenanthrene alkaloids, namely 8-acetonyldihydronitidine and 8-acetonyldihydroavicine, were purified from *Zanthoxylum tetraspermum* and *Z. caudatum* that possessed note-worthy antibacterial activity (Nissanka et al., 2001). The indole alkaloid found present in the ethanol extract of *Tabernaemontana catharinensis* root bark which showed an antimicrobial activity (Medeiros et al., 2011).

4.4 CONCLUSION

Antimicrobial agents are essential for our healthcare needs as they are lifesaving arsenals. However, uncontrolled use of them has emerged antimicrobial resistance as soon as they were introduced. Increased development of drug resistance in pathogens has become a viable problem for the healthcare system. Antimicrobial-resistant microorganisms have higher morbidity and mortality, and in turn, it leads to higher healthcare

costs. Thus, it is very important that discovery and production pace of new active molecules should be maintained on a scale to fulfill increased demand for antibiotics. The pharmaceutical companies are in search of novel antimicrobial agents, but they are non-productive. However, it indicates that health care will face an antibiotic crisis. To deal with the global crisis of antibiotics, coordinated efforts should be made to implement new policies, and research efforts on novel antimicrobial drugs. Natural small molecules, which are produced by plants, animals, and microbes, could be the alternative of source of antimicrobial agents.

KEYWORDS

- **acetyltransferases**
- **adenosine monophosphate**
- **antibiotics**
- **bacteriophages**
- **integrons**
- **lipopolysaccharide**
- **morbidity**
- **plasmids**
- **resistance transposons**

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CHAPTER 5

Personalized Medicine: Understanding the Individual Intricacies

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ABSTRACT

Personalize medicine/precision medicine/stratified medicine/targeted medicine/pharmacogenomics, as the name suggests, refers to the medicines that are designed and formulated based on specific characteristics of a particular person, which in simple terms can be defined as the right drug providing for the right patient at the right dose at the right time. The concept of personalized medicine (PM) came when the sequencing of the entire human genome began in the 21st century. The developments in biotechnology have revolutionized the science in the modern world and allowed scientists to elucidate the role of genetics in behaving with chemotherapy which in turn gave birth to pharmacogenomics. PM has the potential to match therapy with a therapeutic response with the lowest toxicity to ensure better patient care. PM promises to eradicate health-related problems by enabling early diagnosis, risk assessments, and optimal treatments for each particular patient (Vogenberg et al., 2010). Personalized medicine provides a platform to explore the agents that are targeted to patient groups that do not respond to conventional therapies and where the therapies have failed. The most important in Personalize medicine is to elucidate the variations in DNA and RNA characteristics

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with the drug response, seeking to understand the expression of mRNA, which affects the body's response to the medications.

5.1 INTRODUCTION

The best patient care is achieved by having the best response and highest safety margin which becomes possible by the potential of matching therapy with patient characteristics through personalized medicine (PM), and it allows the particular patient to be looked for early-stage diagnosis, risk assessments, and proper treatment. The lowering of economic costs with an overall improvement in health is the main characteristic promise that has been made by it (Vogenberg et al., 2010). For medical applications like patient care, including disease markers for targeted chemotherapy and early disease diagnosis and treatment matching then, how individual genes have to be studied is defined by medical genetics, which is rapidly revolutionizing healthcare, making it more personalized than ever (Offit, 2011). In the National Institute of General Medical Sciences, 2013 the first human genome was completely mapped down and cataloged all the genes in a human (Blix, 2014). The Human Genome Project (HGP) was completed in 2003 and approximately 21,000 genes in a human were cataloged, respectively (Stratton et al., 2009). Researchers in medical genetics have found that not only single genes predict disease but there are other factors which are equally important. Also medical genomics is a broad term which includes the study of individual genes as well as factors (Epigenetic and environmental factors) that have active participation in inheritance patterns and how health or medical care can be matched to the individual genetic makeup (Blix, 2014). The nurses need to be stay informed about the health/medical care to be delivered in which PMs represent a major change like the clinical usage, pharmacogenomics, and the social impact, respectively (Blix, 2014). Genetics plays a great role fundamentally in cancer so, advances in genetics have a prominent impact on cancer therapeutics, and nurses as well as other members of the health care team will be looked at by the patients more importantly (Riley et al., 2012). For obtaining approval of PMs from regulatory agencies like the Food and Drug Administration (FDA) there have been many challenges (Goetz & Schork, 2018). Physicians, health care executives, insurance companies, and finally patients have many issues in accepting

PMs (Goetz & Schork, 2018). Our understanding of Human diseases has advanced more significantly with more focus on the sequencing of the human genome, particularly over the past 10 years (Tremblay & Hamet, 2013). Advances in the technological process in analyzing intergenic variants like SNP (single nucleotide polymorphisms), millions of genes and copy number variations per person have enhanced our understanding of inter person differences in the overall genetic profile (Tremblay & Hamet, 2013). The common genetic differences associated with many diseases have been successfully identified by GWAS (genome-wide association studies) (Tremblay & Hamet, 2013). There occurs a change in the way of new drugs that are being discovered from all the knowledge that we have gained so far, particularly to treat cancer (Tremblay & Hamet, 2013). Diagnosis, prognosis, risk assessment, and treatment are the main application of genomics in PM (Tremblay & Hamet, 2013).

The classical examples in personalized medicines of the genomic investigation areas:

i. Fragile X syndrome, the severity of which is predicted by the number of tri-nucleotide repeats (Sherman et al., 2005).
ii. Risk assessment of breast cancer through BRC1/2 (Tremblay & Hamet, 2013).
iii. Diagnosis of breast cancer subtypes via gene expression analysis (Sørlie et al., 2001).
iv. The women possessing HER2-positive breast cancer, Herceptin® also called trastuzumab administration is confined based on diagnosis (Piccart-Gebhart et al., 2005; Romond et al., 2005).
v. Diminished therapeutic response due to CYP2C19 variants (Shuldiner et al., 2009).

5.1.1 NEED FOR PERSONALIZED MEDICINE (PM)? WHY?

- Subjects having similar symptoms but distinct disorders;
- Genetics will help in medical intervention more specifically;
- In some subjects, drugs work while not in others;
- To reduce allergic and adverse events;
- Design best target based therapies;
- Need for evidence-based medicines;

- A good of percentage of drugs available are not efficacious in treating ailments;
- Some subjects respond to less dose, some to more dose, some to normal and some do not respond at all (Figure 5.1).

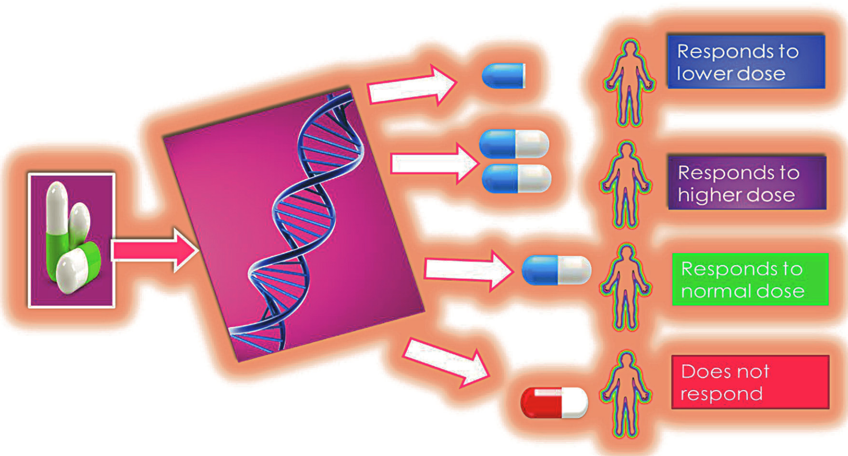

FIGURE 5.1 Pictorial overview of drug responses with different genetic profiles.

5.2 ENVIRONMENTAL FACTORS INFLUENCING GENOMICS

Environmental influences many biological pathways and are responsive to environmental stimuli in the most complex and polygenic diseases, like diabetes, hypertension and atherosclerosis, which are heritable (Hunter, 2005). Cigarette smoking constitutes one of the big environmental risk factors for atherosclerosis (Tremblay & Hamet, 2013). The carrier state of the ApoE epsilon 4 allele with cigarette smoking is having a synergistic effect and increases the atherosclerosis risk, respectively (Mertens, 2010). A group of genomic markers that were related to smoking cessation was identified by GWAS, allowing to develop a quit-success genotype score a prototype by its authors (Rose et al., 2010). Now the data revealed provide ways of smoking stoppage which may be based on genotype score, nicotine dependence, or carbon monoxide observance (Tremblay & Hamet, 2013). A personalized and adaptive approach to smoking cessation cure needs support as the study has not been replicated in the independent cohorts and based on genotypic as well as phenotypic characteristics of a

particular smoker, PM offers smoking cessation ways (Tremblay & Hamet, 2013). Computational modeling needs to be developed to investigate how polygenes talk with factors associated with the environment to perturb the phenotype expression as the gene environmental interactions are complex in nature (Tremblay & Hamet, 2013). Genes play a varying role in both females as well as males in the same families when the interaction between current and post smoking with cardiovascular responses to stress, hypertension, obesity, and BMI (body mass index) modulated by synaptic plasticity was recently described (Nikpay et al., 2012).

5.3 GENOMIC STUDIES: INSIGHTS

Genetic makeup makes humans distinct from one another, simultaneously it also plays a role in the susceptibility to various disorders/diseases (Jiang & Wang, 2010). New technological advances in genomic sequencing have revolutionized the studies on patterns of gene expression, miRNA expression, DNA copy number variations, single nucleotide polymorphisms, and factors assessed by utilizing multiple pathological and clinical parameters, respectively (Panel, 2001; Crivellari et al., 2003). Two gene prognosis signatures panels for the prognosis of breast cancer were developed independently and were validated by two teams respectively one gene signature consisting of 76 genes and the other was comprised of 70 genes (Van De Vijver et al., 2002; Wang et al., 2005). Prediction success was better in both gene signature panels for distant relapse risk than that of adjuvant online software (Jiang & Wang, 2010). In terms of prediction ability of distant relapse risk of breast cancer both gene signature panels show similarities and in the two-breast cancer prognostic gene signature panels there were few genes that overlapped (Jiang & Wang, 2010). Due to differences in the characteristics of the patient population, there was a small overlap. The patients included were of under 55 years of age in 70-gene identification study and patients ages were under 40–70 years old with an average of 54 years in Wang et al. study (Panel, 2001; Wang et al., 2005).

The reference or standard human genome sequence completion has facilitated the cataloging and discovery of differences/variations in the genome sequences among subjects including both diseased as well healthy and in different populations (Ginsburg & Willard, 2009) (Figure 5.2).

About 10–15 million genomic sequence variants have been estimated and are of sufficient frequency in one or more populations to be considered genetically polymorphic in humans (Ginsburg & Willard, 2009). In a single or few subjects, countless rare variants exist and will be accessible via direct genome sequencing (Geoffrey & Huntington, 2009). A key question is to what extent genome impact the likelihood of disease onset, alarm the natural history of the disease, or gives relevant clues about the management of the disease if we talk in the context genomic PM (Geoffrey & Huntington, 2009).

5.4 PHARMACOGENOMICS IN PERSONALIZED MEDICINE (PM)

Pharmacogenomics allows the use of genetics to optimize the drug discovery and development process. The main focus is too tailor-made drugs in which right drugs are developed for the right patient and we can say that it is just a marriage between molecular pharmacology and functional genomics (Norton, 2001; Fakruddin & Chowdhury, 2013). Pharmacogenomics aims to discover the correlations of subject's genotype with their therapeutic response to tailor the specific genetic makeup (Torres et al., 2003). The overall process involves genes encoding proteins are identified that can be used as potential drug targets and to analyze or understand the variations in the genes (Fakruddin & Chowdhury, 2013). The growing evidence that the genetic makeup is a key predictor of how effective drugs will behave has gained the interest and funding (Fakruddin & Chowdhury, 2013) (Jiang & Wang, 2010). One of the best examples is a breast cancer treatment in which gene expression analysis data has been exploited clinically for the determination of the prognosis of a breast cancer patient. Luminal, HER2 positive, and basal-like breast cancers were classified as three major subtypes via gene expression profiling studies which are based on the differentiation status and tumor grades, expression levels of the HER2 (human epidermal growth factor receptor 2), positive or negative lymph node status (Jiang & Wang, 2010). Patients classified into subtypes can help in the prediction of treatment outcomes (Jiang & Wang, 2010). The patients who present the same pathological and clinical manifestation cannot predict the patient's treatment accurately based on traditional prognostics.

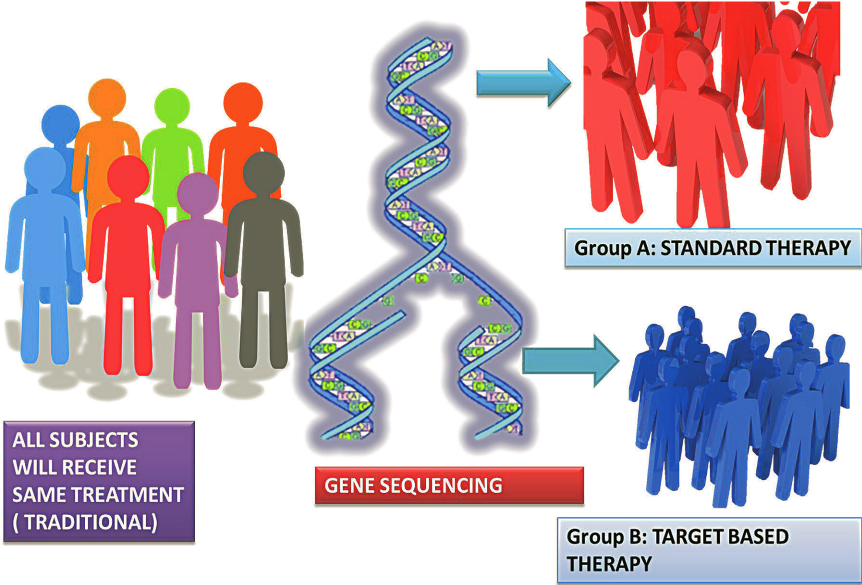

FIGURE 5.2 The overview of pharmacogenomics where the main focus remains to tailor-made right drugs for the right patients.

5.4.1 APPLICATIONS IN PHARMACOGENOMICS

The DNA variations in genes that code for CYP 450 (cytochrome P) family of enzymes which metabolizes 30 different classes of drugs can influence their ability to metabolize certain drugs and the enzymes which are unable to metabolize can cause drug overdose problems in patients, for example, less active enzymes/inactive enzymes are unable to break down some drugs which ultimately lead to drug overdose (Association et al., 1999; Fakruddin & Chowdhury, 2013). Nowadays, genetic tests are being performed in clinical trials by researchers for checking variations in CYP 450 genes for the purpose of screening and monitoring patients (Fakruddin & Chowdhury, 2013) and chemical compounds developed in pharmaceutical companies are screened with variants of CYP enzymes to elucidate how they are getting metabolized. TPMT (thiopurine methyltransferase), an enzyme that breaks down thiopurines, a class of therapeutic compounds, but in case of Caucasians a small percentage of the population have variations in genetic makeup that make them unable to produce an active form of this TPMT protein which results in the accumulation of thiopurines

leading to toxicity (Pistoi, 2002). The pharmacogenomics mainly focuses on three key goals, including reducing risk, increasing efficacy, and developing diagnostic tools that can impact therapeutic decisions and improve the clinical outcomes. The various clinical pharmacogenomics studies are described in Table 5.1.

TABLE 5.1 Clinical Pharmacogenomics Studies

Disorder	Drug	Genetic Polymorphism	Result	References
Schizophrenia	Clozapine	5-HT2A protein C102 allele	The homozygotes of C102 response to atypical antipsychotics like clozapine	Arranz et al. (1995); Masellis et al. (1998); Joober et al. (1999)
Atherosclerosis	Pravastatin	TaqB1alleles of B1 and B2 site in CETP (cholesteryl-ester transfer protein), LDL (lipoprotein lipase), and β-fibrinogen.	B1B2 heterozygotes have less better response as compared to homozygotes with pravastatin	Kuivenhoven et al. (1998)
Alzheimer	Tacrine	ApoE4 protein	APOE4 in subjects having other than homozygous alleles show better response.	Poirier et al. (1995); Richard et al. (1997); Rigaud et al. (2000)
Asthma	Montelukast and Zileuton	ALOX5 genotype	In heterozygotes, there is reduced response	Drazen et al. (1999)

5.5 THE CONCEPT OF BIOBANKS IN PERSONALIZED MEDICINE (PM)

In PM, based on different biomarkers in the patient's blood and tissue therapies are selected (Hewitt, 2011). The bio-specimens required for research purposes to detect useful biomarkers are provided by biobanks, PM shortly will focus on the translation of fast-growing knowledge about disease processes into useful therapies. The path of developing a medical product is highly challenging which is inefficient and costly in today's world, and only a concerted effort in modernizing the critical path of developing a new medical product will succeed (Hewitt, 2011). The FDA critical path initiative identified a better evaluation tool like a biomarker

for the concerted effort of developing medical products (Hewitt, 2011). The recommendation of biomarkers came from AACR–FDA (American Association for Cancer Research), NCI (US National Cancer Institute), and the aim was given on the necessity of the need for bio-banking services as well as bio-specimen quality control, respectively (Khleif et al., 2010). Concerning the cancer research area, in particular, accelerating PM the improvement of bio-banking services is high on goal (Hewitt, 2011). The term biobank has been described in several ways, one of the definitions describe it like an organized collection of a human biological sample and related information stored for one or more research purposes (Cambon-Thomsen et al., 2007; Kauffmann & Cambon-Thomsen, 2008). The collection of non-human samples like a plant, microbe, environmental, and animal may also be explained as biobanks (Hewitt, 2011). Biobanks of human material fall into many categories, depending on design and purpose and it includes population-based biobanks, twin cohort studies and disease-oriented biobanks (Gottweis & Zatloukal, 2007; Hewitt, 2011). The disease-oriented biobanks are the most frequent hospital-based and consist of tumor banks, collection of blood, and other samples from different diseases together with normal control groups (Hainaut et al., 2009; Bevilacqua et al., 2010). The ethical discussion in bio-banking has focused specifically on the validity of general consent (Cambon-Thomsen et al., 2007). The information covers all aspects related specifically to the personal choice then the consent of the person is appropriately informed (Forsberg et al., 2009). The bio-banking collection model a move towards general consent for future research use is considered as a trend in bio-banking ethics (Cambon-Thomsen et al., 2007; Forsberg et al., 2009).

5.6 NEXT-GENERATION SEQUENCING (NGS): IMPLICATIONS IN PERSONALIZED MEDICINE (PM) AND PHARMACOGENOMICS

The recent trends in genetics have dramatically enhanced efforts to unravel the mechanism of human diseases (Rabbani et al., 2016). In many cases, the phenotypic consequences of various disorders are affected by the genotype and searching the casual variants may lead to the development of novel paths of medical intervention. The pharmacogenetics research involvement in the clinical practice could significantly change in the way of medications are consumed or in another way, it can improve the health

care globally and will help in the administration of drugs (Farberov et al., 2013; Goodhead et al., 2013; Girault et al., 2014; Rabbani et al., 2016). The international scientific research effort called HGP aimed to sequence and map down the human genome by using the Sanger sequencing method and the sequence of the human genome was published in 2003 (Cheung et al., 2001; Consortium, 2001; Sequencing, 2004). Generally, the Sanger approach is having excellent accuracy and reasonable read length but low throughput and expensive to sequence down all the genes; however, it's a robust method for clinical application (Rabbani et al., 2016). Next-generation sequencing (NGS) platforms from the last 10 years now provide affordable and high throughput sequencing for the evaluation of functional variations in DNA in many disorders consisting of both monogenic as well as polygenic phenotypes like diabetes, cancer, neurological, and cardiovascular disorders or the regulation of normal human body functions like height, BMI, blood pressure (Metzker, 2010; Bamshad et al., 2011; Kingsmore & Saunders, 2011; Diaz-Horta et al., 2012; Rabbani et al., 2012, 2014). Nowadays, there is increasing demand to apply personal genome sequencing for predicting risk, medical care, lifelong well-being, and therapeutic response in the era of PM (Rabbani et al., 2016). Millions of DNA variants in different populations have been identified by DNA sequencing, mainly SNV (single nucleotide variants) (Altshuler et al., 2012; Consortium, 2010). About 38 million single-nucleotide variants, 14,000 large deletions, and 14 million bi-allelic deletions or insertions have been discovered (Altshuler et al., 2012). The methodology of GWAS was established by the use of a haplotype map of the human genome in HapMap and with the help of GWAS several SNVs associated with cancer, type 2 diabetes (T2D), asthma, and common traits like fat mass, height can be determined (Easton et al., 2007; Moffatt et al., 2007; Sladek et al., 2007; Stacey et al., 2007; Todd et al., 2007; Sanna et al., 2008).

5.7 TRENDS OF PM WITH REFERENCE TO DIABETES

Diabetes is a metabolic disorder characterized by increased blood glucose levels. Approximately 382 million subjects are suffering from diabetic disorder globally and it's an estimate in the near future that it will increase by 50% in the next two decades (Kleinberger & Pollin, 2015). The implementation of PM in Diabetes intervention is very small. The American Diabetes Association classifies diabetes into four classes:

1. **Type 1 Diabetes Mellitus (T1DM):** It refers to an auto-immune disease in which autoantibodies against pancreatic beta cells results in mass cell destruction causing a decrease in insulin production, reduction in weight, polyuria, polydipsia, and make the subject dependent on external insulin (Chiang et al., 2014; Kleinberger & Pollin, 2015). The complex causative factors responsible for T1DM has involved about 40 genes with unknown clinical implications (Chiang et al., 2014).
2. **Type 2 Diabetes Mellitus (T2DM):** It generally refers to a metabolic disorder with a reduction in insulin secretion or increased resistance to insulin (Association, 2017). About 80 susceptible genetic locations have been detected to understand the genetic cause of T2DM (Hara et al., 2014). Genetic testing can revolutionize the diagnosis process in subjects having diabetes or pre-diabetes and can help to intervene in lifestyle change or therapeutic regimen to prevent it at an early stage.
3. **Monogenic Diabetes:** The third category of diabetes has clinical presentation the same as T1DM and T2DM. The clinical presentations are almost the same as T1DM as well as T2DM and are having known genetic and non-genetic causative factors. Monogenic diabetes with genetic testing has proven the ability for enhanced treatment; however, diagnosis is not made yet (Shepherd et al., 2009; Kleinberger & Pollin, 2015).
4. **Gestational Diabetes:** The causative factors may be the same as the other three diabetes subtypes described and may designate expression of elemental vulnerability certified by pregnancy prompted insulin resistance (2014). There is an immense need for development in the field of personalized diabetes in order to curb the growing concern. Almost 13 types of MODY (maturity-onset diabetes of the young) are categorized based on the non-functional gene causing the phenotype of which is one well-elucidated form of monogenic diabetes. Before the age of twenty-five MODY extant in skinny subjects and is inheritable; however, the subject has yet signed of β cell function (Turner et al., 1999). In the UK (United Kingdom) MODY3 is the most prevalent form of MODY consisting of 52% of total percentage however the presence of it changes by ethnicity as well as area (Shields et al., 2010). HNF1A gene which encodes HNF1-α (transcription factor hepatic nuclear

factor 1-α) which is responsible for transcription of many genes that are associated with insulin release, breakdown of glucose and synthesis of insulin. HNF1-α has 55% resemblance with HNF4-α which is mutated in MODY1 and MODY1 comprising 10% MODY in UK (Shields et al., 2010). The HNF1-α as well as HNF4-α have been demonstrated to talk in a way to suppress each other's effect (Boj et al., 2010). The diagnosis of subjects suffering from MODY1 and MODY3 is very crucial for therapeutics because they are more sensitive that is hypersensitivity to sulfonylureas (Pearson et al., 2000; Ješić et al., 2008; Shepherd et al., 2009). The reduction in HNF1-α and HNF4-α genes expression in the liver is responsible for hypersensitivity to sulfonylureas which leads to minimal uptake and causes the persistent enhancement of its blood levels (Boileau et al., 2002). So, the subjects will require only 1/10 dose of sulfonylurea and makes sulfonylureas as the drugs of choice for MODY3 and MODY1. In the above discussion, it's obvious how the genetic makeup plays a role in patient response towards drugs so the application of PM will assist in deciding the right drug for the right patient at the right time and with the right dose.

The other contributing factor is mutations in glucokinase (GCK) gene encoding GCK enzyme which is critical for glucose metabolism leading to MODY2. The mutations are responsible for a reduction in the activity of GCK enzyme that is important for pancreatic β cell supervision at circulating glucose levels. Subjects suffering from MODY2 usually have a mild increase in blood sugar levels and don't proceed to complications like micro-vascular or macro-vascular, So MODY2 subjects don't require any treatment (Ajjan & Owen, 2014). Although certain examinations have demonstrated that a mild increase in blood glucose levels can result in insulin resistance (Clement et al., 1996) and regarding the carriers the gestational diabetes mellitus is a prime example of having GSK gene mutations (Ellard et al., 2000; Shehadeh et al., 2005).

5.7.1 PHARMACOGENETICS OF SOME T2DM MEDICATIONS

Subjects suffering from T2DM contribute to the highest percentage of cases with diabetes mellitus. Metformin being the drug of choice for

T2DM is considered the first-line drug which operates by reducing gluconeogenesis in the liver. The class of drugs like sulfonylureas, meglitinide, glucagon-like peptide (GLP-1), thiazolidinediones, and DPP4 inhibitors (dipeptidyl peptidase inhibitors-4) are considered as second-line classes of drugs (2014). The examinations of the genetic variations that could change the therapeutic response to anti-diabetic drugs have demonstrated moderate effects, and few are totally opposite/contradicting. These examinations describe a judgment that may give us a clue about the upcoming pharmacogenetic recommendations for oral anti-diabetic drugs.

5.7.1.1 METFORMIN

The mechanism of metformin is poorly understood, and its target receptors have not been elucidated for pharmacogenetic variations. The GWAS elucidated that SNP rs11212617 close to ATM locus was connected with a decrease in HbA1c in response to metformin (van Leeuwen et al., 2012). The phosphatidylinositol 3-kinase family having a member namely ataxia telangiectasia mutated gene encoded by ATM is very crucial for cell cycle regulation and repairing of DNA. One of the meta-analysis replications of this very study has shown that the correlation was accredited however 1 of the 3 cohorts demonstrated no correlation (Zhou et al., 2011). The DPP (Diabetes Prevention Program) revealed that there was no correlation between rs11212617 as well as progression from impaired sugar tolerance (Florez et al., 2012). The single nucleotide polymorphism requires more accreditation and exploring more pathways for confirmation and upcoming studies into metformin therapeutic mode of action (a mechanism).

5.7.1.2 SULFONYLUREAS

The sulfonylureas act by ligating with sulfonylureas receptor subunit 1 (SUR1) (which is encoded by ABCC8 gene) near to ATP sensitive K^+ inward rectifying channel responsible for depolarization which is then followed by Ca^{++} influx which results in insulin secretion. The other subunit of the potassium ATP sensitive channel is kir6.2 (which is encoded by KCNJ11) very near to the ABCC8 gene having chromosome number 11 (Inagaki et al., 1995). The high dose of sulfonylurea is given to treat neonatal diabetes mellitus caused by stimulating mutations in KCNJ11

as well as ABCC8 although the low dose is administered as the first line or drugs of choice for the subjects suffering from MODY1 and MODY3. Investigations of varying shapes of these genes have revealed a familiar haplotype of E23K in KCNJ11 and S1369A which are affiliated to T2D mellitus (Florez et al., 2007). Path-clamp technique has revealed that sulfonylureas are less responsive to this haplotype (E23K in KCNJ11 and S1369A) (Lang et al., 2012). The polymorphisms have contradicted correlation with T2D mellitus and sulfonylurea therapeutic effectiveness (Sesti et al., 2006; Sato et al., 2010).

5.7.1.3 MEGLITINIDES

The meglitinides are oral anti-diabetic drugs that usually act by inhibiting potassium sensitive ATP channels causing depolarization leading to insulin release. They are metabolized in the liver (McLeod, 2004) which is the principal organ for drug metabolomics. Meglitinides are transported to the liver by a protein SLCO1B1 and the c.521T>C polymorphism has demonstrated a reduction in the rate of breakdown of meglitinides, but the changed pharmaco-kinetics have a less biological effect on sugar levels (Phillips et al., 2001; Cheng et al., 2013). Repaglinide which is metabolized by CYP2C8 and CYP3A4 differs from Nateglinide which s metabolized by CYP2C9. The genetic polymorphism in metabolic proteins may change the pharmacokinetics of the drugs but it doesn't demonstrate any significant effect on glucose levels of subjects (Kirchheiner et al., 2004).

5.8 THE OPPORTUNITIES FOR PHARMACOGENOMICS IN T2DM

T2D mellitus promotes to consume the pharmacogenomics for the production and development of new drug therapies in addition to how the drug action is affected by genetic polymorphisms. A specific genetic variation could give enough information about the pathophysiology of a disease. The most important prime discovery in pharmacogenomics is the nonsense mutation in SLC30A8 that is a safeguard contrary to T2D mellitus that could be consumed for drug development. So, by shooting SLC30A8 for prevention of up-regulation could assure T2D mellitus nonsense variants. The other prime genetic variant example is APOC3 nonsense variant R19X that could bring the potential upcoming treatment ways. The nonsense

variants reduce the blood levels of APOC3, resulting in a reduction of serum triglycerides levels and safeguarding from coronary disaster (Pollin et al., 2008; TG, HDL Working Group of the Exome Sequencing Project et al., 2014). Nowadays the APOC3 has become the shoot point target for the therapeutic inhibition to safeguard from increased triglycerides (hypertriglyceridemia). The common complication of diabetes mellitus is coronary artery disease due to growing hypertriglyceridemia this can be an opportunistic direction for drug development (Fitzgerald et al., 2014). Pharmacogenomics can bring more dramatic changes in the upcoming future to eradicate the conventional means of drug development and will allow scientists to focus more on the target-based approach by elucidating the entire genomic functions.

5.9 TRENDS OF PM WITH REFERENCE TO ASTHMA

Asthma is one of the inflammatory disorders that affects airways by about 300 million people globally. There are many classes of drugs that are being employed to treat asthma targeting distinct biological pathways consisting of leukotriene antagonists, glucocorticoids, anti-cholinergic, beta-2 agonists, phosphodiesterase inhibitors (PDE) and monoclonal antibodies against IgE. Despite the availability of a large number of drug classes against asthma still, there are refractory asthmatic patients who do not respond to either of the treatments (Chan et al., 1998; Society, 2000). The classes of drugs targeting many pharmacological pathways have adverse and sometimes fatal adverse threatening reactions. The genes which regulate the multiple pharmacological pathways that utilize by anti-asthmatic drugs have many variations that in-turn alters the therapeutic efficacy and form the basis of development of genetic profiles for PM (Castle et al., 1993; Nelson et al., 2006; Salpeter et al., 2006). The genetic investigations of susceptibilities, severity, and therapeutic responses to anti-asthmatics can result in pathways to build up the genetic profiles (Ortega et al., 2015). The ideal genetic profile should be consisting of genetic variants that can predict the drug is choice with maximal therapeutic efficacy, the severity of diseases, the progression of the disease, and predict therapies avoidable for a particular subject. In the case of asthma genetics, the environmental effects intervene in the pharmacological therapy and alter in phenotypic therapeutic response (Figure 5.3). In twin-based clinical trial investigations

in which the heritable pharmacological responses were monitored demonstrated that the therapeutic responses to different therapies within the same subjects vary much less than inter-subject therapeutic responsiveness (Kalow et al., 1998; Drazen et al., 2000). The pharmacogenetic investigations are focused on the genes which regulate the pharmacological pathways utilized by leukotriene, β_2-adrenergic agonists and the glucocorticoids and many genetic locations associated with therapeutic responses in asthma have been identified (Tables 5.2–5.4).

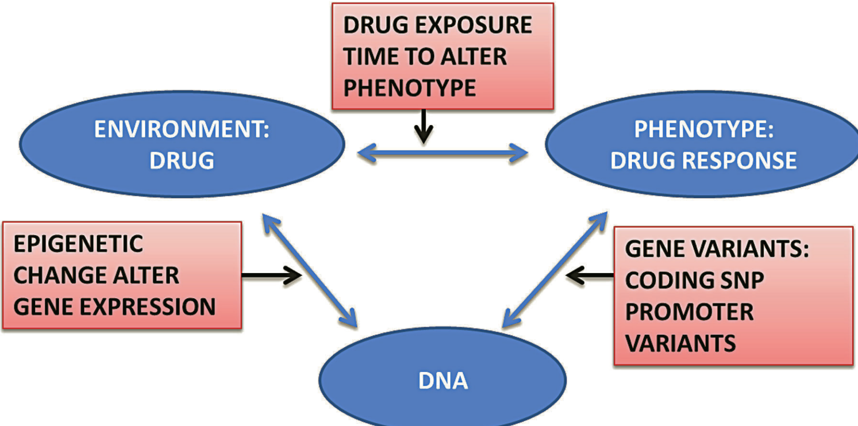

FIGURE 5.3 Pharmacogenomics research in complex disorders. For example, in asthma genetics, the environmental effects intervene in the pharmacological therapy and alter in phenotypic therapeutic response. (Modified from Ortega et al., 2015.)

In the case of lung cancer and cystic fibrosis, the predictive genetic profile is becoming reality in the therapeutic management however not yet in case management of asthma (Ramsey et al., 2011; Rosell et al., 2013). The pharmacogenetic research has identified genes that can affect therapeutic response to the drugs currently under development that shoot specifically pharmacological pathways.

5.9.1 GLUCOCORTICOID PHARMACOLOGICAL PATHWAY (GPP)

The glucocorticoids administered both orally or intravenously are the most common drugs used in inflammatory disorders and are one of the first-line therapies for asthma management along with inhaled

TABLE 5.2 Pharmacogenetic Subject Genes for Glucocorticoid Response in Asthmatics

Class of Drug	Design of Study	Gene	Loci Associated	Phenotype Response	References
Glucocorticoids (inhaled)	Subject gene study	CRHR1	rs242941, rs1876828	Forced expiratory volume in 1 second (FEV_1) response	Tantisira et al. (2004)
		STIP1	rs2236647, rs6591838, rs1011219		Hawkins et al. (2009)
		ADCY9	rs2230739 (Met^{772}Ile)		Tantisira et al. (2005)
	Genome-wide association studies	*GLCCI1*	rs37972=rs37973		Tantisira et al. (2011)
		T GENE	rs6456042, rs3099266, rs2305089, rs3127412		–
–	Subject gene study	CYP3A4	CYP3A4*22 allele	Symptom control	Stockmann et al. (2013)
	Subject gene study	TBX21	rs2240017 (His^{33}Gln)	Broncho-protection	Tantisira et al. (2004); Ye et al. (2009)

TABLE 5.3 Pharmacogenetic Subject Genes for β_2-Adrenergic Agonists Response in Asthmatics

β_2-Adrenergic Agonists	Design of Study	Gene	Loci Associated	Phenotype Response	References
The short-acting beta-agonists example albuterol	Subject gene study	CRHR2	rs7793837	Acute FEV_1 broncho-dilation	Poon et al. (2008); Himes et al. (2012)
		ADCY9	rs2230739 (Ile^{772}Met)		Tantisira et al. (2005); Himes et al. (2012)
		ARG1	rs2781659, rs2781667		Litonjua et al. (2008); Hattersley et al. (2009); Duan et al. (2011); Himes et al. (2012); Iordanidou et al. (2012)
		ARG2	rs7140310, rs10483801		Vonk et al. (2010); Iordanidou et al. (2012)
		NOS3	rs1799983 (Asp^{298}Glu)		Iordanidou et al. (2012)
		THRB	rs892940		Duan et al. (2013)
	Subject gene study and	*ADRB2*	rs1042713 (Gly^{16}Arg)	Acute FEV_1 bronchodilation	Martinez et al. (1997); Taylor et al. (2000); Israel et al. (2004); Choudhry et al. (2005); Duan et al. (2013)
	Genotype-stratified			Long-term peak expiratory flow rate response	
	Subject gene study			Long-term peak expiratory flow rate response	
	Genome-wide association studies	*SLC24A4*	rs77441273 (Arg^{585}Gln)	Acute FEV_1 bronchodilation	Drake et al. (2014)
		SPATS2L	rs295137		Himes et al. (2012); Meyers et al. (2014)
	Admixture mapping	*SLC22A15*	rs1281748, rs1281743		Drake et al. (2014); Flannick et al. (2014)

TABLE 5.3 *(Continued)*

β_2-Adrenergic Agonists	Design of Study	Gene	Loci Associated	Phenotype Response	References
The long-acting beta-agonists examples are salmeterol and formoterol	Subject gene study	*ADCY9*	rs2230739 (Met^{772}Ile)	Long-term FEV_1 response	Kim et al. (2011)
	Subject gene study and genotype-stratified	*ADRB2*	rs1042713 (Gly^{16}Arg)	Long-term peak expiratory flow rate response No effect on peak expiratory flow rate response Bronchoprotection Preference for montelukast or long-acting beta-agonist as an add-on to inhaled corticosteroids	Taylor et al. (2000); Wechsler et al. (2006, 2009); Kleinberger & Pollin (2015); Bleecker et al. (2006, 2007, 2010); Lee et al. (2004); Lipworth et al. (2013)

corticosteroids (ICS) (Peters et al., 2007; Sorkness et al., 2007). A large number of subjects suffering from asthma demonstrate better response with improving their lung functions against ICS and only a few subjects show negative responses (Szefler et al., 2005). The subjects which do not respond to ICS are said to be steroid-resistant or refractory asthmatics which continuously exacerbate the symptoms with no improvement in lung functions (Chan et al., 1998, 2000). The pharma-cogenetic investigations were focused on glucocorticoid pathways based on the biologic subject gene coding the corticosteroid biosynthetic signaling pathway which includes hetero-complex and chaperone proteins (Table 5.2). The biological subject gene investigation of the CRHR1 in 1,117 subjects suffering from asthma randomized to inhalational corticosteroid therapy from the clinical trial cohorts detected 2 CRHRI SNPs associated with lung function response (LFR) (Tantisira et al., 2004). Another subject gene investigation of one of these cohorts assessing different genes coding the glucocorticoid hetero-complex also detected 3 SNPs within STIP1 (heat shock organizing protein gene) associated with LFR amidst inhalational therapy (Vogenberg et al., 2010). The gene variations demonstrated while GPP talks with biological pathways that impact the therapeutic responses against inhalation corticosteroids alone or in combination with SABA or LABA. The ADCY9 gene encodes an enzyme namely Adenylyl cyclase type 9 with coding SNP, $Met^{772}Ile$ (rs2230739), associated with therapeutic bronchodilation effect against SABA, albuterol only in patient's treated with inhalational corticosteroid from CAMP cohort (Tantisira et al., 2005). The ADCY9 gene pathway talk was replicated in an IKTC (independent Korean trial cohort) treated with LABA and formoterol in combination with inhalational corticosteroids (Kim et al., 2011). The gene TBX21 encoding T-box expressed in T-cell transcription which controls native T cell development outside GPP consists a coding single nucleotide polymorphism $His^{33}Glu$ (rs2240017), was involved in the enhancement of bronchial hyper-responsiveness or bronchial-protection during inhalational corticosteroid therapy in the Childhood Asthma Management Program (CAMP) cohort and replicated in IKTC (Tantisira et al., 2004; Ye et al., 2009). An investigation of the genes *CYP3A4*, *CYP3A5*, and *CYP3A7* in 413 inhalational corticosteroid-treated children suffering from asthma identified a specific *CYP3A4* genotype in twenty children's involvement in the enhancement of asthmatic symptom control proposing that the genetic loci are critical for inhalational corticosteroid metabolism

may also serve as biomarkers for therapeutic response (Stockmann et al., 2013).

5.9.2 CYSTEINYL LEUKOTRIENE PHARMACOLOGICAL PATHWAY (CLPP)

The cysteinyl leukotriene-altering drugs are also widely used in the management of asthma; however, they are less effective when compared to inhalational corticosteroids. The cysteinyl leukotriene exists as 5-LO blockers and CLR-1 (cysteinyl leukotriene receptor 1) blockers. The CLGP (cysteinyl leukotriene genetic pathway) is launched and limited by 5-LO encoded by ALOX5 gene followed by a group of proteins that biosynthesize distinct leukotrienes like LTA4H and LTC4S and MRP1 (multiple drug-resistant protein 1) that ship leukotrienes to extra-cellular area to ligate and stimulate CLR (CYSLTR1 and CYSLTR2) (Ortega et al., 2015). The leuckotreine altering drugs used in asthma have been subjected to little replicated pharmacogenetic subject gene investigations mainly due to small patient number although these investigations are beneficial and showed that the variability in pharmaco-therapeutic responsiveness to these drugs in at least fragmentary determined by gene variation (Table 5.4). One of the 1st investigations assessed a TRP (tandem repeat polymorphism) in the RPR (regulatory promoter region) of ALOX5 and demonstrated that this promoter variant was involved with LFR in 144 subjects suffering from asthma treated with 5-LO blocker and ABT-761 (Drazen et al., 1999).

The larger subject gene investigations have demonstrated that ALOX5 promoter variant and other ALOX5 SNPs may also affect the response to LRA (leukotriene receptor antagonist) (Lima et al., 2006; Klotsman et al., 2007; Telleria et al., 2008; Tantisira et al., 2009). The variants in LTC4S and MRP1 have been associated with LFR to another 5-LOI (leukotriene inhibitor) and LRA (Lima et al., 2006; Tantisira et al., 2009). The LPC gene investigations also involved SNPs in LTA4H and CYSLTR1 genes and have demonstrated contradicting results associated with sample sizes (n=61 to n=577) underpowered to consistently identify and replicate pharmacogenetic correlations (Lima et al., 2006; Tantisira et al., 2009).

TABLE 5.4 Pharmacogenetic Subject Genes for LMR in Asthma

Class	Gene	Loci Associated	Design of Study	Phenotype Response	References
LRM: 5-LO inhibitors (ABT-761 and zileuton)	ALOX5	Promoter repeat, rs892690, rs2029253	Subject gene study	Forced expiratory volume in 1-second	Drazen et al. (1999); Telleria et al. (2008)
–	–	rs2115819	–	Response	–
	LTC4S	rs272431			Tantisira et al. (2009)
	MRP1	rs215066, 119774			Tantisira et al. (2009)
Cysteinyl leukotriene inhibitors (montelukast)	*ALOX5*	Promoter repeat, rs2115819			Lima et al. (2006); Klotsman et al. (2007)
	MRP1	rs119774			Lima et al. (2006)
	LTA4H	RS266845		Risk of exacerbation	Lima et al. (2006)
	LTC4S	rs730012			Lima et al. (2006)
	SLCO2B1	rs12422149 (Arg^{312}Gln)		Symptom control and drug levels	Mougey et al. (2009)

5.9.3 B$_2$-ADRENERGIC PHARMACOLOGICAL PATHWAY

The inhaled β_2-receptor agonists are the oldest and most widely used in asthma exacerbations which act through G-protein coupled receptor pathways. There are about three dug classes in this category which consist of SABA, LABA, and ULABA (ultra-long-acting beta-agonists). The asthma death epidemics were associated with a high dose of SABA which includes isoproterenol in the era of 1960s and non-selective fenoterol in the era of 1970s (Crane et al., 1995; Pearce et al., 1997, 1990; Stolley, 1972; Grainger et al., 1991). These death toxicities lead to the withdrawal of drugs from the global market.

The β_2-adrenergic receptor gene ADRB2 I not having any introns; however, a polymorphic gene with more than 49 genetic variants in the MEAC (multiethnic asthma cohorts) evaluated to date (Drysdale et al., 2000; Hawkins et al., 2006; Ortega et al., 2014). The most extensively investigated coding variant AAP 16, Gly^{16}Arg, which is associated with *in-vitro* altered receptor down-regulation. The earlier pharmacogenetic investigation of the ADRB2 gene have shown that GLY^{16} homozygotes have a lesser acute response to SABA bronchodilation compared to ARG^{16} homozygotes which has been verified in extra asthmatic subjects (Lima et al., 1999; Choudhry et al., 2005).

5.10 CONCLUSION

The PM has become popular day by day and in the coming years, it will help to ease the diagnostics as well as therapeutic targeting. The reasons for its popularity are because of the following reasons like:

- Reduce the burden of disease;
- Focuses on prevention;
- Diminishes the duration and severity of illness;
- Decreases health care costs;
- Enhance benefits and reduces risks.

PM has opened the door to integrating genomic-based medicine into a clinical perspective. If efforts, like complemented by well-orchestrated collaborative efforts ranging from stakeholders pursuing the activities designed to accelerate a shared vision of PM remarkable benefits in

genome-informed clinical care, maybe favorable near these 10 years. The healthcare team, patients, and industry should club around meaningful opportunities like patient counseling, concordance in coverage policy, and the appropriate guidelines to facilitate wider adoption of PM.

KEYWORDS

- **biotechnology developments**
- **diagnosis**
- **glucocorticoid pharmacological pathway**
- **next-generation sequencing**
- **optimal treatments**
- **personalized medicine**
- **phosphodiesterase inhibitors**
- **risk assessment**

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CHAPTER 6

Stem Cell Technology and Regenerative Medicine: A Travel Back to Roots

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ABSTRACT

Stem cells are undifferentiated progenitor cells that can produce different cell phenotypes under specific biomolecular stimuli via the differentiation pathway. Stem cells and regenerative medicine are part of an ongoing research study and application process. Overall, increased interest in stem cell research is due to their specific biological nature and their potential to understand the process of aging and degenerative diseases in a better way. Stem cell biology, mainly stem cell-based therapy, has developed as an active field over the past decade. Significant research studies have led to critical breakthroughs in several fields through stem cell technology. Stem cell treatment has made significant strides in customization of therapy, tissue engineering, and disease mitigation in chronic and regenerative diseases. Adult stem cells (ASCs) are promising candidates for disease therapy because of their adaptability, minimal immunogenicity, and great anti-inflammatory capacity. Numerous disorders, including tissue and

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organ damage, cardiovascular, and other functional organ failures, degenerative diseases, and cancer, provide a substantial challenge to the scientific community. Traditional therapies, such as pharmacological therapy and surgery, frequently have little effect on these disorders and fail to meet the growing medical needs. Recent advances in stem cell technology and its regenerative medicine have shown promising results and have given a good hint for their substantial application in disease treatments. Thus, stem cell-based regenerative medicine is expected to become the third treatment option after drug therapy and surgery. This chapter will explore how stem cell technology and regenerative medicine have contributed to disease treatment during the last decade. Moreover, we will also try to highlight the different opportunities and emerging challenges of stem cell-based therapies and regenerative medicine.

6.1 INTRODUCTION

Stem cells are undifferentiated or partially differentiated cells which retain their proliferative capacity throughout the lifetime of an animal (Dalerba, Diehn, Weissman, & Clarke, 2020; Fuchs & Segre, 2000). These cells lack tissue-specific differentiation markers and are present at both embryonic as well as at adult stages of life of an organism and are considered as earliest cell lineages of the body of an organism. Most adult animal cells are no longer capable of proliferation and are arrested at G_0. On their natural death due to aging, cells can be replaced by the proliferation of a subpopulation of less differentiated, self-renewing cells called stem cells (Cable et al., 2020; Dalerba et al., 2020; Gurley & Alvarado, 2019; Slack, 2008). These stem cells are found in most of an organism's adult tissues and are crucial for maintaining the majority of an organism's tissues and organs. However, differentiated cells remain arrested in the G_0 cell cycle stage but resume proliferation as needed to replenish cells lost during injury or death. The cell of this type includes fibroblasts dispersed in connective tissues where they secrete collagen; they are typically arrested in the G_0 stage but rapidly proliferate if needed to repair the damage resulting from cut or wound (Baksh, Song, & Tuan, 2004). The two essential properties of stem cells are self-renewal and potency. Self-renewal is the ability of a cell to go through one are more cycles of cell division to maintain the undifferentiated cell layer or cell mass. At the same time, potency is the

capacity to differentiate into different specialized cell types (Ejtehadifar et al., 2015).

Stem cell biology has received significant interest during the last few decades. As a result, enhanced awareness of its properties and therapeutic potential for its application has been prioritized (Mohammadian et al., 2013). These cells are used in cell therapy and experimental research to treat human problems, including hematology, skin regeneration, and heart disease (Markoski, 2016). In the past, the work related to stem cells was performed by Friedenstein and colleagues in the 1960s. They identified, grew, and differentiated bone marrow (BM)-derived cells from guinea pigs into osteogenic cell lineages (Friedenstein, Chailakhyan, Latsinik, Panasyuk, & Keiliss-Borok, 1974). Their work led to a new perspective on the research related to stem cells. As a result, numerous further studies discovered that the BM contains fibroblast-like cells with congenic potential *in vitro*, capable of forming colonies (CFU-F) (Banas et al., 2007). For more than 60 years, transplantation of hematopoietic stem cells (HSC) approach has been one of the significant curative therapies for various genetic and hematological diseases and disorders (Ghimire, Weber, Mavin, Dickinson, & Holler, 2017). Till and McCulloch in 1963 described a peculiar single progenitor cell type in BM, having the potential to expand clonally and give rise to all hematopoietic cell lineages. This research work represented the first characterization of the HSCs (Becker, McCulloch, & Till, 1963; Volarevic et al., 2014). In addition to this, the recognition of mouse embryonic stem cells (ESCs) in 1981 proved revolutionary in the study of developmental biology (Volarevic et al., 2014). As stem cells can differentiate into specific cell types, it has become a possibility of a renewable source of replacement cells and tissues to treat diseases like leukemia, anemia, spinal cord injury (SCI), stroke, burns, heart diseases, diabetes, osteoarthritis, and rheumatoid arthritis (Lee et al., 2019; Makena, Ranjan, Thirumala, & Reddy, 2020; Tweed, 2019; Witkowski, Lasry, Carroll, & Aifantis, 2019; Zakrzewski, Dobrzyński, Szymonowicz, & Rybak, 2019). The sources of these stem cells can be ESC, somatic stem cells (SSCs) and induced pluripotent stem cells (iPSCs) (Bragança, Lopes, Mendes-Silva, & Santos, 2019). But human embryonic stem cell (hESC) research is ethically and politically controversial as it involves the mass destruction of human embryos like that of abortion. However, in modern times, we have learned a lot about stem cells and are obtained from sources like BM,

umbilical cord blood, adipose tissue, amniotic fluid, and allografts (Acosta & Golub, 2016; McCoy & Sterngass, 2019).

6.2 CLASSIFICATION OF STEM CELLS

Based on differentiation capacity, stem cells are categorized into various types, comprising totipotent, pluripotent, multipotent, and unipotent (Amin et al., 2019). In addition to this, these cells are classified based on evolutionary stages. They include embryonic, fetal, infant, or umbilical cord blood and adult stem cells (ASCs) (Rajabzadeh, Fathi, & Farahzadi, 2019) shows an overview of stem cell classifications based on differentiation potency.

6.2.1 TOTIPOTENT CELLS

These cells can develop any cell of the developing organism, including embryonic and extra-embryonic (e.g., placenta) tissues, for example, 1–4 celled stage of the human zygote (Kalra & Tomar, 2014). During this stage, if cells get separated, they can develop into an entire embryo, including the tissues which support the developing embryo-like, in the natural case of monozygotic twins. Two cells of the single zygote naturally get separated in the mother's body and develop into two separate embryos, including their distinct supportive tissues of extra-embryonic membranes (Kalra & Tomar, 2014).

6.2.2 PLURIPOTENT

These cells can only make cells of the embryo proper, which includes all cells of three germ layers. Therefore, they can make any cell of the body, for example, the inner cell mass of an early embryo (Baker & Pera, 2018).

6.2.3 MULTIPOTENT

These cells can give rise to a few cells on differentiation; for example, HSCs of BM can give rise to different blood cell types (Cheng, Zheng, & Cheng, 2020).

6.2.4 UNIPOTENT

Unipotent stem cells have not received much attention in research compared with that totipotent and pluripotent stem cells. The unipotent stem cell is a cell that can give rise to cells with only one lineage differentiation (Giri, Alexander, Agrawal, Saraf, & Saraf, 2019). Muscle stem cells represent one of the instances of this type of cell (Giri et al., 2019). These cells have the lowest differentiation potential in adult tissues compared to other types of stem cells. More specifically, these cells do not have self-renewal potential. Furthermore, despite their limited differentiation potential, these cells depict their role in treating various disorders (Rajabzadeh et al., 2019).

6.2.5 EMBRYONIC STEM CELLS (ESCS)

Embryonic stem cells (ESCs) are self-renewing cells that originate in the blastocyst's inner cell mass and give birth to all cells during human development (Levenberg et al., 2003). In introduced embryos, these cells can develop into all types of body cells. Due to their unique capacity to generate all somatic cell lineages, these cells may be a good and promising source for cell transplantation and regenerative therapy (Lu, Li, Vida, & Honig, 2004). In other words, ESCs are pluripotent cells capable of differentiating to generate all of the body's specialized cell types (Lu et al., 2004). Additionally, ESCs capture the imagination due to their immortality and near-limitless growth potential. Due to ethical constraints on embryo collection and culture, these cells are employed in research less frequently (AE, 2004).

6.2.6 ADULT STEM CELLS (ASCS)/TISSUE-SPECIFIC STEM CELL

ASCs are undifferentiated cells and are usually located in various body tissues after embryonic development. These cells have the ability to multiplication by cell division and hence contribute significantly to the regeneration of damaged tissues (Mahla, 2016). Specific investigations have demonstrated that these cells can differentiate into cell types from multiple germ layers. For instance, BM stem cells originating from mesoderm can develop into cell lineages derived from mesoderm and endoderm, such

as the gastrointestinal tract, liver, lung, and skin (Krause et al., 2001). Moreover, one more example of ASCs is neural stem cells (NSCs) derived from ectoderm. These cells can differentiate into other lineage such as endoderm and mesoderm (Clarke et al., 2000). The fact that ASCs have therapeutic potential has been proved by cell therapy and regenerative medicine (Mimeault, Hauke, & Batra, 2007).

6.2.7 INDUCED PLURIPOTENT STEM CELLS (IPSCS)

These cells are not present in the body but are created from body cells such as skin cells in a laboratory. Conversion of these somatic cells into iPSCs by gene reprogramming (Karagiannis et al., 2019). In 2006, Takahashi & Yamanaka were able to produce these engineered cells. To date, scientists are in a race to apply these cells in the advanced treatment of deadliest diseases for the substitution of traditional therapies such as drug therapy and surgery. These iPSCs cells have properties similar to embryonic stem cells (ESCs), which can develop into body cells, i.e., pluripotent cells (Karagiannis et al., 2019; Russo, 2017).

6.2.8 CANCER STEM CELLS (CSCS)

Cancer stem cells (CSCs) were primarily identified in acute myeloid diseases by John Dick in the late 1990s. These stem cells are cancerous and are located within tumors or hematological cancers. These cells possess the features of normal stem cells and have the ability to give rise to all other cell types found in the specific cancer sample (Yang et al., 2008). This hypothesis is supported by various evidence as well. In an adult living species, normal stem cells are responsible for repairing and regeneration of damaged and old tissues (Soltysova, Altanerova, & Altaner, 2005). Several research investigations have pointed out that the potential of a tumor to propagate and proliferate depends on the minute or small cellular subpopulation encompassing stem cell-like characteristics often named as CSCs (Li et al., 2007).

6.2.9 MESENCHYMAL STEM CELLS (MSCS)

The connective tissue (i.e., embryonic) consists of mesenchyme. All other hematopoietic and connective tissues originate from the close interaction

of endoderm and ectoderm; however, MSCs do not differentiate into the hematopoietic cell (Fathi & Farahzadi, 2017). Alexander A. Maxi mow in 1924, used a histological detection to recognize a precursor cell within mesenchyme that ultimately develops and leads to various blood cell types (Sell, 2013). MSCs are a kind of cell that has the capacity for multilineage differentiation and self-renewal. They are found in various tissues and organs, including adipose tissue, skin, BM, peripheral blood, cord blood, fallopian tube, lungs, and liver (Ejtehadifar et al., 2015; Mohammadian et al., 2013). Today, stem cells are used for different applications, more importantly in human therapy, such as cell transplantation and cell engraftment. Types of stem cells, their source, uses, and limitations are enlisted in Table 6.1.

6.3 APPLICATION OF STEM CELL TECHNOLOGY AND REGENERATIVE MEDICINE IN DISEASE TREATMENT

Stem cell therapy is the novel process of implanting stem cells to promote the repair response of diseased, dysfunctional, and injured tissues. It is considered a novel substitution for traditional drug therapy and organ transplantation to eliminate these treatments' limitations or shortcomings (Chang, Wu, Harn, Lin, & Ding, 2018). As in the case of drug therapy, there are usually many side effects after treatment. In organ transplantation, it is hardly possible to get a donor. Even if we have the donor, there is unlikely to have a compatible match. In organ transplantation, there is always a chance of rejection from the body, so the person always relies on immunosuppressant drugs throughout life (Holzer, 2019). However, stem cell has shown promise in diseases like leukemia and anemia (BM stem cell transplantation), also in case of baldness and potential treatment in heart strokes (Segers & Lee, 2008), Alzheimer's disease (AD), diabetes, and Parkinson's disease (Duncan & Valenzuela, 2017; Zhu, Uezono, Yasui, & Nakashima, 2018). Stem cell therapy also has shown a lot of promise for wound healing and SCI, and their use can also be sensed in somebody with disabilities like blindness, deafness, and missing teeth (Price, 2020).

Regenerative Medicines are the use of body fluids, cell extracts, cultured stem cells, engineered regenerated tissues and organs for the treatment of severe injuries and chronic diseases to restore and establish back the normal functioning in the state where bodies own regenerative responses do not suffice (Fujita, Kadota, Araya, Ochiya, & Kuwano,

TABLE 6.1 Types of Stem Cells, Their Sources, Uses, and Limitations

SL. No.	Type	Source	Use	Limitation
1.	Embryonic stem cells (ESCs)	Inner cell mass from the blastocyst, an early stage of the embryo.	*Research purpose:* Study of cell commitment differentiation and maturation; drug testing, genomics, growth factor discovery. *Clinical purpose:* Tissue engineering, cell-based therapies.	Ethically exploitation of embryos is not permitted; ESCs can develop into uncontrollable cells if not correctly handled and rejection problems during therapy.
2.	Adult stem cells or somatic stem cells (SSCs)	Endodermal, e.g., gastrointestinal tract. Mesodermal, e.g., hematopoietic stem cells. Ectodermal, e.g., skin stems cells.	*Research purpose:* Drug testing, genomic study, growth factor discovery. *Clinical purpose:* Cell therapies.	These cells are very difficult to get isolated from a simple; difficult to be cultured in laboratory.
3.	Induced pluripotent stem cells (iPSCs)	Engineered in the laboratory from differentiated somatic cells by specific gene expression, e.g., Oct4, Sox2, Klf4, and c-Myc.	*Research purpose:* Disease modeling, drug development, organ, and tissue synthesis, cell type production. *Clinical purpose:* Tissue regeneration, most effective cell-based therapies.	In experimental trials, they have shown to form tumors in mice; mutations occur due to modifications of gene expression by growth factors.
4.	Perinatal stem cells (PSCs)	Cells in amniotic fluid and umbilical cord blood and tissues.	*Research purpose:* Disease modeling, drug discovery. *Clinical purpose:* Cell-based therapies.	–

2018). Regenerative medicine aims to amplify the body's natural healing abilities and encourage the growth of new body fluids, cells, and tissues in the desired body area. This latest and novel process is often used to treat sports injuries, skin burns, cartilage tissue regeneration, wound healing, etc. In the present scenario, donated tissues and organs cannot satisfy the transplantation needs of elderly and ailing populations, prompting the quest for other sources of tissue and organs. Stem cells are characterized by an unlimited capacity for cell division and the ability to transdifferentiate into different types of cells and have emerged as a primary source of regenerative medicine in recent years to repair tissues and organ anomalies caused by congenital disabilities, disease, and aging (Mahla, 2016). Stem cells serve as the basis for all of the body's tissues and organ systems and play a variety of roles in disease progression, development, and tissue repair. ESCs, tissue-specific progenitor stem cells (TSPSCs), mesenchymal stem cells (MSCs), umbilical cord stem cells (UCSCs), bone marrow stem cells (BMSCs), and iPSCs are the several types of stem cells used in regenerative medicine (Kotzabasaki, Sotiropoulos, & Sarimveis, 2020). Some challenging diseases such as AIDS (Figure 6.1) and their treatment by stem cell technology are described in Table 6.2.

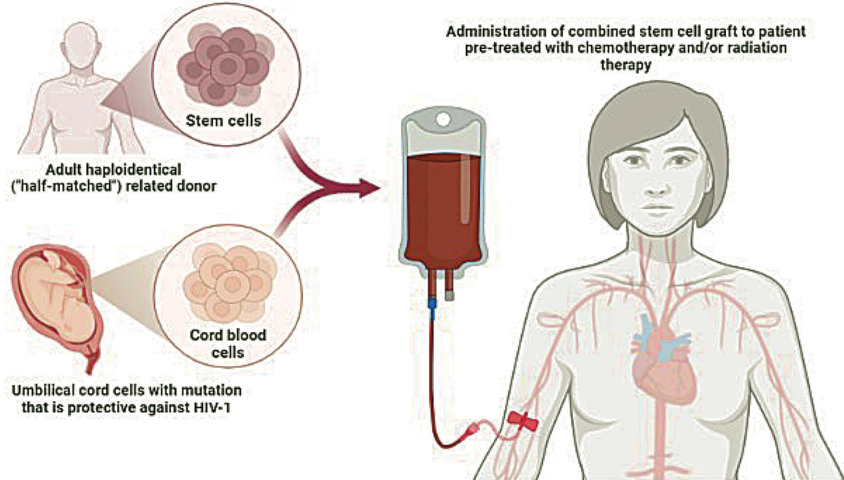

FIGURE 6.1 Role of stem cells in the treatment of AIDS.

TABLE 6.2 Some Challenging Diseases and Their Treatment by Stem Cell Technology

Disease	Factors Causing Disease	Mode of Stem Cells Application	Physiological and Mechanistic Aspects of Stem Cells Therapeutics	Improvements in Disease Signatures and Future Use	Stem Cells Used
Spinal cord injuries	Infection, cancer, accidents	ESCs transplantation to the affected site	Tissue homing is aided by ESCs and releases vasculogenic and neurogenic substances.	Regeneration of spinal tissue and improved balance and sensation	ESCs
Diabetes	Lifestyle, heart defects, and genetics	Transplantation of ESCs-derived PPCs	Progenitors (CD24+, CD133+, and CD49+) differentiate into β-cells, secrete insulin and express PDXL, GCK, and GLUT2	Improvement in glucose levels and obesity can be used for the treatment of T1DM and T2DM	ESCs
Anemia and blood cancer	Injury, genetics, and autoimmunity	Two-step infusion of lymphoid and myeloid	Immunity may be rebuilt by haploidentical BMSCs, which is a crucial step for minorities.	Treatment of aplastic anemia and hematological malignancies	BMSCs
Diabetes	Lifestyle and genetic factors	iPSCs-derived beta-cells transplantation	Skin to beta-cells reprogramming phase through cDE and cPF requires GPs	Treatment of T1DM and T2DM and insulin production	iPSCs
Neurodegenerative disorders	Accidents, age, trauma, and stroke	iGABA-1Ns and cortical spheroid transplantation	iGABA-1Ns secrete GABA; FOXIG cause ASD, spheroid mimics to brain	ASD, Alzheimer’s, seizer, and obstinate epilepsies treatment	iPSCs
Cardiovascular disease	Diabetes, drugs, genetic factors, and lifestyle	ESCs-derived CMs and biomaterial coaxed ESCs	Cardiomyocytes express GCaMP3, secreting vasculogenic factors, and Tbox3 differentiates ESCs into SANPCs	Suppresses heart arrhythmias. CMs electrophysiologic ally integrates into the heart like a pacemaker	ESCs
Lung degeneration	Tuberculosis, cancer, and fibrosis	Biomaterial coaxed iPSCs transplantation	Miniature iPSCs lung resembles airways and alveoli, model drug testing	Regeneration of lung tissue	iPSCs
Congenital heart defects	Developmental errors	Transplantation of fibrin-coaxed AFSCs	The addition of VEFG to PEG-coaxed AFSCs promotes organogenesis	Regeneration of tissue repair for treatment of heart defects	UCSCs
AIDS	HIV1 infection	Transplantation of HIV1 resistant CD4+ cells	Anti-HIV1 CD4+ cells express HIV1 anti-RNA, which restricts HIV infection	Treatment of AIDS as an alternative to antiretroviral	BMSCs

Autologous, allogenic, or syngeneic stem cell transplantation can be used to induce tissue regeneration and immunological lysis of pathogen or cancer cells. To reduce the complications associated with host-versus-graft rejection, tissue typing of human leucocyte antigens (HLA) for tissue and organ transplantation and immunosuppressive agents are suggested. Stem cells exhibit the major histocompatibility complex (MHC) receptor at low levels. They secrete chemokines that attract endothelial and immune cells to the graft site, facilitating tissue tolerance (Morales-Molina et al., 2018). Current methods to stem cell regenerative medicine are based on tissue engineering technologies that integrate concepts of cell transplantation, material science, and microengineering to create organoids that may be utilized to restore the physiological function of damaged tissue and organs. Tissue engineering is a technique that creates nascent tissue on biodegradable three-dimensional scaffolds. The ideal scaffolds promote cell adhesion and ingrowth, match the mechanics of target tissue, promote angiogenesis and neovascularization for adequate tissue perfusion, and are non-immunogenic to the host, hence eliminating the need for systemic immune suppression (Rajabzadeh et al., 2019). In the upcoming section, we will discuss the approach of stem cell technology and regenerative medicine in treating a wide variety of diseases.

6.3.1 SPINAL CORD INJURIES (SCIS)

Spinal cord injury (SCI) is a devastating injury resulting in permanent neurological impairment. It occurs in young and adult people due to accidents, infections, and diseases (Mataliotakis & Tsirikos, 2016). Surgical spinal fixation and subsequent rehabilitation are the standard of care for acute SCI, and no neuroprotective and regenerative therapies capable of directly producing beneficial effects are available (Nagoshi & Okano, 2017). High-dose methylprednisolone has been used frequently. There is excellent room for stem cell treatment in this disease, as seen in treating rats and primates *in-vitro* experiments. Recent progress in stem cell research may pause such disease treatment by proper development of neural precursor cells (NPCs) and their proper therapy to the point of disease (Vincent, 2017). NPC cells can be developed by two means, one from the fetal tissues or ESCs and the second from the somatic mesodermal cell-like adipose cell (Radhakrishnan et al., 2019; Becker &

Mcdonald III, 2012) first demonstrated the efficacy of NPCs transplantation in SCI. They induced NPCs from mouse ESCs and transplanted them into rat spinal cord 9 days post-injury. The grafted cells differentiated into neurons, oligodendrocytes, and astrocytes in the damaged tissue. Transplanted cells showed mitotic neurogenesis and formed synapsis with host neurons in the injured spinal cord. The differentiated cells migrated along the rostro caudal axis from the lesion epicenter. After that, the analysis showed improved control and coordination of limbs due to motor function recovery (Venkatesh, Ghosh, Mullick, Manivasagam, & Sen, 2019).

6.3.2 DIABETES

Diabetes is defined as the loss of pancreatic B-cell bulk or function, which results in insulin insufficiency relative to the body's metabolic requirements. Historically, diabetic management has focused on medication, as demonstrated by insulin replacement therapy, to address the hormone's peripheral activities (Chen, Cohrs, Stertmann, Bozsak, & Speier, 2017). With an increased understanding of diabetes, new therapies to repair and restore failed B-cell activity are increasingly being examined as adjuncts to conventional diabetes treatment regimens. The emphasis is on exogenous pancreas/islets or artificial islets transplantation, improved proliferation and maturation of endogenous B-cells, prevention of B-cell loss, or accelerated regeneration of B-like cell populations from stem cell pools and non-B-cell sources (Sordi et al., 2017). There are two types of diabetes; Type 1 diabetes (T1D) is characterized by insulin insufficiency caused by autoimmune destruction of islet B-cells. Type 2 diabetes (T2D) is characterized by the insulin secretory capability gradually failing to meet peripheral insulin requirements. Exogenous insulin injections are essential for persons with T1D and advanced T2D (Gupta et al., 2018). Glucagon-like peptide-1 (GLP-1) analogs have been developed to stimulate insulin production while preserving B-cell function. Alternatively, DPP-4 inhibitors have been utilized to enhance endogenous insulin production in T2D by preventing GLP-1 inactivation. ESCs induce pluripotent cells' spontaneous differentiation potential via embryoid body formation, followed by selection for cells expressing B-cell/B-cell progenitor markers. It has been demonstrated that using insulin-producing cells generated from ESC offspring and chosen for B-cell-specific gene expression may correct

hyperglycemia in diabetic mice (Meng, 2016). Additionally, stepwise differentiation techniques for each lineage have been adopted substantially from *in vitro* B-cell developmental blueprints. Thus, directed differentiation of ESCs resulted in the generation of insulin-producing cells; however, subsequent research cast doubt on the authenticity of generated progeny (Sackett et al., 2016).

6.3.3 ALZHEIMER'S DISEASE (AD)

Alzheimer's disease (AD) is a progressive neurodegenerative condition characterized by memory loss and cognitive impairment caused by synaptic dysfunction and an abnormal buildup of misfolded proteins (Castellazzi et al., 2019). Numerous fruitless attempts to produce novel chemicals or antibodies to intervene in the disease's etiology have been attempted. As a replacement or regeneration approach for AD, stem cell-based treatments offer fresh hope. AD is the most common kind of dementia, characterized by gradual memory loss and impairment of cognitive functioning. AD was first described in 1907 by Alois Alzheimer. Because AD is complex, it is difficult to pinpoint the precise pathophysiologic process. However, synaptic failure is the primary symptom of neuronal death in the cortex and hippocampus due to an abnormal buildup of neurofibrillary tangles and β-amyloid (Aβ) plaques (Kwak, Lee, Yang, & Park, 2018). AD is classified into two subtypes: familial and sporadic. Familial AD accounts for around 5% of cases and is caused by a unique autosomal genetic mutation in the amyloid precursor protein (APP), presenilin-1 (PSEN-1), or presenilin-2 (PSEN-2).

In comparison, the majority of occurrences of AD are sporadic. It often manifests itself late in life and is likely to be caused by interactions between a detailed genetic profile (including apolipoprotein [ApoE4]) and environmental variables such as cardiovascular disease, depression, and smoking (Stoychev et al., 2019). iPSCs were first developed from mouse fibroblasts in 2006. These cells are reprogramed into a state of pluripotency similar to that of ESCs. IPSCs are thought to be able to differentiate into various cells, including neurons and neurospheres. So neuronal subtypes can be generated and automated using iPSCs. For example, iPSC-derived glia could be used for research regarding inflammatory response in AD (Hunsberger et al., 2016). The workflow for iPSC-based cell therapy is shown in Figure 6.2.

FIGURE 6.2 The workflow for iPSC-based cell therapy.

Additionally, MSCs have garnered considerable interest in the therapy of AD due to their outstanding accessibility, the relative simplicity of handling, substantial research, and a broad range of differentiating potential (including neuronal cells). Additionally, MSCs are beneficial as cell-based therapeutics since they may be injected intravenously, penetrate the blood-brain barrier, are low tumorigenic, and provoke a mild immune response (then do other cell-based therapies). Rather than being used to replace neurons, MSCs appeared to exert beneficial effects via their released factors, which promote the proliferation, differentiation, and survival of the neurogenic niche (Kwak et al., 2018). MSCs are obtained from a broad number of sources. MSCs generated from bone marrow (BM-MSCs) have been the subject of extensive research for an extended period. The immunomodulatory capacity of BM-MSCs is derived from the secretion of soluble factors such as IL-6, IL-10, TGF-β, and PGE2. They have been shown to impair the function of monocyte-derived dendritic cells and change the phenotypic of natural killer cells. Adipose tissue is an excellent source of MSCs. MSCs produced from adipose tissue (AT-MSCs) can differentiate into neuronal and astrocyte-like cells.

Additionally, AT-MSCs release a variety of neurotrophic factors that are required in the neurogenic niche. Finally, MSCs produced from

umbilical cord blood (UCB-MSCs) can differentiate into neuron-like cells. These cells have been investigated in a mouse model of AD. One method of action that has been proposed is the activation of M2-like microglia (El-Omar et al., 2014).

6.3.4 APLASTIC ANEMIA (AA)

Aplastic anemia (AA) is a rare and potentially fatal BM failing illness characterized by pancytopenia and hypocellular BM. The majority of instances are caused by an autoimmune attack on BM stem and progenitor cells, which results in pancytopenia (Brodsky & Jones, 2005). While allogeneic BM transplantation may be curative in younger patients, most are ineligible for transplantation due to advanced age or a lack of a matched donor. With regular immunosuppressive therapy (IST), comparable long-term survival is possible in severe AA. However, one-quarter to one-third of patients treated with IST will not respond, and 30–40% of responders will relapse. These individuals may have chronic thrombocytopenia, require frequent platelet transfusions that are costly and inconvenient, and are at risk of developing more major bleeding issues. Thrombocytopenia is a significant cause of morbidity and mortality in patients with AA (Huber, Kumar, & Tefferi, 2003). Almost all patients with AA are thrombocytopenic upon presentation; platelet counts of 50,000 or 20,000/lL are used to diagnose moderate and severe AA. Thrombocytopenia is caused by decreased hematopoietic stem and progenitor cell numbers and function, which results in reduced megakaryocytopoiesis and insufficient mature platelet formation (Mansour et al., 2000).

Allogeneic hematopoietic stem cell transplantation (AHSCT) is the usual treatment case for severe patients, but there is always a risk of immune reaction from the recipient. Cultured hemopoietic stem cells can produce hematopoietic factors, mainly erythropoietic-stimulating agents (ESAs) and G-CSF and GM-CSF and IL-3 treat AA. Still, their infusion does not affect (Ogawa, 1993). The use of umbilical cord multipotent stromal cells and allogeneic HSCs in treating heavily transfused patients with severe AA have shown promise. It opens the door for the future use of umbilical cord cells for anemia disorders (Luan, Chen, Chen, Ding, & Ni, 2015).

6.3.5 LUNG DEGENERATION

Diseases such as TB, cancer, cystic fibrosis, alpha-1 antitrypsin (AAT), and smoking cause lung alveoli tissue degradation. Tissue degeneration can indicate reversible cell damage or necrosis, which indicates irreversible cell damage. Cell enlargement, cytoplasmic vacuolation, apparent perinuclear gaps, cytoplasmic bleb development, and cilia loss are all examples of reversible cell injury. Pyknosis, karyorrhexis or karyolysis, cell enlargement, and cell fragmentation are examples of permanent cell injury (Kumar, Abbas, Aster, & Deyrup, 2020).

Using stem cell technology, damaged pulmonary tissues can be regenerated. As previously stated for the use of mesodermal cells in the development of iPSCs for stem cell technology, here also the generation of disease-specific iPSCs from individuals with a variety of lung diseases, as there is a significant lack of conventional therapies or human model systems for several inherited or degenerative lung diseases (Cazzaniga, 2017). It has been hypothesized that pluripotent stem cells or differentiated offspring are promising options for reconstituting wounded lung tissues *in vivo* or simulating the etiology of lung disease *in vitro*. Therefore, scientists attempted to reprogram fibroblasts extracted from mice with lung disorders affecting the three major cell lineages of the adult lung: lung epithelium, lung endothelium, and lung interstitium (Karagiannis et al., 2019). CF or AAT deficiency-related emphysema (which affects the lung interstitium and epithelium)-affected mice were used to collect fibroblasts. The researchers focused on reprogramming dermal fibroblasts derived from 6-mm skin punch biopsies of mice with a hereditary CF mutation (homozygous DF508 mutant CFTR genotype). In one research work, researchers also obtained additional banked, frozen CF or AAT-deficient fibroblasts from the cell repositories. They used a single lentiviral "stem cell cassette" (STEMCCA) to encode all four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc, in a single polycistronic vector. By combining all reprogramming transgenes in a single cassette, STEMCCA accomplished reprogramming of postnatal mouse fibroblasts with high efficiency and allowed the derivation of mouse iPSC containing a single viral integration of the hSTEMCCA-loxP vector allowed efficient generation of iPSC clones from all samples, regardless of the age of the individual from which the cells originated (Ahmed, 2016). Finally, to functionally assess pluripotency of the disease-specific iPSC

cells generated with this method, researchers subdermally transplanted three representative iPSC cell lines into immunodeficient mice and found the cells gave rise to teratomas comprised of differentiated tissues characteristic of the three primary germ layers, ectoderm, mesoderm, and endoderm (Miranda, Fernandes, Diogo, & Cabral, 2020).

6.3.6 TOOTH REGENERATION

Various studies have been done on dental reconstruction with the help of MSCs. One of the studies has reported that dental pulp-derived stem cells (DPSCs) could promote periodontal regeneration in a canine model. Also, it was shown that canine DPSCs were successfully isolated and had a rapid proliferation and multi-lineage differentiation capacity (Khorsand et al., 2013).

6.3.7 WOUND HEALING

A chronic wound is one of the most frequent ailments and causes distress (Mansoub et al., 2018). MSCs generated from dental tissue are reliable donors of the cytokines and growth factors that promote wound healing. Several research findings indicated that stem cells produced from the horse's deciduous teeth might be a unique technique for wound care and could be used clinically to treat non-healing wounds (Srionrod, Bootcha, & Petchdee, 2016). However, additional study is required to understand the underlying mechanisms of beneficial growth factors that contribute to wound healing processes when using stem cells produced from deciduous teeth (Amirkhani et al., 2016). This early discovery suggests that stem cells made from deciduous teeth can improve wound healing in rabbit excisional wound models (Srionrod et al., 2016). In addition, one of the researchers experimented with a rat model and found that ADSCs provide a potentially suitable matrix for full-thickness wound healing (Lin et al., 2013).

6.4 CONCLUSION

The human body is strengthened by specialized cells collectively called stem cells, which can self-renew and develop into various cell types such as adipocytes, osteocytes, chondrocytes, and neurons. Additionally, to the qualities outlined above, these cells are readily separated, may be safely

transplanted to wounded locations, and possess immune regulatory capabilities. Numerous *in vitro* and *in vivo* experiments using animal models have effectively proved the promise of multiple kinds of stem cells for treating various illnesses; nevertheless, clinical outcomes have been mixed. According to research, stem cells have a wide range of applications in treating disorders such as heart failure, wound healing, and tooth regeneration. Additionally, these cells are critical in the therapy of sub-branch neurodegenerative illnesses like Alzheimer's and Parkinson's disease.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ETHICAL STATEMENT

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

KEYWORDS

- **cancer stem cells**
- **embryonic stem cells**
- **induced pluripotent stem cells**
- **perinatal stem cells**
- **plasticity**
- **regenerative medicine**
- **stem cell therapy**

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CHAPTER 7

CRISPR-Cas Genome-Editing Tool: A Prospective Application to Study Human Diseases

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) is a prokaryote-derived genome editing tool acquired from the defense system of prokaryotes. CRISPR-Cas has become an indispensable genome editing tool with many applications in biotechnology and medical research to treat various human diseases, including neurodegenerative disorders, cardiovascular diseases, and cancers. CRISPR-Cas no longer remained merely a gene-editing tool, but its applications diversified due to the reprogramming capacity of Cas9, including epigenetic editing, gene regulation, and chromatin engineering.

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CRISPR-Cas is also used to develop animal models for studying and developing possible treatments for human genetic diseases. This study aims to overview the wide range of applications of CRISPR-Cas in various fields of biotechnology.

7.1 INTRODUCTION

Genome editing holds enormous value in the different areas of molecular biology, biotechnology, and in the field of medicines. DNA of eukaryotes consists of billions of nitrogenous bases, and the ability to alter these DNA bases at particular positions harbors tremendous significance. Discoveries of restriction enzymes fueled the recombinant DNA technology era and enabled researchers to manipulate the DNA at predetermined locations (Kelly & Smith, 1970; Smith & Wilcox, 1970; Danna & Nathans, 1971). Consequently, several targeted nucleases for genome editing emerged, which involves transcription activator-like effector nucleases (TALENs), meganucleases, and zinc-finger nucleases (ZFNs). However, these techniques were relatively low in target efficiencies and time-consuming, limiting their widespread usage (Chandrasegaran & Carroll, 2016). In this context, CRISPR has revolutionized the entire genome-editing field in terms of efficiency, flexibility, and more straightforward in use (Lino et al., 2018). This genome editing technique consists of endonuclease whose DNA cutting specificity is determined by a short guideRNA (gRNA). For decades, CRISPR had been known as a typical DNA repeat element in prokaryotes before it was discovered as an adaptive immune system in prokaryotes and subsequently developed as a robust genome-editing tool (Adli, 2018).

The Clustered Regularly Interspaced Short Palindromic Repeat DNA Sequences are responsible for immunity in about 40% of bacterial species, including *Escherichia coli*, Streptococcus pyogenes, Staphylococcus aureus, Streptococcus thermophiles, Streptococcus mutans, Staphylococcus epidermidis, Corynebacterium diphtheria, Neisseria meningitides, and in about 90% of archaeal species such as *Archaeoglobus fulgidus*, *Methanocaldococcus jannaschii*, *Pyrococcus furiosus*, *Haloferax mediterranei*, *Methanothermobacter thermoautotrophicum*, and *Sulfolobus solfataricus*. Immune system of prokaryotes is capable of responding and consequently eliminating the viral genetic elements by this gene-editing process (Horvath et al., 2008; Horvath & Barrangou, 2010; Jinek et al., 2012; Sangal, Fineran, and Hoskisson, 2013; Zhang et al., 2013; Ran et al., 2015).

CRISPR-Cas is a component of bacterial immune systems that can cut the DNA at predetermined locations with a pair of molecular scissors (Cas) directed by the programmable gRNA sequence. CRISPR locus is flanked by conserved protein-coding genes, which vary in their order and direction of orientation. Based on sequence similarity, about 93 *cas* genes are classified into 35 families, out of which 11 families encode the proteins which constitute *cas* core, including the families encoding Cas9 nuclease. It has been found that a complete CRISPR-Cas locus contains at least one gene from the *cas* core (Hille & Charpentier, 2016).

Cas nucleases are endonucleases that binds to DNA and create double-stranded breaks. At the time of viral infection, the bacterium utilizes Cas nucleases to cut off a fragment of viral DNA known as protospacer which is reserved in the genome of the bacterium with the fragments from the other viruses that have earlier infected the bacterium. These viral spacer pieces are positioned in the middle of repeated palindromic sequences resulting in the formation of spacers and palindromic repeat arrangements from where the CRISPR has derived its name (Figure 7.1). CRISPR-Cas system comprises of two indispensable components: CRISPR-associated (Cas) endonuclease, which binds to DNA and creates DSBs, and a gRNA sequence, which directs target of Cas nucleases (Richter, Chang, and Fineran, 2012).

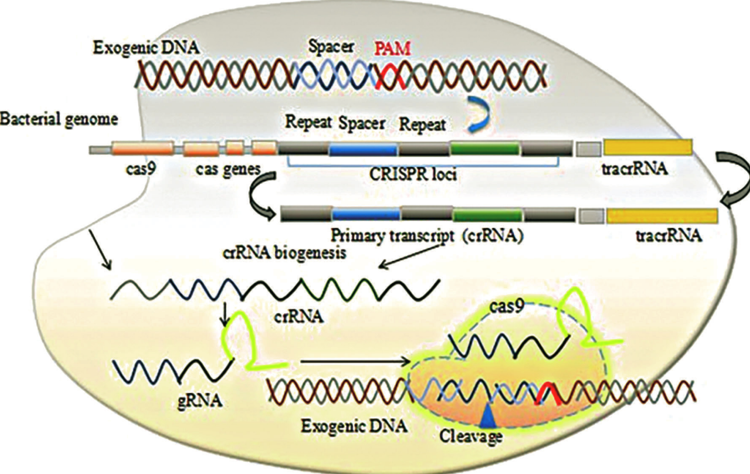

FIGURE 7.1 Molecular mechanism of CRISPR/Cas9.

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Reinfection of bacteria with the same virus is recognized and subsequently destroyed by Cas9. Activity of Cas9 relies upon CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) (Chylinski, Le Rhun, & Charpentier, 2013). During the course of the original infection, the genetic element which was stored in CRISPR locus serves as crRNA which is complementary to the viral spacer, while tracrRNA acts as a scaffold; complex of these pair of RNAs is known as guideRNA (gRNA) directing molecular scissors Cas9 to introduce a cut. Prior to the introduction of breaks, Cas9 scans the foreign DNA for protospacer adjacent motif (PAM), located downstream of the target site (Gleditzsch et al., 2019). After recognizing PAM, endonuclease Cas9 scans upstream region where if it detects the target pointed by the gRNA will introduce a double-strand break (DSB) inactivating the virus since viruses do not possess DNA repairing mechanisms (Chylinski, Le Rhun, & Charpentier, 2013; Gleditzsch et al., 2019).

7.2 GENOME EDITING UTILIZING CRISPR-CAS9

The complex of gRNA can be specifically engineered into single chimeric guideRNA (sgRNA) directing Cas9 to the predetermined locations to introduce DSBs offering a simple and cost-effective method of genome editing. Depending upon the presence of correct PAM sequence, the only required thing is to provide a new gRNA and then Cas9 can introduce breaks at a compass of targeted sites in the genome of any organism (Dong, Gou, & Lian, 2022). As it was originally detected in bacterial defense systems, wherein the DNA of any invading bacteriophages (virus) is broken down into fragments rendering them inactive due to lack of DNA repairing machinery (Rath et al., 2015). Once the mechanism underlying its DNA cleavage activity was determined, it was swiftly developed as genome editing tool. This genome editing tool (CRISPR-Cas9) takes the advantage of DNA repair pathways after the introduction of DSBs. At the cellular level, several DNA repairing pathways are operational among them only a few are used in gene editing, including non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is commonly used in gene inactivation, while HDR is used to insert new fragments of genetic material (Xue & Greene, 2021).

7.3 MOLECULAR MECHANISMS OF CRISPR GENOME EDITING

CRISPR is a robust genome editing technology with a wide range of applications. Some of them are described in subsections.

7.3.1 CRISPR GENE KNOCKOUT

Since Cas9 creates DSBs and the most likely DNA repair mechanism by which these DSBs are repaired is through NHEJ. However, as known NHEJ is error-prone, and usually ends up the insertion or deletion mutations (indels) in the region of the gene being repaired. Frameshift mutation commences if the insertion or deletion mutations (indels) takes place within the coding region of the gene, leading to the non-functionality of the gene commonly called as gene knockout (KO). Gene KOs are the backbone of many research areas such as pathway analysis, functional genomics, screening, disease modeling and drug discovery. Targeting various regions of the gene by using multiple gRNAs certifies high efficiency gene knock outs (Ishibashi et al., 2020).

7.3.2 CRISPR KNOCK-IN

DSBs created by Cas9 can also be repaired by HDR and this repair mechanism offers a window of opportunity to insert a new piece of DNA or whole gene. This process of insertion is known as gene knock-in. In order to achieve gene knock-in, a DNA template known as donor template must consist of a sequence of gene of interest flanked by homology regions that must match on either side of the DNA break. This donor template along with other editing machinery of Cas9 and sgRNA is delivered to cells to achieve desired results. Gene knock in has achieved key position in biotechnology, including gene therapies to correct genetic mutations, diseases modeling, and recombinant protein production (Banan, 2020).

Gene knock-ins are more challenging in comparison to the gene KOs, because HDR is very less occurring pathway as compared to NHEJ, as it occurs only in certain phases of cell cycle. Moreover, HDR-mediated knock-in efficiencies are very less. In order to overcome such issues people started experimental optimization, synchronization of cell cycle

and various treatments that either disable NHEJ or boost HDR (Song et al., 2016).

7.3.3 CRISPR ACTIVATION (CRISPRa) AND CRISPR INTERFERENCE (CRISPRi)

CRISPR-Cas9 can be employed not only to insert or delete the genes, but it can also be used to regulate gene expression as well which is known as CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi). Upregulation and down regulation of gene expression can be performed by using CRISPRa and CRISPRi, respectively, by using an engineered variant of Cas9 called as catalytically dead Cas9 (dCas9), therefore cannot cut the DNA. Integrating dCas9 with transcriptional effectors can modulate the expression of the target gene. Unification of dCas9 with transcriptional effectors simply leads to the delivery of transcriptional effectors to the gene of interest and modulate their expression as dCas9 can't cleave the DNA. Modulation of gene expression has tremendous applications in biotechnology, developmental biology, functional genomics, infectious diseases, and screening for identification of genetic elements that imparts resistance to various drugs. Since CRISPR-Cas9 is highly efficient, therefore screening studies for the discoveries of drugs has become simple and time-efficient. Screening by CRISPR technique involves the formation of a huge sgRNA library, targeting various genes to analyze phenotypic effects of these wide range of edits delineating the relationship between genotype and phenotype. One is able to identify the genes that are actually involved in disease pathogenesis by performing knock out studies on normal cell line. Similarly, one can identify the drug targets in a diseased/cancerous cell line using the same methodology. CRISPR screening is more accurate with comparatively less off targets than RNAi screens of drug discovery (Kampmann, 2018).

7.3.4 CRISPR-CAS BASE EDITING AND PRIME EDITING

Recently, CRISPR methods have been adopted as base and prime editing, both of which are based on the same principle and both of these methods do not cleave the target DNA. Instead, base editing involves the use of

either catalytically dCas9 or a nickase Cas9 (nCas9) While the dCas9 is catalytically dead therefore cannot cleave DNA, nCas9 is capable of producing either nick or single-strand breaks (Figure 7.2). Therefore, a fusion of either of the two to the DNA-modifying enzyme can modify nucleotides at specific points (Anzalone, Koblan, & Liu, 2020). However, base editing cannot be performed to alter every possible nucleotide this led to the discovery of prime editing.

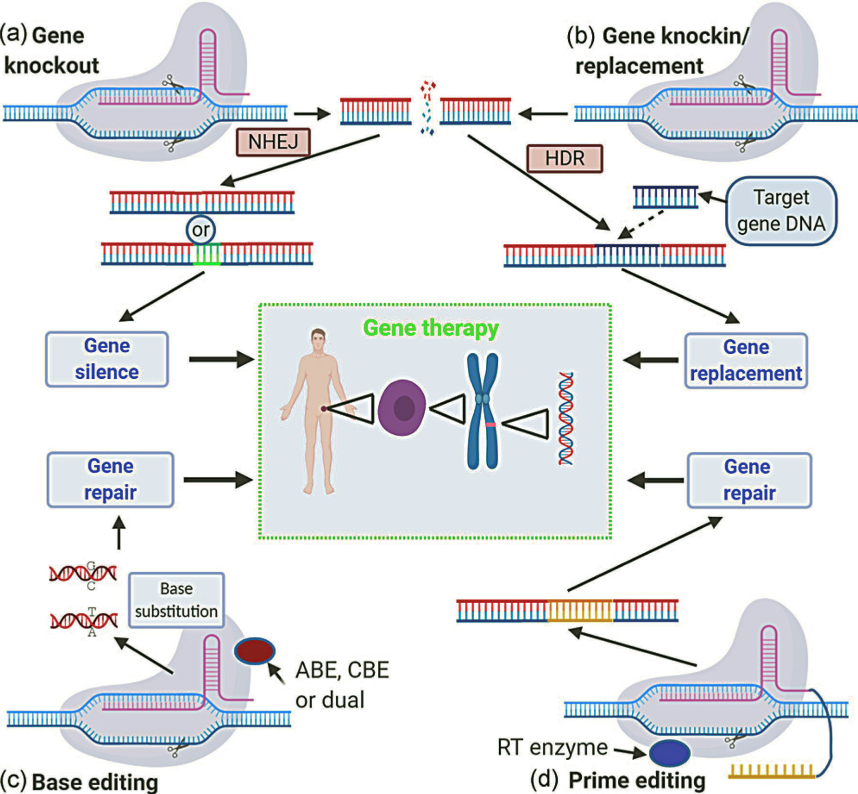

FIGURE 7.2 CRISPR/Cas9-based gene therapy. (a) Gene knock-out; (b) gene knock-in/ replacement; (c) base editing; and (d) prime editing – can be employed to correct and/or replace the disease-related genes in human genome.

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Prime base editing involves the fusion of designed reverse transcriptase with nickase nCas9 and prime editing guideRNA (pegRNA). This

prime editing gRNA pegRNA has two parts which performs two different functions. One guides it to the desired region while other harbors desired substitution mutations to be incorporated after the repair of single-stranded break that has been produced (Nelson et al., 2022). After the alteration of one strand by the prime editor, the complementary strand can also be modified by an additional gRNA and nCas9 that will induce a nick followed by its repairing with earlier edited strand as template. It has been predicted that prime editing can treat about 89% of genetic mutations in humans (Kantor, McClements, & MacLaren, 2020).

Base Editing	Prime Editing
• It Involves substitution mutations. • It is reliable, predictable, and efficient genetic outcomes. • It does not require template-based HDR. • It avoids undesirable double-stranded DNA breaks and oversized genomic rearrangements.	• It Involves insertion and deletion mutations. • It has low error rates. • It can perform transition and transverse mutations. • It can insert up to 45 bps and can delete up to 80 bps.

7.4 APPLICATIONS

7.4.1 CELLULAR AND GENE THERAPIES

CRISPR has enormous capability to transform to the field of medicine, it has ability to treat wide range of diseases such as cancer, blood disorders, ocular disorders, and neurodegenerative diseases (Liu et al., 2021) (Figure 7.3). First cell therapy utilizing CRISPR methodology was performed to treat patients with sickle cell diseases restoring the fetal hemoglobin, thus eliminating the requirement of a functional copy of adult hemoglobin (Park & Bao, 2021). Recently, in 2021, an important trail of CRISPR was performed to treat transthyretin amyloidosis, a neurodegenerative disease, displayed very encouraging results (Gillmore et al., 2021). Not only has the field of medicine, CRISPR also transformed the field of pediatrics. CRISPR has been used to treat pediatric cancer by the generation of chimeric antigen receptor (CAR) T cells, a kind of immunotherapy utilized in treatment of cancer. Lymphocytes T-cells are engineered after their extraction from a person to express CAR before injecting back to the

patients. These T cells expressing CARs helps in identifying and eliminating the specific type of cancer efficiently from which a patient suffers (Razeghian et al., 2021).

CRISPR technique is still in its early phases of clinical trials, and this technique has the capability to treat a wide range of genetic conditions in the near future, including ovarian and breast cancer associated with BRCA mutations, cystic fibrosis, beta-thalassemia, Alzheimer's, and Huntington's disease (Hirakawa et al., 2020).

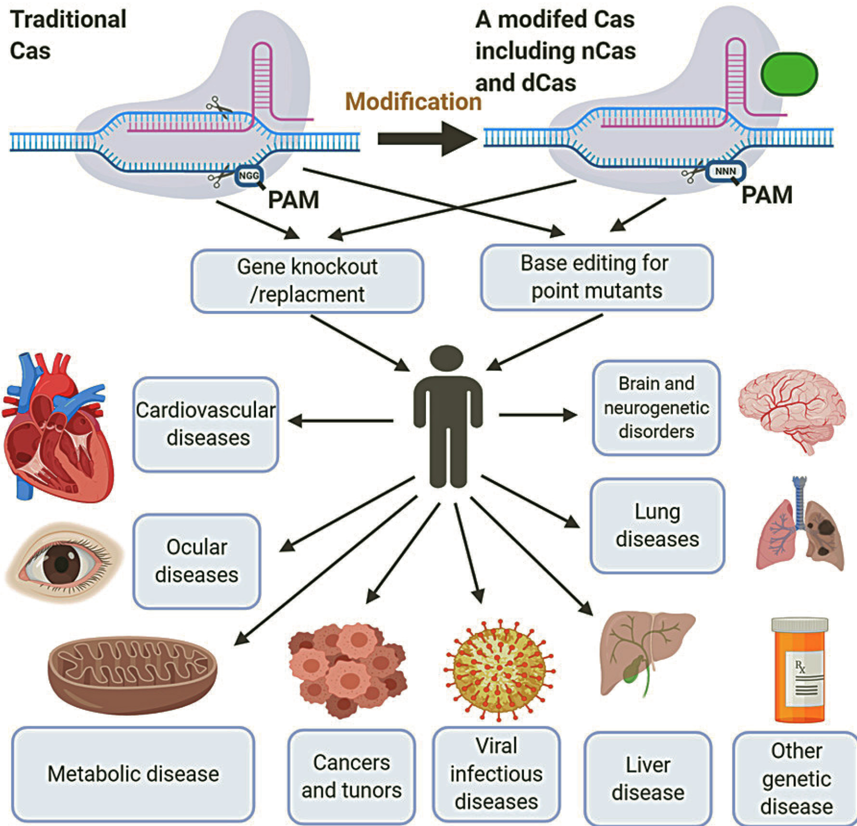

FIGURE 7.3 CRISPR-Cas genome editing tool has a huge capability to treat various genetic diseases, including cardiovascular diseases, brain and neurodegenerative disorders, respiratory diseases, hepatic diseases, and malignancies, as well as metabolic syndromes and virulent infectious diseases.

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7.4.2 DIAGNOSTICS

CRISPR can be used not only as a therapeutic tool but also as a diagnostic tool as well. For example, during COVID-19 pandemic (Dara and Talebzadeh, no date), CRISPR was used as a diagnostic tool such as CRISPR SARS-CoV-2 SHERLOCK™ was permitted for emergency use authorized by federal authorities. Another CRISPR-based COVID-19 kit DETECTOR was developed by Mammoth Biosciences, which uses Cas9's scan function to search and detect genetic material of Coronavirus (Joung et al., 2020). Utilizing the search function of Cas9, many other diagnostic tools have been engineered to identify other infectious and genetic diseases.

7.4.3 AGRICULTURE

CRISPR-Cas has also widespread application in agriculture as well and scientists are claiming that CRISPR-modified foods will be available within the next decade. It is possible because CRISPR can be used to develop drought and disease resistance plants (Joshi, Bharat, & Mishra, 2020). It has the capability to increase the shelf-life of perishable foods, thereby increasing the access to healthy foods at low cost and reducing the food wastage.

7.4.4 CRISPR IN DISEASE MODELS AND ANIMAL MODELS

As compared to the traditional gene targeting methods of development of animal models, the CRISPR-Cas system has provided cheaper and faster development of animal models with a high degree of precision. It also has potential to provide more precise disease models. Some of them are described in subsections.

7.4.4.1 PRIMARY CELLS

Human primary cell lines are very important for disease modeling as they produce more reliable data as compared to immortalized cell line. Primary cells edited by the CRISPR technology can be used for cell therapy and disease modeling. Genetic manipulation of primary cells poses significant challenges; however, scientists have developed methods to increase CRISPR editing efficiency in primary human cells, which involves use

of chemically modified sgRNAs, synchronizing the cell cycle, generating ribonucleoprotein (RNP) complex for delivery, and cell-specific optimization of delivery methods which leads to the production of many cell therapies, including T cell immunotherapies (Mangeot et al., 2019).

7.4.4.2 STEM CELLS AND ORGANOIDS

CRISPR system has revolutionized the world of stem cell research. As known stem cells are pluripotent in nature, i.e., they can differentiate into any type of cell, making them extremely beneficial in the field of medical research. The emergence of induced pluripotent stem cells (iPSCs) was played a vital role because they are adult cells that can reprogramed to become pluripotent.

Human iPSCs are very difficult to grow and reprogram, because they are quite resistant to genetic reprogramming, however CRISPR has made it easier and also delivery efficiency is much greater as compared to earlier gene-editing tools and techniques used to manipulate iPSCs (Ben Jehuda, Shemer, & Binah, 2018).

Three-dimensional models of human organs that are developed in the lab are known as organoids and the CRISPR-Cas9 edited iPSCs can be used to produce such organoids. Organoids are more readable to study human diseases because they provide complexity related to the real human organs, thus providing a good model to analyze response to treatments and mimic the progression of diseases more accurately than cell cultures which are grown as monolayers or suspensions (Driehuis & Clevers, 2017). Recently, brain organoids are generated using CRISPR edited iPSCs to study the evolution of the human brain (McTague et al., 2021).

7.4.4.3 ANIMAL MODELS

Development of animal models that mimic human diseases is imperative in the studies of various diseases but do have some drawbacks, including a lack of translational capacity. CRISPR-edited transgenic animal models are closer to mimic human diseases and are comparatively easier and more efficient than earlier genome manipulation technologies (Shrock & Güell, 2017). It has the capacity to edit at any desired location and has also the ability to generate multiple edits within the same organism. Humanized knock-ins in animals are generated by the use of CRISPR technology

which involves deletion of a particular region of the gene or whole gene followed by replacing it with a human version. Humanized knock-ins are key in understanding as well as treating human diseases (Artegiani et al., 2020). Recently a significant breakthrough has been achieved through gene-edited models of Duchenne Muscular Dystrophy in treating this disease, giving rise to the development of a possible gene therapy (Choi & Koo, 2021). CRISPR has also been used to generate a wide variety of neurodegenerative animal models as well (Yang et al., 2016).

7.5 CONCLUSION

The development of genome editing tool CRISPR-Cas has revolutionized the whole field of genomic research from last few years, enabling the advancement in basic genomic research for a wide range of applications. Also, CRISPR-Cas-based technologies are used to develop both *in vitro* and *in vivo* diseases models. CRISPR-Cas is now regarded as an umbrella term and is believed to be a promising approach to treat various human diseases. Continuous progression of CRISPR-based technologies not only revolutionizes the medical research but also offers various therapeutic opportunities to treat various diseases. No doubt CRISPR technology has proven a game-changer for genome engineering, but it's just the beginning, more possibilities and valuable benefits of this technology will be seen in the near future.

KEYWORDS

- **animal model**
- **clustered regularly interspaced short palindromic repeats**
- **CRISPR-Cas**
- **gene therapy**
- **genetic disorders**
- **genome editing**
- **protospacer adjacent motif**
- **transcription activator-like effector nucleases**

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CHAPTER 8

Emerging Immunotherapies for Healthcare Systems

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ABSTRACT

The human immune system is equipped with the ability to fight against invading pathogens. It has been an exciting area to target this ability of the immune system for improving human health. Recent technological advances have greatly accelerated the research toward the development of targeted therapeutics. The steadfast progress for the development of immunotherapies has paved the way for better understanding and fine-tuning of the immune system for fighting against different diseases. Considering the important role of T-cells in driving immunological responses, their differentiation and functional subsets immunotherapies are designed with an aim to release the brakes on the immune system. Immunotherapy uses a wide variety of immunomodulators that augment immune responses using various strategies ranging from altering the cultured cells to monoclonal antibodies and autologous immune enhancement therapies. This chapter aims to provide a comprehensive outlook on the pre-clinical and clinical findings for the development of different immunotherapeutic approaches for the management of different diseases.

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8.1 INTRODUCTION

The host immune system is the key determinant of the infection outcome (i.e., disease progression/regression), so harnessing the immune responses for fighting against diseases remains the hottest areas of biomedical research. Immunotherapy is a treatment approach that harnesses the host's own immune system to combat infections or diseases. It harnesses innate and adaptive arms of immunity for fighting the disease and/or enhance immune resistance against pathogens.

The history of modern immunotherapy began back in the late 19th century, when Dr. William B Coley first attempted to harness the host immune system to fight against cancer, when most of the cancer cases underwent remission and developed erysipelas. Dr. Coley attempted to treat these subjects by injecting a mixture of live but inactivated *Streptococcus pyrogens* and *Serratia marcescens* that yielded durable and complete remission in the sarcoma, lymphoma, and testicular carcinoma cases. Unfortunately, the mechanism behind the action of Coley's toxin was not deciphered and potential risks associated with injection of pathogenic bacteria instigated the oncologists to opt for surgery and radiotherapy as the standard treatment option in early 20th century (Decker & Safdar, 2009). The use of attenuated bacteria for treating malignancies re-emerged in 1976, when BCG (Bacillus-Calmette-Guerin) was successfully used for preventing recurrence of bladder cancer (Morales et al., 1976) and has been used till date (Morales et al., 2017). In the same year, IL-2 was identified, that was used for metastatic cancer cases for enhancing the T-cell population, that eventually gained Food and Drug Administration (FDA) approval in 1991 for treatment of metastatic kidney cancer and for treating metastatic melanoma (in 1998) (Rosenberg et al., 1985). In 1970, Kohler and Milstein used hybridoma technology for developing monoclonal antibodies and later, research on antibody-based therapies led to the development of rituximab that targeted the immature B-cells (Rudnicka et al., 2013). In 1997, FDA approved monoclonal antibody-based therapy for the treatment of non-Hodgkin's lymphoma. In 2010, FDA approved sipuleucel-T, as the first vaccine for prostate cancer. Another breakthrough in immunotherapy came after the discovery of immune checkpoint molecules, in 2011 and 2014, when Ipilimumab (targeting CTLA-4) and Nivolumab (targeting PD-1), respectively, were approved for treating melanoma cases. Further, promising results have been obtained with the

use of MPDL3280A (anti-PD-L1 antibody) in melanoma, lung cancer and bladder cancer (Rosenberg et al., 2016; Patel et al., 2017).

In recent years, immunotherapy has become the hottest areas of biomedical research for treating several diseases. With the aim of manipulating host immune responses to fight diseases, immunotherapy is also referred to as the biological therapy, due to its ability to use substances called biological response modifiers (BRM). Human body usually produces small amount of BRM in response to invading pathogens however, in laboratory conditions these substances are produced in large quantities for therapeutic purpose. These BRMs or immunomodulators have fewer side effects and lesser potent in terms of creating drug resistance in disease causing microbes (Masihi, 2001).

Based on the mechanism of action immunotherapies can be broadly categorized into two classes: passive immunotherapy and active immunotherapy. Passive immunotherapy (also called adoptive immunotherapy) employs donated or laboratory-made immune cells and/or recombinant molecules (monoclonal antibodies) for fighting against diseases. This approach can be specific (monoclonal antibody therapy) or non-specific (for example, injection of patient's cultured T-cells, after *in vitro* expansion and or activation). Active immunotherapy directly triggers host immune system/immune response to fight infections. It is again divided into specific (generates cell-mediated and antibody responses) and non-specific (elicits cytokine and other cell signaling molecules) arms. Overall, immunotherapies can be agent based or cell based that aims to trigger the host immune responses by targeting different virulence factors and brings about immunological manipulations for therapeutic benefits.

8.2 TYPES OF IMMUNOTHERAPEUTIC AGENTS

As discussed in the above sections, there are two types of immunotherapeutic approaches – the specific and non-specific. In this section, we discuss the targeted therapeutic approaches for stimulating immune responses. Over the decades, there have been diverse therapies that block the biochemical pathways and have been successfully used for fighting against several diseases. This chapter aims to give a comprehensive outlook on different approaches that have revolutionized the biomedical sector.

8.2.1 SPECIFIC IMMUNOTHERAPIES

Treatment for several diseases includes the use of specific immunotherapies that aims to target particular immune cell population and/or molecule for immunomodulation. Recent specific immunotherapies are focused on modulating the signaling pathways/molecules as PD-1, CTLA-4, etc., for altering the host immune responsiveness.

8.2.1.1 mRNA VACCINES

Nucleic acid therapeutics has been one of the best innovative alternatives to the conventional vaccine approaches, which was successfully used in animal model (in 1990) (Wolff et al., 1990). Later in 1992, documented the effect of vasopressin encoding mRNA injection elicited the physiological responses (Jirikowski et al., 1992). But the instability of mRNA, inefficient *in vivo* delivery and high innate immunogenicity restricted subsequent research for mRNA-based therapeutics. Recent technological advances have again brought the mRNA-based therapeutic strategies to the limelight for vaccinology. RNA-based vaccines remains potentially advantageous as compared to the DNA-based vaccination approaches in terms of its safety (non-infectious), it can be easily degraded during normal cellular processes and easy modification options provide flexibility in regulating its half-life during *in vivo* administration (Kariko et al., 2008; Kauffman et al., 2016; Thess et al., 2015). Furthermore, the easy manufacturing and production of mRNA vaccines makes this field exciting avenue to explore for different diseases. Therefore, RNA vaccines fulfill different criterion for an ideal vaccine so, vast array of clinical trials are undergoing for translation of these trials for clinical success.

Broadly speaking, there are two forms of RNA vaccines available against infectious agents: self-amplifying (replicating) vaccines and non-replicating vaccines. Self-amplifying vaccines (SAV) are alpha virus genome-based formulation, where genes for RNA replication machinery remain unaffected while the genes for structural proteins are replaced with the desired antigenic fragment. SAVs facilitates the production of large amount of antigen from small vaccine dosage, that eventually elicits stronger T- and B-cells mediated immune responses against diverse range of viral infections (Fleeton et al., 2001; Geall et al., 2012; Bogers et al.,

2015) and bacterial infections (Maruggi et al., 2017). Further, SAVs create their own adjuvants (double stranded RNA structures) that improve the vaccine efficacy; however, greater size and higher immunogenicity restricts its repeated usage. Conversely, non-replicating mRNA vaccines can further be divided into two sub-classes, depending on their mode of delivery: *ex vivo* loading of DCs or direct injection method.

Ex vivo DCs loading method remains the choice for generating cell-mediated immune responses for cancer, HIV (electroporation of autologous DCs with mRNA for HIV antigens). The intervention was found to successfully elicit CD4 and CD8 T-cells responses in HIV cases; however, it didn't confer any clinical advantage to the cases (Routy et al., 2010; Allard et al., 2012). Similarly, DC-based vaccination for cytomegalovirus (CMV) in stem cell recipients induced cell-mediated immune responses (Van Craenenbroeck et al., 2015).

Recently, non-replicating mRNA-based directly injectable vaccines are emerging as simple and easy administrable option in resource poor settings. This vaccine format was first demonstrated against influenza virus infection in 2012 (Petsch et al., 2012). Thus, mRNA vaccines in its uncomplexed form were sufficiently immunogenic for eliciting protective responses.

Similarly, protamine-based RNActive platform encoding for rabies virus induced protective cell- and antibody-mediated immune responses in animal model (Schnee et al., 2016). The prophylactic vaccine was also tested in humans where needle-free delivery method induced elicited variable levels of functional antibody responses against the virus, with reasonable tolerability profile (Alberer et al., 2017).

Different formats of mRNA vaccines are available that use lipid- or polymer-based delivery platforms. In mice model, cationic 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) lipid-complexed mRNA vaccines against HIV are known inducers of antigen-specific CD4+ and CD8+ T-cell responses (Pollard et al., 2013). Similarly, PEI-complexed mRNA vaccine (against HIV) and lipid complexed vaccine (against influenza) successfully induced T-cell activation and responses (Kranz et al., 2016; Zhao et al., 2016).

Nucleoside-modified mRNA vaccines have been the new innovation; however, limited data on its efficacy has been reported. First data on the successful use of the vaccine was demonstrated for Zika virus in mice and macaques (Pardi et al., 2017). A subsequent study reported for the efficacy

of vaccine in terms of moderate immune responses however, the booster dose elicited protective immune responses in vaccinated group (Richner et al., 2017). Another trial against influenza virus, reported for protective efficacy of low-dose LNP-complexed mRNA vaccine. The successful pre-clinical findings paved the way for human trials where the vaccine was potently immunogenic and elicited protective antibody responses (Bahl et al., 2017). Presently, several vaccines are entering clinical trials and are expected to deal with a diversity of antigens for fighting against infectious diseases.

In context with the cancer vaccine, mRNA-based vaccines aim to target tumor or its associated antigens. Mostly, cancer vaccines are therapeutic rather than prophylactic and the idea of RNA-based cancer vaccines emerged about two decades ago (Boczkowski et al., 1996). DCs based mRNA vaccines were potent inducers of protective responses that can be further enhanced with the use of mRNA-encoded adjuvants. Several reports about the use of costimulatory molecules (CD83, OX40, and 4-1BB) significantly enhanced the immunostimulatory potential of DCs based vaccines (Dannull et al., 2005; Aerts-Toegaert et al., 2007; De Keersmaecker et al., 2011). Similarly, mRNA encoding for pro-inflammatory cytokines and trafficking molecules have also been successful in inducing anti-tumor T-cells and NK cells responses (Bontkes et al., 2008; Dorrie et al., 2008). Furthermore, a cocktail of mRNA-encoded adjuvants (named TRiMix, includes CD70, CD40L, and TLR4) has also shown promising results in fighting cancer in several pre-clinical studies (Bonehill et al., 2008; Van Lint et al., 2014). The cocktail serves as a potent inducer of DC activation and triggers TH1 type immune responses that eventually cause tumor regression in different cancer cases (Wilgenhof et al., 2013; Wilgenhof et al., 2016; Batich et al., 2017). Therefore, there has been tremendous progress in writing the success story of RNA vaccines however, this is not the end since, dozens of clinical trials for different diseases are still ongoing and potency of RNA vaccines against several infectious pathogens are still under exploration.

8.2.1.2 DNA VACCINES

DNA vaccination approach although first used in the early 90s, has recently been evolving as a smart strategy for the development of

immunotherapeutics, with the aim to stimulate the humoral as well as the cellular immune responses. In animal models, DNA vaccines have been successfully used to prevent or treat some infectious diseases, cancer, autoimmunity, and allergies (Ulmer et al., 1996).

DNA vaccines are aimed to deliver one or several antigen encoding genes (under the control of appropriate promoter) with the aim to elicit antigen-specific immune responses. With the direct injection of genetic material, a relatively smaller number of cells incorporates the genetic material in them that eventually leads to inappropriate expression of the introduced gene that has important immunological consequences. The inappropriate gene expression forms the key for immune activation since most of the cells do not present the protein to the T-cells that eventually triggers the CTL responses by CD8+ T-cells (if presented to the immune cells with the MHC-I) that inhibits viruses. Conversely, CD4+ T-cells are activated (if the peptide fractions are presented in context with MHC-II by the antigen presenting cells). CD4+ T-cells (Th1 type) stimulate the B-cells and the antibody production (Encke et al., 1999). These vaccines have significantly gained attention in recent years due to their stability, improved safety, and feasibility for large scale manufacturing. Furthermore, strategies aiming at improving their immunogenicity and antigen presentation by employing innovative delivery platforms, adjuvants, immune-stimulatory molecules, or blockade of immune checkpoint molecules are still being underway.

The success of DNA vaccines has been seen in the clinical settings, where several trials are ongoing. Clinical trial targeting the breast cancer has validated the safety and efficacy of DNA plasmid encoding for Her-2 gene, where remittent cases (stage II, III, and IV) upon vaccination with DNA along with cytokine adjuvant and GM-CSF yielded humoral and cell-mediated immune responses. It is well known that the success of DNA vaccine regimen usually depends on the preventing the disease recurrence, this formulation was successfully known to elicit immunological memory (Disis et al., 2004).

A veterinary DNA vaccine for West Nile virus safety in horses has been already approved. Some previous studies show that intramuscular injection of 'naked' plasmids produces immunogens that would elicit immune responses against influenza virus antigens in mice (Ulmer et al., 1993). Plasmid DNAs which express the influenza virus hemagglutinin

(HA) glycoproteins to increase protective immunity against has also been investigated (Fynan et al., 1993).

HA, the primary viral antigen in influenza, is commonly used in DNA vaccines against influenza virus. Other viral proteins are being used infrequently, usually in combination with HA, but sequences encoding PB1, M2, NP, and M1 have also been used in DNA vaccines (Patel et al., 2012; Lim et al., 2013; Lee et al., 2018). Plasmids encoding for NP and M2 have been shown to minimize the viral load and increase survival in BALB/c mice (Ulmer et al., 1993; Tompkins et al., 2007). Fusion protein antigen (combined use of can be used to expand the range of host responses (Lee et al., 2018). In mice, plasmid-encoded chimeric proteins of H1N1 HA and the conserved M2-ectodomain increase cross-reactivity of antibody response compared to when using HA alone viruses in DNA vaccine (Park et al., 2011). The USDA approved conditionally, the first commercial DNA vaccine against H5N1 in chickens. Effective plasmid delivery and the use of suitable adjuvants are still major obstacles to overcome before influenza DNA vaccines can be used in humans.

In SARS-CoV-2 infection antibodies are formed against the P and S protein. The N protein is covering the viral genome as well as it helps in the release of virus particles from cells. The S (S1, S2, and S2') protein, on the other hand, plays an important role in pathogenesis through binding to the host cell via its receptor-binding domain and thus inducing infection (Zhang et al., 2022). All DNA vaccines currently being under clinical trials have used the S protein as their antigen. One of them is "Study of COVID-19 DNA Vaccine (AG0301-COVID-19)" (ClinicalTrials.gov Identifier: NCT04463472). In this study they used two experimental groups: low dose group and high dose group. In both cases they recruited 15 people aged between 20 to 65 years and expected date of completion of the project is July 31, 2021 (ClinicalTrials.gov) (Table 8.1).

8.3 STEM CELL THERAPY

Stem cells have the capacity of proliferation, migration, and differentiation, making them a perfect candidate for potential therapeutic applications in tissue regeneration and repair. This self-renewable capacity is also the characteristics of tumor cell, but the difference is that stem cell divides in a very controlled manner while tumor cell divide in an uncontrolled

TABLE 8.1 . Recent COVID-19 Nucleic Acid Vaccine Clinical Trials

Estimated Study Start Date	Estimated Study Completion Date	Project Title	Clinical Trials. gov Identifier	Phase	Subject (Age)	Number of Subjects	Study Location
June 17, 2020	June 17, 2022	Safety and Immunogenicity Study of GX-19, a COVID-19 Preventive DNA Vaccine in Healthy Adults	NCT04445389	Phase I/II	18 to 50	210	Korea
December 30, 2020	March 30, 2022	Safety and Immunogenicity Study of GX-19N, a COVID-19 Preventive DNA Vaccine in Healthy Adults	NCT04715997	Phase I/II	18 to 55	170	Korea
February 25, 2021	June 2022	Safety and Immunogenicity of COVID-eVax, a Candidate Plasmid DNA Vaccine for COVID-19, in Healthy Adult Volunteers	NCT04788459	Phase I/II	18 to 65	160	Italy
December 30, 2020	May 2022	CORVax12: SARS-CoV-2 Spike (S) Protein Plasmid DNA Vaccine Trial for COVID-19 (SARS-CoV-2)	NCT04627675	Phase I	18 and older	36	United States
March 2021	August 2022	A Clinical Trial of a Plasmid DNA Vaccine for COVID-19 [Covigenix VAX-001] in Adults	NCT04591184	Phase I	18 to 84	72	Canada
August 31, 2020	September 30, 2021	Study of COVID-19 DNA Vaccine (AG0302-COVID-19)	NCT04527081	Phase I/II	20 to 65	30	Japan
June 29, 2020	July 31, 2021	Study of COVID-19 DNA Vaccine (AG0301-COVID-19)	NCT04463472	Phase I/II	20 to 65	30	Japan
November 23, 2020	March 31, 2022	Phase II/III Study of COVID-19 DNA Vaccine (AG0302-COVID-19)	NCT04655625	Phase II/III	18 and older	500	Japan
February 15, 2021	December 31, 2022	The Safety and Immunogenicity of a DNA-based Vaccine (COVIGEN) in Healthy Volunteers	NCT04742842	Phase I	18 to 75	150	Australia

Source: Assess at ClinicalTrials.gov as of March 22, 2021.

manner. Using this property stem cells are used in therapy called stem cell therapy in which stem cells used to cure, prevent a disease or disorder (Mahla, 2016). The most common problems that can benefit from this type of treatment are strokes (Liu, 2013), diabetes (Shahjalal et al., 2018), macular degenerations (Sun et al., 2017) and osteoarthritis (Gangji et al., 2011).

Extensive research in stem cell provides clue about its usage for treating cancer patients due to its characteristics like self-renewal, modulatory effects on other cells, directional migration, and self-differentiation, which can be used as drug targeting, immune cell modulation, and therapeutic carriers. Embryonic stem cells (ESCs) can give rise to any form of cell except those in the placenta. We can make pluripotent cells directly from mouse embryonic or adult fibroblast cultures, which we call induced pluripotent stem (iPSCs) cells when cultured in defined factors (Oct3/4, Sox2, c-Myc, and Klf4) (Takahashi & Yamanaka, 2006). Both hESCs and iPSCs have recently been used to induce effector T- and NK cells, and also to produce anti-cancer vaccines (Matsushita et al., 2014; Kooreman et al., 2018). The second type of stem cell is adult stem cells (ASCs) is a group of three types of cell mesenchymal stem cells (MSCs), neural stem cells (NSCs) and hematopoietic stem cells (HSCs) used in cancer treatment (Table 8.2). Another Third type of stem cell is called as the CSCs (cancer stem cells) which is produces by epigenetic mutations in natural stem cells or progenitor/precursor cells and play important role in growth of cancerous cell, its metastasis, recurrence, and treatment resistance (Chang, 2016). Therefore, targeting CSCs could be a good option for cancer treatment.

In tumors, patients need intravenous infusion of autologous or allogeneic HSCs. These HSCs are thought to have a homing mechanism that allows them to migrate quickly into established stem cell niches in the bone marrow (BM) where interaction of the CXCR4 receptor (present on stem cells) and the gradient of SDF-1 (secreted by cells lining of BM stem cell niches) taken place (Juarez et al., 2012). Some other examples of molecular signaling of HSC homing process are Ceramide-1 phosphate and sphingosine-1-phosphate (Seitz et al., 2005; Massberg & von Andrian, 2009), calcium sensing receptor (Adams et al., 2006), proton sensing GPCR (Okajima, 2013). HSCs migrate across the bloodstream and through the endothelial vasculature to various organs and in the BM niche via interaction between endothelial (through LFA-1, VLA-4/5,

CD44) and secretion of matrix degradable enzyme MMP-2/9 (Lapidot et al., 2005) (Table 8.2).

TABLE 8.2 Different Types of Stem Cells and Their Therapeutic Utility for Cancer

Cell Type	Location	Role	Use in Cancer
Hematopoietic stem cells (HSCs)	Bone marrow	Can form all mature blood cells	Multiple myeloma, leukemia, and other cancers
Mesenchymal stem cells (MSCs)	Bone marrow, adipose tissue, and fetal tissue (umbilical cord)	Tissue repair as well as regeneration	Liver, lung cancer, pancreatic tumors, and other cancers
Neural stem cells (NSCs)	Central nervous system	Generate new neurons and glial cells	Metastatic breast, lung, and prostate cancers and other cancers

A large number of registered clinical trials using MSCs to treat solid tumors are currently underway. The world's first gastrointestinal tumor clinical trial (phase I/II) using genetically engineered MSCs is successfully completed in humans (TREATME1) (Niess et al., 2015). There are other trials have also been completed and many more clinical trials currently underway (Table 8.3).

MSCs are extensively studied stem cell which is widely being used to treat autoimmune disorders, involvement in hematopoietic stem cell transplantation (HSCT), and treatment of infections and their complications, including septic shock and acute respiratory distress syndrome. BM-MSCs may protect against infectious challenges by activation of the host immune responses and modulating proinflammatory host immune response (Auletta et al., 2012).

Stem cell therapy has recently been developed to treat parasitic infections. *In vivo*, MSCs have been shown to strengthen the liver damage caused by *S. japonicum* infection, and this effect is improved when MSCs are combined with praziquantel. These results indicate that treating *S. japonicum*-induced liver injury and fibrosis with MSCs is a novel therapeutic strategy (Xu et al., 2012). In mice infected with *Plasmodium berghei*, massive recruitment of MSCs has been observed in secondary lymphoid organs, and transplantation of these cells into naive mice provides host resistance against malaria infection (Thakur et al., 2013). BM mononuclear cells have been shown to reduce inflammation

TABLE 8.3 Some Current Ongoing Stem Cell Therapy Clinical Trials in Cancer

Estimated Study Start Date	Estimated Study Completion Date	Project Title	Clinical Trials. gov Identifier	Phase	Subject (Age)	Number of Subjects	Study Location
August 18, 2018	October 22, 2021	Mesenchymal Stem Cell Therapy for Liver Cirrhosis: A Phase I/II Study	NCT03626090	Phase I/II	21 to 69	20	Singapore
January 11, 2007	April 24, 2013 (completed)	Adoptive Cell Therapy for B-Cell Malignancies After Allogeneic Hematopoietic Stem Cell Transplantation with Costimulated, Tumor-Derived Lymphocytes	NCT01445132	Phase I	18 to 75	11	United States
April, 2017	July 2023	Efficacy of Doxycycline on Metakaryote Cell Death in Patients with Resectable Pancreatic Cancer	NCT02775695	Phase II	18 years and old	12	United States
March 5, 2019	September 1, 2025	Targeted Stem Cells Expressing TRAIL as a Therapy for Lung Cancer	NCT03298763	Phase I/II	18 and old	46	United Kingdom
January 16, 2019	July 31, 2024	A Phase 1 Study of IDH1 Inhibition Using Ivosidenib as Maintenance Therapy for IDH1-mutant Myeloid Neoplasms Following Allogeneic Stem Cell Transplantation	NCT03564821	Phase I	18 and old	22	United States
February, 2018	November, 2021	High-dose Chemotherapy with Autologous Hematopoietic Stem Cell Transplantation as Adjuvant Treatment for Triple Negative Breast Cancer Patients Without Complete Pathological Response to Neoadjuvant Chemotherapy	NCT02670109	Phase II	18 years to 60	20	Mexico
November 2005	December 2022	Hematopoietic Cell Transplantation in the Treatment of Infant Leukemia and Myelodysplastic Syndrome	NCT00357565	Phase II	Up to 3 Years	20	United States

Source: Assess at ClinicalTrials.gov as of March 22, 2021.

and fibrosis in mice infected with *Trypanosoma cruzi* in mouse models of Chagas disease (Soares et al., 2004).

Targeting MSCs or NO appears to be an effective therapeutic intervention for the development of new prevention strategies for *Mycobacterium tuberculosis* (TB). Combining standard anti-TB chemotherapy with autologous MSC transplantation could eventually improve anti-TB treatment efficacy in MDR-TB patients. Studies have shown that MSCs are vulnerable to infection by viruses from the herpes family such as Epstein-Barr virus, HSV-1, HSV-2, and CMV and when MSCs are infected with herpes viruses, they lose their ability to function. The highly pathogenic avian influenza A/H5N1 infection is permissive to human MSCs, and infection can cause apoptosis and loss of immunomodulatory function of MSCs. MSCs secrete extracellular vesicles (ECVs) that have anti-inflammatory and anti-influenza properties hence it can be used as a therapeutic agent (Khatri et al., 2018). In mice, Treatment with MSCs reduces inflammation and mortality associated with the Japanese encephalitis virus, which is the most common cause of viral encephalitis in Asia (Bian et al., 2017) (Table 8.4).

8.4 REGULATORY T-CELL THERAPY

Tregs are a type of T cell that helps to regulate immune responses. The main function of Treg is to suppress a variety of pathological and physiological immune responses in the host, allowing immune homeostasis to be maintained (Sakaguchi, 2000). Despite the fact that Tregs are characterized as T cells that suppress immune responses, it has been established that regulatory T cell populations are diverse; some are induced in response to infectious challenge, while others are considered natural regulators (Belkaid, 2007). These inducible Tregs cells produces inhibitory cytokines such as IL-10 and (TGF)-β. Tregs are CD4+CD25+ T cells that make up about 10% of total peripheral CD4+ T cells in both mice and humans (Lee et al., 2011). Foxp3 transcription factor is highly expressed in the CD4+CD25+ population (Bacchetta et al., 2007), which is required for Treg cell differentiation, function, and programming of suppressor T cell function (Fontenot et al., 2003; Haque et al., 2011). Impaired regulatory T-cell function is linked to autoimmune, infectious, and allergic diseases in humans (Taams et al., 2006). Taking all together, these cells play a vital role in the maintenance of both innate and acquired immune responses.

TABLE 8.4 Some Current Ongoing Stem Cell Therapy Clinical Trials in Different Infectious Diseases

Estimated Study Start Date	Estimated Study Completion Date	Project Title	Clinical Trials. gov Identifier	Phase	Subject Age (Year)	Number of Subjects	Study Location
April, 2020	September 2020 (completed)	Clinical Use of Stem Cells for the Treatment of COVID-19	NCT04392778	Phase I/II	40 to 60	30	Turkey
June 1, 2020	June 30, 2021	Efficacy of Intravenous Infusions of Stem Cells in the Treatment of COVID-19 Patients	NCT04437823	Phase II	30 to 70	20	Pakistan
January 4, 2019	October 2021	Antigen Specific Adoptive T Cell Therapy for Adenovirus Infection After Hematopoietic Stem Cell Transplantation	NCT03378102	Phase 1	3 Months and old	20	United States
October 2016	December 2022	CMV Specific T Cell Therapy After Allogeneic Stem Cell Transplantation	NCT03067155	Phase II	16 to 75	20	Belgium
January 15, 2020	June 30, 2022	Escalation Antifungal Prophylaxis for Patients Undergoing Allogeneic Hematopoietic Stem Cell Transplantation	NCT04273178	Phase II	16 to 65	175	China
May 21, 2017	December 30, 2020	Effectivity of Mesenchymal Stem Cell on Vertebral Bone Defect Due to *Mycobacterium tuberculosis* Infection	NCT04493918	Phase II	15 to 64	20	Indonesia

Source: Assess at ClinicalTrials.gov as of March 22, 2021.

In lung, pancreatic, breast, liver, and skin cancer patients having high numbers of CD4+ CD25+ Tregs in the blood or in the tumor itself (Liyanage et al., 2002; Woo et al., 2002; Wolf et al., 2003; Ormandy et al., 2005). These Treg cells stop NK cell mediated cytotoxicity and also the production of IFN-γ from CD4+ and CD8+ T cells. Treg cells are found abundantly in the tumor microenvironment, where they suppress antitumor immune responses, promoting tumor growth and progression. Infiltrating lymphocytes from Hodgkin lymphoma contain both CD4+ CD25+ and IL-10-producing Tregs, which suppress mitogen- and antigen-specific peripheral blood mononuclear cell responses (Marshall et al., 2004). Chemokines are a type of secreted chemotactic protein that regulates leukocyte migration by binding to G protein-coupled receptors on target cells. They are involved in the recruitment of inflammatory cells as well as immunomodulatory cells, such as Treg cells, for antitumor responses, and play an important role in selective homing of neoplastic B and T cells (Nagarsheth et al., 2017).

The immune response to infection is a delicate balancing act between the successful induction of proinflammatory responses and the anti-inflammatory responses needed to limit tissue damage. Tregs (CD4+ CD25+ Treg and IL-10-secreting Treg) undoubtedly play a key role in maintaining this balance during infection, with outcomes ranging from highly harmful to the host to highly beneficial to both the host and the pathogen (O'Garra et al., 2004). Studies have shown that in comparison to uninfected controls, infected patients with *Helicobacter pylori* have more CD4+ CD25+ FOXP3+ T cells in the stomach and duodenal mucosa (Lundgren et al., 2005). There are some more examples of infectious dieses in which Treg cells might contribute to chronic infection (*Helicobacter hepaticus, Listeria monocytogenes, Leishmania major, Schistosoma masoni,* herpes simplex virus, cytomegalovirus) (Belkaid & Rouse, 2005).

Targeting Treg cells can be used as the main therapeutic approach against both cancer and infectious diseases. In mice, Treg cell depletion can elicit highly effective antitumor immune responses in preclinical cancer models (Onda et al., 2019). CD25 can be blocked or used to deplete Treg cells depending on the interaction of CD25-specific antibodies with the Fc receptor (Huss et al., 2016). An alternative strategy, use of a recombinant IL-2 diphtheria toxin fusion protein (ONTAK) significantly decreases Treg cells in peripheral blood (Cheung et al., 2019). Treg cells in tumors may have a unique chemokine receptor expression pattern that can be used

for therapeutic purposes. CCR4 is highly expressed on Treg cells and other CD4+ T cell subsets, is essential for Treg cell migration, and its ligands are expressed by a variety of TME cells (Faget et al., 2011). Further comparisons of Treg cells from malignant and normal tissues showed that CCR8 is a more specific marker for tumor-resident Treg cells than CCR4 and has a much more limited expression pattern (Magnuson et al., 2018). Similarly, CCR4 antagonists could be used to improve cellular and humoral immune responses to antigens from *Mycobacterium, Haematopinus suis*, HBV, and *Plasmodium*. High cell surface expression of molecules like PD-1, ICOS, TIGIT, LAG3, TIM3, TNFR2, and 4-1BB could be used to antibody development for Treg-specific targeting (Bour-Jordan & Bluestone, 2009). Inhibition of the PD1PDL1/PDL2 pathway increases the IFN reaction to *M. tuberculosis in vitro* (Stephen-Victor et al., 2015) (Table 8.5).

8.4 CHECKPOINT INHIBITORS

This type of immunotherapy blocks the proteins that serve as the brake for the immune system for fighting against the invading pathogens or tumors. During chronic infections and cancer, the immune system is manipulated to stop and/or slow down its functions so that pathogen/tumor can establish a niche for its survival. Checkpoint inhibitors are designed to release the brakes on the immune system and enhance the existing immune responses to promote the elimination of cancer cells and/or pathogens. In 2011, FDA approved the first immune checkpoint inhibitor therapy for treating cancer – the CTLA4-blocking antibody ipilimumab. Many other checkpoint inhibitors are currently under clinical trial or in different phases of pre-clinical trials alone and in combination with other treatment regimens. These molecules have revolutionized medical oncology field and the discovery of their therapeutic utility won the Nobel prize for Medicine for Prof. J. Allison and T. Honjo in 2018. Till 2020, immune checkpoint inhibitors are amongst the most successful immunotherapies developed so far.

Impairment of the T-cells functions remains the hallmark for several diseases; CTLA-4, PD-1, and other immune checkpoint molecules are known to regulate the magnitude of T-cell responses. During malaria infection, checkpoint molecules are upregulated on T-cells that induces CTL dysfunctions. PD-1 deficient mice have shown to exhibit improved

TABLE 8.5 Some Current Ongoing Regulatory T-Cell Clinical Trials in Different Cancers and Infectious Diseases

Estimated Study Start Date	Estimated Study Completion Date	Project Title	Clinical Trials. gov Identifier	Phase	Subject Age (Year)	Number of Subjects	Study Location
July 1, 2020	December 31, 2022	PD-L1-Expressing Regulatory T Cells in Localized Prostate Cancer Patients Undergoing Iodine-125 Permanent Brachytherapy	NCT04369508	–	30 years to 90	20	Beijing
February 22, 2018	February 2022	Allogeneic Immunotherapy for Hematological Malignancies by Selective Depletion of Regulatory T Cells (DLI-Boost)	NCT03236129	Phase III	Child, adult, older adult	–	France
July 8, 2019	May 31, 2024	A Phase 1 Trial of CD25/Treg-depleted DLI Plus Ipilimumab for Myeloid Disease Relapse After Matched-HCT	NCT03912064	Phase I	18 years and old	25	United States
May 5, 2020	April 2021	Effector and Regulatory T-Cell Receptor Repertoire Analyzes in Patients Affected by COVID-19 (CovRep)	NCT04379466	–	18 years to 75	60	France
March 2021	September 2022	Safety of T-Regulatory Cell Therapy in Subjects With COVID-19 Induced Acute Respiratory Distress Syndrome	NCT04737161	Phase I	18 years to 75	20	United States

Source: Assess at ClinicalTrials.gov as of March 22, 2021.

parasitic clearance and monoclonal antibody-based therapy targeting PD-1 accelerated the reduction of parasitemia (Butler et al., 2011). In contrast, anti-CTLA4 and anti-PD-L1 antibody are known to predispose the *Plasmodium berghei* infected mice to fatal cerebral malaria (Hafalla et al., 2012). This indicated for the varying tissue-specific effects of checkpoint inhibition therapy. A similar increase of the checkpoint molecules expression has been observed in visceral leishmaniasis (VL), where these molecules abrogate the long-term parasite control by blocking the CTL functions. PD-L1 blockade is known to improve the CTL survival and functions by inducing the parasite clearance from the spleen. Similar effects were observed with anti-CTLA-4 therapy, which markedly reduced the parasitic burdens in the liver and spleen in mice model (Joshi et al., 2009; Singh et al., 2019). Cutaneous form of leishmaniasis is also known to upregulate the expression of PD-1, which impairs the IFN-γ production, proliferation of TH1 cells while, its blockade is associated with reversal of the adverse immunological outcomes (Mou et al., 2013).

Anti-PD-1 therapy in macaques, is known to enhance the antigen-specific CTL responses, viral clearance, improved survival and attenuated the excessive immune activation (Dyavar Shetty et al., 2012; Mylvaganam et al., 2018). The therapy was known to enhance the viral control after the therapy was discontinued. The use of checkpoint therapy for treating HIV-infected malignancy cases reported for its safety without interfering efficacy of the antiviral therapy (Chang et al., 2018). In case of hepatitis B infection (HBV), there is a strong association between the polymorphism in checkpoint molecules has been found to be associated with the viral clearance and clinical outcomes (Chihab et al., 2018). Checkpoint molecules are usually upregulated during acute HBV infection and declines once the viral load is cleared (Zhang et al., 2008). Conversely, during chronic infection, checkpoint molecules are upregulated, monoclonal antibody-based blockade of these molecules substantially improves the antibody production, CTL proliferation and their functions (Fisicaro et al., 2010; Schurich et al., 2011; Salimzadeh et al., 2018). Similar to HBV, hepatitis C virus (HCV) clearance is also regulated by the polymorphisms in the checkpoint molecules that eventually determines the clinical outcomes (Xiao et al., 2015; Sepahi et al., 2017). High levels of PD-1 have been observed on the CTLs that possessed exhausted T-cells phenotype and were not able to clear the infection, blockade of PD-1 was found to relieve the intrahepatic T-cell exhaustion (Golden-Mason et al., 2007; Nakamoto et al., 2008).

Similarly, blockade of CTLA-4 was found to significantly improve the infection outcomes (Sangro et al., 2013). Checkpoint molecules are also known to be induced during several acute respiratory viral infections; however, it does not always result in T-cell exhaustion. Administration of anti-PD-1 monoclonal antibody to influenza infected mice, significantly improved the CTL number and virus clearance (Rutigliano et al., 2014).

In bacterial infection, during gastrointestinal *H. pylori* infection, monoclonal antibody therapy targeting CTLA-4 has been known to reduce gastric inflammation without reducing the bacterial load (Watanabe et al., 2004; Lee et al., 2010). Conversely, PD-1 deficient mice have shown potential improvement in the survival rates (reduced mortality) and bacterial clearance abilities (Yao et al., 2009). PD-1 expression is also known to be associated with reduced proliferative abilities of T-cells, poor T-cell infiltrations and impaired effector functions which can be reversed by blocking PD-1/PD-L1 (Day et al., 2018). Anti-CTLA-4 based therapy has also been efficacious in improving the T-cells functions without affecting the granuloma formation or IFN-γ expression (Kirman et al., 1999). Checkpoint molecules (PD-1 and CTLA-4) have also been important determinants of lepromatous immunological responses. These are upregulated on cutaneous T-cells in lepromatous and tubercular leprosy (Palermo Mde et al., 2012). Since these infections are poorly responsive to antimicrobial therapies; therefore, novel therapeutic strategies like checkpoint blockade can serve to open ways for disease management.

Conventional anti-fungal therapies have been used for fungal infections, however, histoplasmosis (caused by *Histoplasma capsulatum*) affecting the advanced AIDS cases and SOT exhibited marked enhancement in the expression of PD-1 (Lazar-Molnar et al., 2008). Genetic deficiency of PD-1 in mice model was found to significantly improve the survival rates by inducing fungal clearance, reducing airspace edema and necrosis. The mortalities due to invasive candidiasis is significantly higher (Pappas et al., 2018), anti-CTLA-4, anti-PD1 and anti-PD-L1 monoclonal antibody therapies have been shown to markedly improve the survival rates. The checkpoint molecule-based therapy upregulates the MHC-II expression by APCs and IFN-γ production (by T-cells) that eventually skews the immune response to TH1 type immunity (Chang et al., 2013; Roussey et al., 2017). Unfortunately, excessive TH1 type immune responses are associated with the development of immune reconstitution inflammatory syndrome (IRIS) (Balasko & Keynan, 2019) therefore, future studies

warrant the understanding of the impact of therapy and development of adverse events. Overall early pre-clinical and clinical success of checkpoint inhibitor therapy in different diseases has paved the path for adapting the checkpoint-based therapy for management of different pathologies.

8.5 AUTOLOGOUS IMMUNE ENHANCEMENT THERAPY (AIET)

Autologous immune enhancement therapy (AIET) is the type of immunotherapy where the cells from the patients' blood are isolated, cultured, and processed to activate them until their resistance to fight against the disease/cancer is enhanced. The cultured cells are then added back in the patient to immune system to clear off the infection/cancer. The cells, antibodies, and organs of the immune system defend the body against invading micro-organisms and cancer. AIET was first used for the treatment of cancer, in the late 1980s, when Rosenberg et al. reported that the immunotherapy lowered the tumor regression rates (2.6–3.3%) in metastatic cancer cases (Rosenberg, 1984). AIET along with chemotherapy/radiotherapy was considered as one of the biggest revolutions in the field of cancer management. Till date, different strategies of autologous and allogenic immune cells, NK cells, CTLs, DCs, and genetically manipulated autologous as well as allogenic immune cells have been widely used in the immunotherapeutics. The current technology of AIET uses the autologous NK cells and activated T-cells for treating cancers. Though the treatment concept emerged in 1980s, but it was started to be used since early 1990s, after several successful clinical trials in gastric, lung, ovarian, and liver cancers (Egawa, 2004). When this cell-based immunotherapy is combined with the conventional therapeutic approaches, it showed marked improvement in the treatment efficacy by 20–30% and it was also efficacious for treating relapse cases (Egawa, 2004; Manjunath et al., 2012).

Adoptive transfer of tumor infiltrating T-cells (TILs) cultured in the presence of recombinant IL-2 induced the lysis of autologous tumors, improved the survival rates, and regressed the tumors in a variety of cancers (1483950). TILs are also known to induce cell-mediated immune responses by immune activation reactions in tumor cases (Rosenberg, 1991). AIET with IL-2 and lymphokine activated killer (LAK) cells has been used for treating the malignancies, when used in combination with chemotherapy/radiotherapy improved the survival in lung carcinoma cases

(Kimura & Yamaguchi, 1997). Another piece of evidence, reported for the adoptive transfer of TILs after chemotherapy as an advantageous approach for the complete cure of the ovarian cancer cases (Fujita et al., 1995). Adoptive immunotherapy has also been shown to be safe and potential treatment option for reducing the post-surgical recurrence and improving the recurrence free surgical outcomes in hepatocellular carcinoma cases (Takayama et al., 2000). Similarly, AIET with tumor-associated lymphocytes in combination with chemotherapy (cisplatin/5-fluorouracil) effectively prolonged the survival in stage IV gastric cancer cases (Kono et al., 2002). AIET has mostly been used for cancer management, it has also been reported in infectious diseases however, in majority of clinical situations, the host immune responses take the charge and resolve the infection, and therefore, AIET is generally not the preferred treatment choice. Though AIET cannot be the preferred option for viral disease management however, there are clinical scenarios (solid organ and BM transplantation) where complete ablation/suppression of host T-cell immunity is needed for preventing the allograft rejection. This increases the risk for reactivation of persistent viruses such as cytomegalovirus (CMV) and Epstein Barr virus (EBV), which can have life threatening consequences in patients' (Barnes & Stallard, 2001; Sivaraman & Lye, 2001). AIET has shown potential applications for controlling viral diseases, EBV-specific T-cells have shown an important role in the resolution of infection and long-term viral control. Adoptive transfer of T-cells in immunosuppressed patients restored the protective immune functions and polyclonal T-cells populations which eventually was proved beneficial for treating virus-induced lymphoproliferative syndromes (Reddehase et al., 1987; Riddell et al., 1992; Papadopoulos et al., 1994).

CD25 immunotoxin-based depletion of T-cells *ex vivo* and allo-depleted haplodeficient donor cells has served to provide momentum to the anti-viral immune responses in the recipients (Andre-Schmutz et al., 2002; Amrolia et al., 2006). AIET trial of infusion of adenoviral specific CD4+ and CD8+ T-cells in adenovirus infected children after hemopoietic stem cell therapy led to sustained decline in the viral loads and eventually resolution of infection (Feuchtinger et al., 2006). Therefore, AIET based therapy has now found widespread application in different diseases and opens the ways for application of similar strategies for other infectious diseases.

8.5.1 NON-SPECIFIC IMMUNOTHERAPY

Cytokines are essential regulators of the immune system that alters the properties of cells to determine the infection outcomes. These are produced by leukocytes and act other leukocytes; therefore, they are also referred to as the interleukins (ILs). Interferons are another class of signaling molecules which are released in response to invading pathogens and help in their elimination from the body. Interferons are named after their ability to interfere with the viral replication and fight against the pathogens (Nora et al., 1975). Cytokines have been developed as the protein therapies as a means to boost host immune responses and circumvent infections.

8.5.2 INTERFERONS

Cytokines are known modifiers of cellular proliferation and differentiation. The synergy and antagonism between different cytokines remain the key determinant of the immunological outcomes. The story of cytokines dates back to last century when interferons were discovered. Interferons are categorized into three classes: α-interferons, β-interferons, and γ-interferons. Interferon-α (IFN-α) and β are class I, i.e., virus-induced interferons, which shares several structural and biological properties and compete for binding to the same receptor. IFN-α are produced by leukocytes in response to viruses or double stranded RNA. It was one of the earliest cytokines, known to exert anti-tumor activity that was further explored in several pre-clinical and clinical trials during the 1980s and 1990s (Gresser & Bourali, 1970). There are two recombinant forms of human IFN-α that are approved by FDA (in 1995) for licensed use in clinics: IFN-α2a (Roferon-A or Sylatron) and IFN-α2b (Intron-A). IFN-α is the most commonly used interferons for the treatment of more than 14 different types of cancers, including hematological malignancies and solid tumors (as melanoma, Kaposi's sarcoma, etc.) (Pfeffer et al., 1998). Before the discovery of imatinib, IFN-α was the standard therapy for chronic myeloid leukemia (CML). Later research focused on improving the efficacy of imatinib by combining with pegylated-IFN-α, that yielded significant improvement in the treatment of the cancer cases (Simonsson et al., 2011; Johnson-Ansah et al., 2013; Talpaz et al., 2013).

Clinical trial for IFN-α2a significantly improved the survival rates of high-risk melanoma patients and was found to be beneficial as compared to ganglioside vaccine and it was also found effective for treating inoperable melanomas (Livingston et al., 1994; Creagan et al., 1995). The existing cancer treatment are associated with a high risk of relapses and mortality that accentuated the need for adjunctive therapy. High dose of IFN-α2b based therapy has also been tested in several clinical trials for improving the survival rates and promoting the relapse free survival in cancer cases (Livingston et al., 1994; Kirkwood et al., 1996). The high-dose treatment was associated with significant toxicity; therefore, low-dose interferon therapy was tested as an adjunctive option for treating high-risk melanoma cases. Unfortunately, the trial reported for no significant improvements in the relapse free survival rates of the melanoma cases (Pehamberger et al., 1998). Recombinant IFN-α has also been used for viral diseases; in HCV treatment, pegylated IFN-α was introduced in the clinics in 2001, that required a single weekly injection rather than thrice a week. Pegylated IFN-α was used for some time as the standard therapy for chronic HCV infection in combination with ribavirin (Zeuzem et al., 2003; Aghemo et al., 2010). Unfortunately, severe side effects associated with the therapy brought up other interferons as albumin IFN-α, IFN-λ and consensus IFN (CIFN) as potential options.

Similar to HCV, pegylated IFN-α2a was shown to be less successful for HBV with development of relapses in 5–10% of cases. Since the side effects can easily be managed, therefore, despite of the potential side effects, it remains the standard of care for the HBV infections (Huang et al., 2013; Wang et al., 2014).

Albumin IFN-α based phase III trial raised concerns regarding its safety which led to its failure to gain FDA approval (Balan et al., 2006; Zeuzem et al., 2010). CIFN is a recombinant protein that contains the common amino acids from different type I interferons. It has shown higher anti-viral ability than the conventional IFNs thus, it is required in low dose that overcomes the potential side effects associated with higher doses. Though useful in low doses, its short half-life, rapid absorption, and fast elimination from urine (similar to IFN-α) called upon for daily injections, however, successful phase III trials won it approval for preferred treatment option (Sjogren et al., 2007; Bacon et al., 2009).

Sub-cutaneous injections of IFN-α2b have also been used in combination with the standard lopinavir for management of novel coronavirus

infection (COVID-19) that shortened the hospitalization duration and accelerated the viral clearance and recovery in the cases (Pereda et al., 2020).

IFN-β is produced by fibroblasts and epithelial cells. It shares approximately 25% amino acid sequence homology with IFN-α. It is mostly used for the treatment of multiple sclerosis and as an anti-viral therapy (Ahn et al., 2009; Inoue et al., 2009). The efficacy of IFN-β alone or in combination with standard therapeutic regimen has been reported in HCV infection and for patients who are poorly responsive to IFN-α2b/ribavirin treatment alone (Ishikawa et al., 2012). Another study reported for the efficacy of two weeks of IFN-β/ribavirin treatment followed by pegylated IFN-α/ribavirin treatment that significantly reduced the viral loads as compared to the cases who were treated with pegylated IFN-α/ribavirin alone. Furthermore, IFN-β is known to lower the incidence of treatment-associated adverse events as compared to IFN-α alone (23173698). Immunotherapy aimed to block the type I interferon on inflammatory cells is in the early phase of development, likewise, type I IFN blocking antibody has been used in systemic lupus erythematosus (SLE) cases (Yao et al., 2009). Thus, IFN-α and IFN-β are prototypic immunotherapies that has remarkably improved the clinical outcomes in cancer cases due to their ability to control diverse cellular events. Recent research has elucidated their role as therapeutics however, further insight into their role in regulating the immunological outcomes is warranted.

The second class of cytokine, IFN-γ is structurally and functionally distinct from other interferons. It is produced by the T-cells activation, in response to mitogenic stimulation or non-specifically in response to IL-2. IFN-γ is one of the critical cytokine that determines the success of immunotherapy, in response to several immune checkpoint therapies IFN-γ and signature genes (HLA-DR, IDO1, STAT1, CXCL10, CD274, CXCL9) are the read-outs for the successful therapeutic outcomes in different cancers (Higgs et al., 2018; Karachaliou et al., 2018; Ni & Lu, 2018). The combination of checkpoint therapy with IFN-γ has shown to improve the efficacy of cancer immunotherapy. The combination of nivolumab or pembrolizumab along with IFN-γ (NCT02614456 and NCT 03063632) is still ongoing (Mojic et al., 2017).

IFN-γ has direct anti-tumor effects due to its anti-angiogenic, pro-apoptotic, and immune stimulatory abilities (Pujade-Lauraine et al., 1996; Wall et al., 2003; Marth et al., 2006). It has been used in combination with

chemotherapy to induce apoptosis in ovarian cancer cells and it has shown to improve the response rates (from 56% to 68%) and prolong the progression free survival rates in cases (Windbichler et al., 2000). It has also been under trial for its use as an adjuvant for vaccine and chemotherapies (NCT00428272, NCT00499772, NCT00824733, NCT00004016).

In a targeted clinical trial using IFN-γ in the form of aerosol for tuberculosis cases resulted in a marked decline in the mycobacterial burden in the sputum, increased body mass index (BMI) and reduced cavitary lesion sizes. IFN-γ based adjunctive therapy has shown to activate the signal transduction and gene expression in alveolar macrophages and improve the disease related clinical outcomes (Condos et al., 1997; Suarez-Mendez et al., 2004). In chronic HBV infection, IFN-γ1b alone has no impact on viral infection but exerts an immunomodulatory effect (Lau et al., 1991). Similar findings were reported for HCV infection (Muir et al., 2006), but the pre-treatment with IFN-γ prior to IFN-α treatment significantly enhanced immunological responsiveness in HCV cases which could be attributed to the interferon mediated enhanced viral clearance (Saez-Royuela et al., 1991; Katayama et al., 2001). Adeno-IFN-γ (non-replicating adenovirus with IFN-γ cDNA insert) has been tested in cutaneous lymphoma cases, after the success of phase I trial, the drug has now been tested under phase II trial (Dummer et al., 2004; Dummer, 2005).

Anti-IFN-γ based monoclonal antibody treatment (Fontolizumab and AMG 811) has also been used for management of multiple sclerosis (Skurkovich et al., 2001; Skurkovich & Skurkovich, 2003), inflammation associated with Crohn's disease (Hommes et al., 2006), rheumatoid arthritis (Sigidin et al., 2001) and lupus (Machold & Smolen, 1990; Theofilopoulos et al., 2001).

Usually, interferon therapy is an immunoregulatory strategy that affects a vast variety of immune cells, and their clinical application is yet to be explored for the management of different diseases.

8.5.3 INTERLEUKINS (ILS)

Recent advances in understanding the immune responses have encouraged newer strategies to come over the horizon for fighting against different diseases. Immunity against invading micro-organisms can be enhanced by inducing the expression of cytokines that stimulates the immune activation

and differentiation of immune cells to fight against invading microorganisms or suppressing the negative regulatory cytokines. Different recombinant interleukins (ILs) have been undergoing trials while others are under development for potentiating the immune responses by improving the antigen priming and orchestrating the pharmacological properties of the chemotherapy for better immunological outcomes.

IL-7 is an important cytokine in reinforcing the immune system, which can restore normal T-cells population that wanes naturally over the period of time. It can be used in patients who have undergone thymic involution (thymus becomes less efficient in T-cell generation), chemotherapy, HIV cases and BM transplantation. IL-7 is known trigger for the expansion of different T-cells and increases overall T-cell repertoire diversity (Sportes et al., 2008; Parrish et al., 2009; Levy et al., 2012). Unlike, naïve and memory cells, regulatory T-cells, effector memory cells and senescent T-cells minimally expand in response to the therapy emphasizing for the fact that IL-7 enhances the T-cells reactivity against chronic infections (Rosenberg et al., 2006). Increasing dose of recombinant IL-7 is well-tolerated, with non-significant adverse events associated with the therapy in the clinical trial (Rosenberg et al., 2006; Sportes et al., 2008, 2010). It has been tested in cancers, idiopathic lymphopenia, chronic viral infections, immunodeficiencies, and/or BM transplantation. It has also been tested as adjuvant for improving the vaccine, adoptive cell transfer therapies and chemotherapeutic efficacy (Mackall et al., 2011).

IL-2 is another crucial cytokine that has been widely used for different diseases including chronic myelogenous leukemia (CML) cases after HSCT, where low dose of IL-2 with Treg infusion significantly improved the clinical effects of HSCT (Pulster et al., 2009). IL-2 based therapy for the management of HCV related vasculitis was not associated with any adverse events and it significantly improved the vasculitis and attenuated the signatures associated with inflammation and oxidative stress (Saadoun et al., 2011). Administration of IL-2 has also raised promising results for the treating several forms of cancers (Chavez et al., 2009), graft-versus-host disease (GVHD) (Koreth et al., 2011; Kennedy-Nasser et al., 2014), SLE (Mizui & Tsokos, 2016; Humrich & Riemekasten, 2019) and type 1 diabetes (T1D) (Long et al., 2013; Dwyer et al., 2016).

IL-15, produced by myeloid cells has immune potentiating effects and has shown anti-tumor activity by inducing the expansion of NK cells and CD8+ T-cells in head and neck cancer, renal cell carcinoma (RCC) and

melanomas (Miller et al., 2018) however, the toxicity associated with the treatment led to its limited its use as a therapeutic agent. It has also been thought of as a possible alternative for rheumatic disorders and Kawasaki disease (McInnes & Gracie, 2004).

IL-21, relatively newcomer in the field of cytokine has also shown potential anti-tumor effects in early trials in humans. It has been tested across different cancer lines including RCC, melanoma, and breast cancer, adenocarcinoma, etc. Similar to the IL-15, it mediates the antitumor effects via NK cells (mediated by antibody dependent cell mediated cytotoxicity and cytokine production) and CD8+ T-cells. The effects were further enhanced when it was used in combination with monoclonal antibody therapy (as Rituxan) (Bhave & Carson, 2009).

IL-1β is another cytokine that has a crucial role to play in inflammatory disorders. Recombinant IL-1 receptor antagonist (Anakinra) has been an FDA approved drug for treating rheumatoid arthritis. It has been used for NOMID cases (where blockade of receptor helped in restoring hearing and vision) thus prevents organ specific damage and disability (Goldbach-Mansky, 2009).

Ustekinumab, anti-IL-12 monoclonal antibody has been used for inhibiting inflammatory cell infiltration and epidermal hyperplasia in psoriasis cases and has recently been approved by FDA. Apart from chronic psoriasis, it has also been used for treating the psoriatic arthritis and Crohn's disease (20074279). Systemic IL-12 administration in cancer cases led to systemic toxicity and remained fatal in some cases, thus, ruled its use in cancer treatment (Brunda et al., 1993; Jenks, 1996); however, recent use of nanogold delivery system has been used for improving the therapeutic index (Gasparri et al., 2019).

These are just to name a few; however, different cytokine immunotherapies have been used for treating different diseases and several other therapies are under pre-clinical and clinical trials.

8.5.4 RISKS OR SIDE EFFECTS ASSOCIATED WITH IMMUNOTHERAPY

Immunotherapy has been the best choice for certain diseases as cancer; however, potential risks associated with the therapy limits its usage. The time duration of manifestation of side effects varies but mostly the patients' complaints of potential problems in first weeks to months.

In case of checkpoint-based therapy, the overactive T-cells are the cause for the side effects seen as tiredness, dry and itchy skin, diarrhea, breathlessness, or dry cough due to lung inflammation. Usually, the side effects depend on the type of immunotherapy; top side effect associated with immunotherapy is inflammation and fatigue. Additionally, immunotherapies are associated with development of flu like symptoms including fever, chills, weakness, dizziness, nausea, sinus congestion, low/high blood pressure and fatigue. Conversely, pain, swelling, redness, itchiness, rash, diarrhea, heart palpitation, stuffy head and weight gain due to deposition of extra fluids in the different parts of the body can be observed in many treated cases. However, the symptoms subsides and/or ease up after the first treatment dose. The potential side effects associated with immunotherapy can also manifest after its usage in combination with other drugs leading to serious pathological outcomes including diabetes and inflammatory arthritis. Some immunotherapies have been to be associated with severe or even fatal allergic and inflammatory reactions however, the occurrence is rare. Serious outcomes can be controlled by using steroid and other treatment options that potentially helps in inhibiting the signals on cells that causes damage (Michot et al., 2016; Spain et al., 2016). Furthermore, potential side effects accentuate the need for research to avoid these issues.

8.5.5 MANAGEMENT OF SIDE EFFECTS OF IMMUNOTHERAPY

Early reporting of the potential side effects remains the key for its management, if left untreated the symptoms may get worse and even be life threatening in some cases. With proper treatment the side effects wanes in one to three weeks. However, it is important to get monitored for any symptoms of unwellness before self-medication. Usually, the side effects are characterized by the grading system on the scale of 1–4 that greatly facilitates its management, depending on the severity. Mild to severe side effects are graded in the range of 2 to 4, that can be managed with the steroids such as prednisone. The management of severe side effects (with a grading score of 3–4) relies on immediate hospitalization of the patient for intravenous steroid treatment/other medicines and immediately terminating the immunotherapy till the resolution/control of the side effects. Conversely, in case of the persistence of severe side effects, it is usually recommended to permanently terminate the immunotherapy. It is assumed

that the previous immunotherapy doses can "train the immune system" to recognize the cancer cells and fight against them. Despite the risks associated with severe side effects, most of the patients on immunotherapy experiences only mild side effects. Guidelines are available for managing the immunotherapy associated adverse events. This includes the laboratory tests for adrenocorticotrophic hormone (ACTH), cortisol, thyroid profiles, and testosterone in males to identify endocrine dysregulations. If hypophysitis is suspected, with headache and/or visual symptoms, then magnetic resonance imaging (MRI) scans are recommended. If adrenal anomalies (dehydration, hypotension) are suspected, then administration of stress-dose steroids and hormone replacement therapy is recommended in an inpatient setting along with a delay/discontinuation of therapy (Weber et al., 2016).

Dehydration, hypotension, and/or shock out of proportion to the current illness, administer stress-dose steroids in an inpatient setting, delay or discontinue treatment with anti-PD-1 therapy, and consider hormone-replacement therapy. Hypothyroidism can also be preceded by the signs and symptoms of hyperthyroidism over a period of days to weeks, which mimics the clinical presentation of Hashimoto's thyroiditis.

Guidelines are available on how to appropriately manage endocrine AEs following immune checkpoint inhibition. Laboratory testing for adrenocorticotropic hormone (ACTH), cortisol, T4, thyroid-stimulating hormone, and testosterone in males, may help identify endocrine dysfunction. If hypophysitis is suspected, especially with a headache or visual symptoms, consider a MRI scan of the brain with pituitary cuts and visual field testing. Treatment with immune checkpoint inhibition may continue with low-grade endocrine toxicity, although patients should be closely monitored.

8.6 CONCLUSION

The main challenge faced in the field of immunotherapy remains to be the low rates of participation for clinical trials, only 3% of cancer cases willingly participate while 40% of trials fails to achieve minimum number of enrollments for their trials. Secondly, the high cost (approx. 1 million per patient per year) of the treatment restricts its application in underdeveloped nations. Thirdly, the therapy is successful for a certain number of cases

while others fail to respond to the treatment; overall, the success rates of participation remain to be 15–20%. Unfortunately, it has still been an enigma to dissect who will respond to the therapy and who won't respond. Thus, these challenges call upon for much-needed effort for successfully establishing immunotherapy as a first-line treatment option.

KEYWORDS

- **adoptive cell transfer**
- **autologous immune enhancement therapy**
- **biological response modifiers**
- **cytomegalovirus**
- **hemagglutinin**
- **immunotherapy**
- **monoclonal antibody**

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