ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 September 20; 11(5): 231-277

Published by Baishideng Publishing Group Inc

World Journal of Methodology

Contents

Bimonthly Volume 11 Number 5 September 20, 2021

FRONTIER

REVIEW

SYSTEMATIC REVIEWS

World Journal of Methodology

Bimonthly Volume 11 Number 5 September 20, 2021

Contents

ABOUT COVER

Editorial Board Member of *World Journal of Methodology*, Tommaso Vincenzo Bartolotta, MD, PhD, Professor, Department of Biomedicine, Neurosciences and Advanced Diagnostics - Section of Radiology, University Hospital, via Del Vespro 129, Palermo 90127, Italy. tommasovincenzo.bartolotta@unipa.it

AIMS AND SCOPE

The primary aim of *World Journal of Methodology* (*WJM*, *World J Methodol*) is to provide scholars and readers from various fields of methodology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJM mainly publishes articles reporting research results obtained in the field of methodology and covering a wide range of topics including breath tests, cardiac imaging techniques, clinical laboratory techniques, diagnostic self-evaluation, cardiovascular diagnostic techniques, digestive system diagnostic techniques, endocrine diagnostic techniques, neurological diagnostic techniques, obstetrical and gynecological diagnostic techniques, ophthalmological diagnostic techniques, otological diagnostic techniques, radioisotope diagnostic techniques, respiratory system diagnostic techniques, surgical diagnostic techniques, *etc*.

INDEXING/ABSTRACTING

The *WJM* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
September 20, 2021

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION ETHICS
https://www.wjgnet.com/bpg/GerInfo/288

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 September 20; 11(5): 231-242

DOI: 10.5662/wjm.v11.i5.231

ISSN 2222-0682 (online)

FRONTIER

Genomics in medicine: A new era in medicine

Vishwanath Pattan, Rahul Kashyap, Vikas Bansal, Narsimha Candula, Thoyaja Koritala, Salim Surani

ORCID number: Vishwanath Pattan 0000-0002-7077-8331; Rahul Kashyap 0000-0002-4383-3411; Vikas Bansal 0000-0001-6047-5559; Narsimha Candula 0000-0002-5949-6974; Thoyaja Koritala 0000-0002-1020-9882; Salim Surani 0000-0001-7105-4266.

Author contributions: Pattan V, Kashyap R and Surani S were involved with idea origination, writing and review of manuscript; Bansal V, Candula N and Koritala T were involved in writing and review of literature.

Conflict-of-interest statement: None of the authors have any conflict of interest.

Manuscript source: Invited manuscript

Vishwanath Pattan, Division of Endocrinology, Wyoming Medical Center, Casper, WY 82601, United States

Rahul Kashyap, Vikas Bansal, Department of Anesthesiology and Peri-operative Medicine, Mayo Clinic, Rochester, MN 55905, United States

Narsimha Candula, Hospital Medicine, University Florida Health, Jacksonville, FL 32209, United States

Thoyaja Koritala, Hospital Medicine, Mayo Clinic Health System, Mankato, MN 56001, United States

Salim Surani, Department of Internal Medicine, Texas A&M University, Corpus Christi, TX 78405, United States

Corresponding author: Salim Surani, FACC, FACP, FCCP, MD, Professor, Department of Internal Medicine, Texas A&M University, 701 Ayers Street, Corpus Christi, TX 78405, United States. srsurani@gmail.com

Abstract

The sequencing of complete human genome revolutionized the genomic medicine. However, the complex interplay of gene-environment-lifestyle and influence of non-coding genomic regions on human health remain largely unexplored. Genomic medicine has great potential for diagnoses or disease prediction, disease prevention and, targeted treatment. However, many of the promising tools of genomic medicine are still in their infancy and their application may be limited because of the limited knowledge we have that precludes its use in many clinical settings. In this review article, we have reviewed the evolution of genomic methodologies/tools, their limitations, and scope, for current and future clinical application.

Key Words: Genomic medicine; Medical genetics; Gene sequencing; DNA sequencing; RNA sequencing; Clustered regularly interspaced short palindromic repeat; Gene based therapy; Genomic tools; Genome editing

Core Tip: The field of Genomics is the future of medicine, as evidenced by the

Specialty type: Medical laboratory technology

Country/Territory of origin: United States

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 12, 2021
Peer-review started: January 12, 2021
First decision: June 17, 2021
Revised: June 18, 2021
Accepted: July 19, 2021
Article in press: July 19, 2021
Published online: September 20, 2021

P-Reviewer: Taheri S
S-Editor: Gao CC
L-Editor: A
P-Editor: Guo X

unprecedented research and clinical application which pushed the time boundaries for the coronavirus disease 2019 mRNA vaccines. However the path to unleashing the potential from genomic tools is far from perfect. A thorough research with international collaboration and cooperation is a necessity and the need of the hour.

Citation: Pattan V, Kashyap R, Bansal V, Candula N, Koritala T, Surani S. Genomics in medicine: A new era in medicine. *World J Methodol* 2021; 11(5): 231-242
URL: https://www.wjgnet.com/2222-0682/full/v11/i5/231.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i5.231

INTRODUCTION

Understanding the human genome has come a long way since the initial discovery of DNA structure by Watson and Crick in 1953[1]. The genome study and reference used to be a very specialized area, but lately with the advent of the messenger based RNA vaccine have brought the concept of genetics even to the lay public. In the 1970s, the ability to manipulate DNA with recombinant DNA technology increased the horizon. Our understanding of medical genetics began with inheritance patterns of single-gene diseases. The database of Mendelian Inheritance in Man (MIM) was initiated in the early 1960s by McKusick[2]. As of January 5, 2021, 4368 genes were mapped to phenotype-causing mutations[3]. However, only a small portion of diseases have a monogenic cause. The majority of the common diseases are polygenic, and elucidation of their mechanism has remained elusive.

The human genome project, which was completed in 2003, revolutionized the understanding of the human genome and served as a turning point to fast forward the genomic methodologies. However, the clinical application of findings from these genomic studies is still in its infancy. This is largely because we still have not understood or made complete sense of the available information. That is, the sequence data have been difficult to correlate to functional outcomes, making it difficult to understand the genetic basis of diseases and the complex gene-lifestyle-environment influences or their interaction. Moreover, most of the initial focus of the research had been on coding regions of DNA which comprises approximately 2% of the DNA and the knowledge about specific implications of non-coding DNA regions (98% of DNA) are largely unknown[4,5].

Remarkably, the human genome and the closest related species chimpanzees differ in single nucleotide alterations by a mere 1.23% and in deletions, insertions, and copy number variations by 3%[6]. In humans, the genomes of any two individuals are about 99.9% identical. However, a mere 0.1% variation allows for changes in a massive number of nucleotides because the human genome has approximately 30 billion base pairs (3.3×10^9)[7].

In this review, we will discuss the evolution in genomic methodology, limitations, and their scope for current and future clinical application.

GENOMIC TOOLS AND THEIR EVOLUTION

DNA sequencing

After the initial DNA sequencing method by Maxam and Gilbert[8] in 1977, the chain-termination DNA sequencing method developed by Sanger *et al*[9] in 1977 was used for the next few decades. It relied on the template DNA strand and had limited capacity for sequencing gene panels. Subsequently, with commercial production of high throughput technologies or next-generation sequencing (NGS) revolutionized the DNA sequencing by 2007[10]. Also called as massively parallel sequencing, NGS does parallel sequencing of millions of small DNA fragments. Each DNA fragment is fixed at a unique location on the solid support. While the sample of the patient's DNA which serves as a template in NGS is amplified and fragmented, the third-generation sequencing uses single DNA molecules rather than the amplified DNA as a template thus eliminating errors from DNA amplification processes. The NGS can be used for whole-genome sequencing, exome sequencing, or targeted gene panels comprising

Table 1 Characteristics of commonly used genomic tools

Tools for genomics	Principle of use	Pros and application	Limitation
Genome-wide association studies (GWAS)	Gene mapping study using DNA microarray to identify the association between SNP and specific risk alleles that are more prevalent in cases than in controls, *via* linkage disequilibrium	Has potential for population-based application. Example — The Severe COVID-19 GWAS Group[34] studied patients with respiratory failure from severe COVID-19 and narrowed down the genetic susceptibility locus to a gene cluster on chromosome locus 3p21.31. They also verified the potential involvement of the ABO blood group system	Does not establish causality but only an association with SNP; Missing heritability- cannot explain variance in complex traits or genes with a small effect size; Does not account for epigenetic changes and epistasis (gene-gene interaction); GWAS data catalog mostly from individuals of European descent which may limit application in minority population [35]
Expression quantitative trait loci (eQTL) analysis	Links SNPs to changes in gene expression by measuring the expression of many genes simultaneously in microarrays. Helps to narrow down to SNPs more likely to impact the disease condition	Provides better insight into specific causal mechanisms[36]; Liver eQTL — useful in pharmacogenomic studies by analyzing Epistatic eQTL Interactions [37]	Limited tissue interrogation will give misleading biological interpretations about the gene mediating the regulatory effect to increase disease risk[38]
Deep sequencing or Next-generation sequencing	**Exome sequencing:** 85% of known disease-causing mutations in Mendelian disorders are found in exons. Exome sequencing is a useful tool to find the causal genes for Mendelian disorders	Reduced cost and limited data to interpret; Linkage study design is unsuitable for extremely rare and sporadic Mendelian disorders for which exome sequencing would be more practical[39]	**Exome sequencing**: It can miss pathogenic variants in a non-coding region. Repetitive regions (*e.g.*, pseudogenes) can confound results in whole-exome sequencing[41]; Potentiate technical biases regarding exon capture limiting its use in detecting copy-number variants as well as in genomic regions where capture is less efficient[42]
	Whole-genome sequencing: Can sequence every nucleotide base in the human genome (approximately 3.3×10^9 base pairs)	**Whole-genome sequencing**: Avoids inherent biases of exome capture	**Whole-genome sequencing**: Too much data but little clinical knowledge available to interpret; Higher cost compared to clinical utility
	Targeted gene panel: Provides information on prespecified disease-associated genes	**Examples:** Rapid whole-genome sequencing to investigate extensively drug-resistant (XDR) tuberculosis[40]	
RNA-seq	Uses NGS to analyze RNA expression patterns or transcriptome profiling by reverse transcription of RNA sample to complementary DNAs (cDNA) and PCR amplification	**Can be used:** to analyze RNA expression profile at single cell level or quantify gene expression[43]; to obtain data on novel transcripts and is not limited by availability of reference genome data[44]; to identify alternatively spliced genes; to detect allele-specific gene expression[44]	cDNA synthesis and PCR amplification steps can introduce bias and errors[44]
Epigenomics	Epigenomics involves methods used to identify DNA methylation and histone modifications. Sodium bisulfite can identify unmethylated cytosines due to its ability to convert unmethylated cytosines to uracil. However the methylated cytosine is resistant to this conversion. Methylation-dependent restriction enzymes are used for DNA methylation analysis[45]. Chromatin immunoprecipitation (ChIP) is used for the investigation of histone modifications	ChIP allows precise mapping of the DNA-protein interaction in living cells. Cross-linked protein-DNA complex can be treated with exonucleases to remove cross-linked DNA sequences that are not avidly bound to protein of interest. This is called ChIP-Exo. This allows mapping of *in vivo* protein occupancy at single nucleotide-level resolution[47]	Needs design of antibodies specific to DNA-bound protein of interest which could be modified histone or transcription factors
	Immunoprecipitation techniques: ChIP on Chip; ChIP-Seq. Chromatin is isolated from the sample and the DNA involved in DNA protein cross-linked complex is isolated using antibodies specific to the DNA-bound protein. The isolated DNA is amplified using PCR and analyzed using gel electrophoresis imaging, microarray hybridization (ChIP-chip), or direct sequencing with NGS (ChIP-Seq)[46]		
Transcriptomics	**Northern blot:** RNA molecules separated by gel electrophoresis by size and subsequently hybridized with labeled complementary ssDNA and detected using chemic luminescence or autoradiography	Northern blot can both quantify the amount of RNA and also determine the size of mRNA transcript. Can detect transcript variant of genes[49]	Northern blot-need radioactive probes and has lower sensitivity
	Ribonuclease (RNase) protection assay: Differs from northern blot by use of antisense RNA probes	**RNase protection assay:** It can simultaneously detect and quantify	**RNase protection assay:** Does not provide information on transcript

called riboprobes	multiple mRNA targets in a single RNA sample .It has high sensitivity	size[52]
Real-time RT-PCR: cDNA are synthesized by reverse transcription from the sample RNA identified. The resulting cDNA is amplified by using fluorescently labeled oligonucleotide primers. Fluorescence intensity is monitored and correlated with several PCR cycles	**Real-time RT-PCR:** Allows quantitative genotyping, detection of SNPs and allelic variants or genetic variations even when mutation is found in very small fraction of cells in the sample. Has become clinical standard for diagnoses in Infectious diseases and it's role is evolving rapidly in cancer diagnostics [50]	**Real-time RT-PCR:** The process is complex and any errors in choice of reagents, primers or probes will affect accuracy. There could be risk for errors during data analysis and reporting. The process is expensive [53]
In situ hybridization: Tissue specimen is fixed to preserve morphology and then treated with proteases. A labeled probe is hybridized to the sample and detected using chemiluminescence or autoradiography[48]	**In situ hybridization:** Very useful in diagnostic application when there is limited tissue sample (in embryos and biopsy specimen). Several specific hybridizations can be done on the same sample. Tissue samples can be freeze for future use[48]	**In situ hybridization:** Low diagnostic yield when the sample has low DNA and RNA copies[48]
Spotted DNA arrays: Measures relative expression levels between 2 samples. cDNA probes amplified by PCR are spotted on a glass slide and then mRNAs are isolated from the samples. The mRNA from each sample is labeled with different fluorescent dyes. The samples are mixed, co-hybridized with cDNA probes on glass slides to measure relative gene expression	**Spotted DNA arrays:** The major application of DNA array is measurement of gene expression levels [51]	**Spotted DNA arrays:** DNA array can only detect known sequences, that were used to construct the array. It only gives relative estimate of gene expression and not reliable for absolute quantification. When the genome has multiple related sequences then design of array that distinguishes these sequences is challenging. Difficult to reproduce the array[51]

SNP: Single nucleotide polymorphism; NGS: Next-generation sequencing; PCR: Polymerase chain reaction; RT-PCR: Real-time reverse transcription polymerase chain reaction; ssDNA: Single stranded DNA.

tens to hundreds of genes.

Single nucleotide polymorphism

Single nucleotide polymorphism (SNP) is the variation in genetic sequence by a single nucleotide. It is the most common type of genetic variation in man[11]. It was detected in the 1980s using restriction enzymes[12]. With application of the microarray technology to SNPs, the scope of SNP in clinical practice has widened, especially in oncology. The first SNP array analysis was done in 1998 and the first application of SNP array analysis in cancer was done in 2000[13]. SNP array analysis is used to determine loss of heterozygosity, allelic imbalance, genomic copy number changes, frequency of homozygous chromosome regions, uniparental disomy, DNA methylation alterations and linkage analysis of DNA polymorphisms in cancer cells [13,14].

DNA amplification

Kary Banks Mullis successfully demonstrated polymerase chain reaction (PCR) in 1983 [15]. PCR is a cost-effective method that can amplify a single DNA exponentially[16]. It is a rapid, highly specific, and extremely sensitive method. PCR is being used in SNP genotyping, detection of rare sequences, insertion-deletion variants, and structural variants like copy-number variants.

Linkage and association analysis

Linkage studies have been used for mapping of genes for heritable traits to their chromosomal locations. 1st genetic linkage map was done in 1911 by Sturtevant A[17]. Parametric linkage analysis is used to map the disease-causing gene for monogenic diseases. Here, the logarithm of the odds (LOD) scores and recombination fractions are used to map the gene location. Model-free linkage analysis or non-parametric linkage analysis is used for complex or polygenic diseases, or when the model of inheritance is not known[18]. Linkage analysis of the whole genome can identify large regions of the chromosome with evidence of disease containing the gene[19,20], but this large span of chromosomes can have hundreds of candidate genes.

Linkage studies have been used for mapping Mendelian traits with high penetrance in families and relatives[20]. They are especially useful to identify rare alleles that are present in a small number of families[21], for disease genes with weak effects and polygenic diseases, linkage disequilibrium association mapping has proved to be more

useful. In genome-wide association studies (GWAS), genotyping of hundreds or thousands of SNPs is done in cases and control populations and their association with heritability is analyzed. A combination of linkage and association methodologies helps to identify and characterize the wider range of disease-susceptibility variants[22].

Fluorescence in Situ Hybridization (FISH) was developed in 1987. It is a cytogenetic technique which uses fluorescent DNA probes which are designed to label precise chromosomal locations. The advantage of FISH over conventional cytogenetic metaphase karyotype analysis is lack of cell culture requirement. It can rapidly evaluate interphase nuclei in the fresh or paraffin-embedded sample[23]. However, the resolution of this technique is only as good as that of karyotype bands. Cloned DNA FISH probes of about 100 kb, called bacterial artificial chromosomes, are now available. FISH is being utilized more in making clinical diagnosis among Oncology due to its simplicity and reliability to evaluate the key biomarkers in various malignancies.

Comparative genomic hybridization

Comparative genomic hybridization (CGH) was developed in 1992. CGH can detect DNA copy number changes across the entire genome of a patient sample in a single experiment. It compares the hybridization signal intensity of a test sample (for example tumor sample) against a reference sample along the chromosomes[13].

HAPMAP AND 1000 GENOME PROJECTS HAVE CREATED A CATALOG OF SNPS

The HapMap project was started in 2002 to develop a haplotype map of the human genome. It can also describe the common patterns of human genetic variation[24]. The 1000 Genomes Project comprised a total of 26 diverse population set in which whole-genome sequencing was performed. It also used deep exome sequencing and dense microarray genotyping to give a comprehensive description of common human genetic variation[25].

TARGETED GENOME EDITING OR GENOME ENGINEERING

It involves modification of the genome at a precise, prespecified locus using programmable nucleases. Examples of some of the programmable nucleases include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas (CRISPR-associated) system. These programmable nucleases are designed to impart site-specific double-strand breaks (dsBs) in chromosomal DNA. The cell is therefore forced to use one of the endogenous DNA repair mechanisms — homologous recombination or homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). This enables targeted genetic modifications during the repair process in the living cells (*in vivo*) (Table 1)[26]. ZFNs and TALENS recognize the target sequence through protein-DNA interaction. CRISPR-Cas nucleases recognize target sequences through RNA and DNA base pairing[26].

In the year 2013, Cong *et al*[27] and Mali *et al*[28] showed successful genome editing in mammalian cells using the CRISPR system. In the last 5 years, we have seen a leap in the research interest (both animal and human) in CRISPR genomic editing.

While genome editing holds promise to correct the defective genome in vivo, therapies can also be designed to alter the gene expression without altering the genomic code. For example, anti-sense oligonucleotide can be used to alter the splice points of pre-mRNA to correct for a defective gene or suppress its expression. Examples of drugs which use splice modulation and approved by Food and Drug Administration (FDA) are Eteplirsen (exon skipping, approved for Duchenne muscular dystrophy) and nusinersen (exon inclusion, approved for spinal muscular atrophy)[29].

Table 1 summarizes the commonly used genomic tools, their working principle, advantages/applications and limitations (see Table 1). Table 2 summarizes the major genome/gene editing tools their working principle, advantages/applications and limitations. Table 3 summarizes gene-based therapies that are either FDA approved therapies or investigational therapies showing promise.

Table 2 Characteristics of genome-editing technologies using programmable nucleases

Gene editing	Principle of use	Advantages or application	Limitation
CRISPR-Cas9 guided gene editing: (1)NHEJ; and (2)HDR	Cas9 enzyme (an endonuclease) cleaves ds- DNA at a specific site as determined by the specific sequence of the guide RNA. Genome editing is done when the cell tries to repair the dsB (either *via* NHEJ or HDR)	Has the potential to edit genes in almost any cell type *in vivo*; Has potential in every field, notably infections[54], genetic disease [55], cancer[56] *etc.*; CRISPR-Cas9 can also be used for large scale loss-of-function gene screen: Catalytically inactive Cas9 (dCas9) can be directed by guide RNA, bind to specific genes to reversibly suppress or activate gene transcription (by fusion of transcription activators or suppressors with dCas9)[57]; Epigenetic modulators (*e.g.*, DNA methylase) can also be fused with dCas9 to achieve controlled epigenetic modulations. Cas-9 NHEJ is simpler and efficient; Cas-9 HDR is more precise but lower efficiency than NHEJ. The mutant version of the Cas9 called Cas9 nickase can be used to minimize the risk of off-targets	The off-target activity of RNA-guided endonuclease-induced mutations[58]. Off-target mutations with a frequency below 0.5% cannot be detected by current off-target detection techniques[59]
Augmented CRISPR-Cas12a system	Cas12a cuts target ds- DNA. However, unlike Cas9, Cas12a subsequently becomes activated and causes indiscriminate cleavage of ssDNA causing collateral damage. SARS-CoV-2 RNA DETECTR Assay: samples from upper airway swabs are processed using simultaneous reverse transcription and isothermal amplification with loop-mediated amplification (RT-LAMP). Subsequently the Cas12 enzyme is added	CRISPR-Cas12a system can be used to create new drug or cell delivery systems and bio-sensing (*e.g.*, to detect methicillin-resistant Staphylococcus aureus, Ebola virus[60]. Emergency Use Authorization (EUA) Only for qualitative detection of nucleic acid from the SARS-CoV-2 in upper respiratory specimens[61,62]	Limited research data and application. The technology is still in its infancy
CRISPR-Cas 13	CRISPR-Cas 13 system can be used *via* SHERLOCK technique for ultra-sensitive detection of RNA or DNA from the clinical samples	SherlockTM CRISPR SARS-CoV-2 kit: Emergency Use Authorization (EUA) qualitative for detection of nucleic acid fromSARS-CoV-2 in upper respiratory specimens[63,64]	
Prime editors	It uses a catalytically impaired Cas9 which is fused to an engineered reverse transcriptase and prime editing guide RNA. The guide RNA specifies the target site and encodes the desired sequence	Prime editing is associated with fewer off-target edits when compared with conventional CRISPR-Cas system[65]. Anzalone *et al*[66] applied prime editing in human cells to correct the primary genetic causes of sickle cell disease and Tay-Sachs disease. It does not require double-strand breaks or donor DNA templates	Research literature on application of prime editing is limited. Unlike conventional CRISPR-Cas system prime editing may not be able to provide large DNA insertions or deletions[65]
Zinc finger nucleases	Zinc finger nuclease (dimer of zinc finger hybrid bound to restriction endonuclease) is a programmable nuclease that cleaves specific sites in DNA. They recognize the target sequence through protein-DNA interaction	Potential for plant genome editing for crop improvement[67]	Necessity to engineer novel proteins for each target site: Expensive; Difficult to reproduce
TALENS	TAL proteins have TAL effector DNA-binding domain fused to a DNA cleavage domain. TALENs create dsBs that require repair by NHEJ or HDR	The DNA-binding specificity of TALEs is easier to engineer than zinc-fingerProteins[68]	Necessity to engineer novel proteins for each target site. TALENs are large and pose packaging challenge in viral delivery systems[69]

HDR: Homology-directed repair; NHEJ: Nonhomologous end-joining; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TALENs: Transcription activator-like effector nucleases; dsBs: Double stranded breaks; ssDNA: Single stranded DNA; TAL: Transcription activator-like; SHERLOCK: Specific High Sensitivity Enzymatic Reporter UnLOCKing.

DISCUSSION

The newer genomic technology and tools have broadened the scope and pushed the time limits for development of new diagnostic kits, preventive strategies like vaccines, therapeutic strategies like gene modulation and gene therapy. A lot is yet to be studied in terms of the complex interaction of gene-environment-lifestyle-disease. Knowing the impact of genomics on disease pathophysiology and response to medications[30]. expands the scope of research and clinical application. While genome editing holds promise to correct the defective genome in vivo, therapies can also be designed to alter the gene expression without altering the genomic code (example exon skipping, or inclusion discussed above).

The newer genomic editing tools have showed great potential and promise but they need to be studied extensively before clinical application. Also, uniform international ethical guidelines and guiding principles need to be established so that these genomic technologies are not misused.

Table 3 Gene based therapies: List of Food and Drug Administration approved therapies and investigational therapies showing promise

Therapy or drug	Indication	Mechanism of action	Approval status
Janssen COVID-19 vaccine	Prevention of 2019 coronavirus disease (COVID-19) for individuals 18 yr of age and older	Recombinant, humanadenovirus type 26 vector which expresses the SARS-CoV-2 "S" antigen after entering human cells thus eliciting immune response against COVID-19	Emergency use authorization (EUA) on February 27, 2021[70]. Pause placed on vaccine use on April 13, 2021[71]. FDA lifted vaccination pause on April 23, 2021[72]
Pfizer-BioNTech COVID-19 Vaccine [73-75]	Prevention of COVID-19 for individuals 16 yr of age and older	modRNA forumated in lipid particles when delivered to host cells express SARS-CoV-2 "S" antigen, thus eliciting immune response against COVID-19	EUA on December 11, 2020
Moderna COVID-19 vaccine[76-78]	Prevention of COVID-19 for individuals 18 yr of age and older	modRNA forumated in lipid particles when delivered to host cells express SARS-CoV-2 "S" antigen, thus eliciting immune response against COVID-19	EUA on December 18, 2020
Lumasiran[79]	Primary hyperoxaluria type 1	HAO1-directed small interfering ribonucleic acid	Approved in Nov 2020
Viltolarsen[80]	Duchenne muscular dystrophy	Antisense oligonucleotide directed to exon 53 skipping	Approved in August 2020
Brexucabtagene autoleucel[81]	Relapsed/refractory mantle cell lymphoma	Genetically modified autologous CD19 T cells directed against CD19 expressing cancer cells	Approved in July 2020
Golodirsen[82]	Duchenne muscular dystrophy	Antisense oligonucleotide directed	Approved in December 2019
Givosiran[83]	Acute hepatic porphyria	Double-stranded small interfering RNA that degrades the ALAS1 mRNA in hepatocytes *via* RNA interference	Approved in November 2019
Onasemnogene abeparvovec-xioi[84]	Spinal muscular atrophy (SMA)	AAV9-based gene therapy which encodes the human SMN protein	Approved in May 2019
Inotersen[85]	Polyneuropathy of hereditary transthyretin-mediated amyloidosis	Transthyretin-directed antisense oligonucleotide	Approved in October 2018
Axicabtagene ciloleucel[86]	Relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy	Genetically modified autologous CD19 T cells directed against CD19 expressing cancer cells	Approved in October 2017
Tisagenlecleucel[87]	Refractory or relapsed B-cell precursor acute lymphoblastic leukemia (ALL)	Genetically modified autologous CD19 T cells directed against CD19 expressing cancer cells	Approved in August 2017
Nusinersen[88]	SMA	Survival motor neuron-2 (SMN2)-directed antisense oligonucleotide	Approved in December 2016
Eteplirsen[89]	Duchenne muscular dystrophy	Antisense oligonucleotid that binds to exon 51 of dystrophin pre-mRNA	Approved in September 2016
Talimogene laherparepvec[90]	Genetically modified herpes simplex virus, type 1 used as oncolytic viral therapy	They utilized the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with melanoma who had the recurrence after the initial surgery	Approved in October 2015
Giroctocogene fitelparvovec[91]	Moderately severe to severe hemophilia A	Factor VIII gene delivery using recombinant adeno-associated viruses as vectors	Investigational in phase 3 trial
Inclisiran[92]	Heterozygous and possibly homozygous familial hypercholesterolemia	Small-interfering ribonucleic acid which decreases hepatic production of PCSK9	Investigational phase 3 trial
Volanesorsen[93]	Familial chylomicronemia syndrome	Antisense oligonucleotide that targets the messenger RNA for apo-CIII	Conditional approval by European Medicines Agency's (EMA) but not by FDA
CRISPR-Cas9 gene editing[94]	Sickle cell disease and β-thalassemia	CRISPR-Cas9based allele editing of the BCL11A erythroid-specific enhancer in autologous CD34+ cells	Investigational- FDA Fast Track Designation for CTX001 in sickle cell disease

AAV: Adeno-associated virus; ALAS1: Aminolevulinate synthase 1; BCL11A: B cell lymphoma/leukemia 11A; HAO1: Hydroxyacid oxidase (glycolate oxidase) 1; modRNA: Nucleoside-modified messenger RNA; SMN: Survival motor neuron 1; FDA: Food and Drug Administration.

It is very important to include diverse populations and to represent minority population in the genomic studies, so that results could be generalized and more accurate diagnostic, predictive and therapeutic tools can be developed.

Genomics in medicine is indeed a new era in medicine. Even the control of coronavirus disease 2019 pandemic[31] has just begun at the time of writing of this article with gene based therapies eliciting immune response against severe acute respiratory syndrome coronavirus 2 spike proteins. A unified international collaboration[32,33] is needed to continue expanding gene therapy use in opening new frontiers for fight against novel infections and disease.

CONCLUSION

Genomic medicine holds great promise for providing insight into disease pathophysiology, provide better diagnostic or disease predictive tools, preventive therapies and finally for targeted treatment of diseases. Although some of the newer tools (like CRISPR system) have great potential, more research is needed before these tools can be unleashed to clinical use. Hence there is great need for studies to unravel the mystery of complex interaction of both coding and noncoding genomic regions with environment and lifestyle influences on disease occurrence and management.

REFERENCES

1 **Watson JD**, Crick FH. The structure of DNA. *Cold Spring Harb Symp Quant Biol* 1953; **18**: 123-131 [PMID: 13168976 DOI: 10.1101/sqb.1953.018.01.020]
2 **McKusick VA**. Mendelian Inheritance in Man and its online version, OMIM. *Am J Hum Genet* 2007; **80**: 588-604 [PMID: 17357067 DOI: 10.1086/514346]
3 **Johns Hopkins University**. OMIM Gene Map Statistics. [cited 20 December 2020]. In: Johns Hopkins University [Internet]. Available from: https://www.omim.org/statistics/geneMap
4 **Ling H**, Vincent K, Pichler M, Fodde R, Berindan-Neagoe I, Slack FJ, Calin GA. Junk DNA and the long non-coding RNA twist in cancer genetics. *Oncogene* 2015; **34**: 5003-5011 [PMID: 25619839 DOI: 10.1038/onc.2014.456]
5 **Gloss BS**, Dinger ME. Realizing the significance of noncoding functionality in clinical genomics. *Exp Mol Med* 2018; **50**: 1-8 [PMID: 30089779 DOI: 10.1038/s12276-018-0087-0]
6 **Suntsova MV**, Buzdin AA. Differences between human and chimpanzee genomes and their implications in gene expression, protein functions and biochemical properties of the two species. *BMC Genomics* 2020; **21**: 535 [PMID: 32912141 DOI: 10.1186/s12864-020-06962-8]
7 **Gyles C**. The DNA revolution. *Can Vet J* 2008; **49**: 745-746 [PMID: 18978969]
8 **Maxam AM**, Gilbert W. A new method for sequencing DNA. *Proc Natl Acad Sci U S A* 1977; **74**: 560-564 [PMID: 265521 DOI: 10.1073/pnas.74.2.560]
9 **Sanger F**, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977; **74**: 5463-5467 [PMID: 271968 DOI: 10.1073/pnas.74.12.5463]
10 **Reuter JA**, Spacek DV, Snyder MP. High-throughput sequencing technologies. *Mol Cell* 2015; **58**: 586-597 [PMID: 26000844 DOI: 10.1016/j.molcel.2015.05.004]
11 **Shen LX**, Basilion JP, Stanton VP Jr. Single-nucleotide polymorphisms can cause different structural folds of mRNA. *Proc Natl Acad Sci U S A* 1999; **96**: 7871-7876 [PMID: 10393914 DOI: 10.1073/pnas.96.14.7871]
12 **Gray IC**, Campbell DA, Spurr NK. Single nucleotide polymorphisms as tools in human genetics. *Hum Mol Genet* 2000; **9**: 2403-2408 [PMID: 11005795 DOI: 10.1093/hmg/9.16.2403]
13 **Mao X**, Young BD, Lu YJ. The application of single nucleotide polymorphism microarrays in cancer research. *Curr Genomics* 2007; **8**: 219-228 [PMID: 18645599 DOI: 10.2174/138920207781386924]
14 **Sato-Otsubo A**, Sanada M, Ogawa S. Single-nucleotide polymorphism array karyotyping in clinical practice: where, when, and how? *Semin Oncol* 2012; **39**: 13-25 [PMID: 22289488 DOI: 10.1053/j.seminoncol.2011.11.010]
15 **Fairfax MR**, Salimnia H. Diagnostic molecular microbiology: a 2013 snapshot. *Clin Lab Med* 2013; **33**: 787-803 [PMID: 24267186 DOI: 10.1016/j.cll.2013.08.003]
16 **Wages JM**. Polymerase Chain Reaction. In: Worsfold P, Townshend A, Poole C. Encyclopedia of Analytical Science. Oxford: Elsevier, 2005: 243-250
17 **Griffiths AJ**, Miller JH, Suzuki DT, Lewontin RC, Gelbart WM. An Introduction to Genetic Analysis - NCBI Bookshelf. 7th ed. New York: W. H. Freeman, 2000
18 **Dawn Teare M**, Barrett JH. Genetic linkage studies. *Lancet* 2005; **366**: 1036-1044 [PMID: 16168786 DOI: 10.1016/S0140-6736(05)67382-5]
19 **Ott J**, Wang J, Leal SM. Genetic linkage analysis in the age of whole-genome sequencing. *Nat Rev Genet* 2015; **16**: 275-284 [PMID: 25824869 DOI: 10.1038/nrg3908]
20 **Pulst SM**. Genetic linkage analysis. *Arch Neurol* 1999; **56**: 667-672 [PMID: 10369304 DOI: 10.1001/archneur.56.6.667]
21 **Hinrichs AL**, Suarez BK. Incorporating linkage information into a common disease/rare variant framework. *Genet Epidemiol* 2011; **35** Suppl 1: S74-S79 [PMID: 22128063 DOI: 10.1002/gepi.20654]

22 **Ott J**, Kamatani Y, Lathrop M. Family-based designs for genome-wide association studies. *Nat Rev Genet* 2011; **12**: 465-474 [PMID: 21629274 DOI: 10.1038/nrg2989]

23 **Hu L**, Ru K, Zhang L, Huang Y, Zhu X, Liu H, Zetterberg A, Cheng T, Miao W. Fluorescence in situ hybridization (FISH): an increasingly demanded tool for biomarker research and personalized medicine. *Biomark Res* 2014; **2**: 3 [PMID: 24499728 DOI: 10.1186/2050-7771-2-3]

24 **National Human Genome Research Institute**. The International HapMap Project. Updated 06/04/2012. [cited 10 December 2020]. In: National Human Genome Research Institute [Internet]. Available from: https://www.genome.gov/11511175/about-the-international-hapmap-project-fact-sheet

25 **1000 Genomes Project Consortium**, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. A global reference for human genetic variation. *Nature* 2015; **526**: 68-74 [PMID: 26432245 DOI: 10.1038/nature15393]

26 **Kim H**, Kim JS. A guide to genome engineering with programmable nucleases. *Nat Rev Genet* 2014; **15**: 321-334 [PMID: 24690881 DOI: 10.1038/nrg3686]

27 **Cong L**, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; **339**: 819-823 [PMID: 23287718 DOI: 10.1126/science.1231143]

28 **Mali P**, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering *via* Cas9. *Science* 2013; **339**: 823-826 [PMID: 23287722 DOI: 10.1126/science.1232033]

29 **Lim KRQ**, Yokota T. Invention and Early History of Exon Skipping and Splice Modulation. *Methods Mol Biol* 2018; **1828**: 3-30 [PMID: 30171532 DOI: 10.1007/978-1-4939-8651-4_1]

30 **Pattan V**, Seth S, Jehangir W, Bhargava B, Maulik SK. Effect of Atorvastatin and Pioglitazone on Plasma Levels of Adhesion Molecules in Non-Diabetic Patients With Hypertension or Stable Angina or Both. *J Clin Med Res* 2015; **7**: 613-619 [PMID: 26124907 DOI: 10.14740/jocmr2178e]

31 **Shah A**, Kashyap R, Tosh P, Sampathkumar P, O'Horo JC. Guide to Understanding the 2019 Novel Coronavirus. *Mayo Clin Proc* 2020; **95**: 646-652 [PMID: 32122636 DOI: 10.1016/j.mayocp.2020.02.003]

32 **Walkey AJ**, Kumar VK, Harhay MO, Bolesta S, Bansal V, Gajic O, Kashyap R. The Viral Infection and Respiratory Illness Universal Study (VIRUS): An International Registry of Coronavirus 2019-Related Critical Illness. *Crit Care Explor* 2020; **2**: e0113 [PMID: 32426754 DOI: 10.1097/CCE.0000000000000113]

33 **Walkey AJ**, Sheldrick RC, Kashyap R, Kumar VK, Boman K, Bolesta S, Zampieri FG, Bansal V, Harhay MO, Gajic O. Guiding Principles for the Conduct of Observational Critical Care Research for Coronavirus Disease 2019 Pandemics and Beyond: The Society of Critical Care Medicine Discovery Viral Infection and Respiratory Illness Universal Study Registry. *Crit Care Med* 2020; **48**: e1038-e1044 [PMID: 32932348 DOI: 10.1097/CCM.0000000000004572]

34 **Severe Covid-19 GWAS Group**, Ellinghaus D, Degenhardt F, Bujanda L, Buti M, Albillos A, Invernizzi P, Fernández J, Prati D, Baselli G, Asselta R, Grimsrud MM, Milani C, Aziz F, Kässens J, May S, Wendorff M, Wienbrandt L, Uellendahl-Werth F, Zheng T, Yi X, de Pablo R, Chercoles AG, Palom A, Garcia-Fernandez AE, Rodriguez-Frias F, Zanella A, Bandera A, Protti A, Aghemo A, Lleo A, Biondi A, Caballero-Garralda A, Gori A, Tanck A, Carreras Nolla A, Latiano A, Fracanzani AL, Peschuck A, Julià A, Pesenti A, Voza A, Jiménez D, Mateos B, Nafria Jimenez B, Quereda C, Paccapelo C, Gassner C, Angelini C, Cea C, Solier A, Pestaña D, Muñiz-Diaz E, Sandoval E, Paraboschi EM, Navas E, García Sánchez F, Ceriotti F, Martinelli-Boneschi F, Peyvandi F, Blasi F, Téllez L, Blanco-Grau A, Hemmrich-Stanisak G, Grasselli G, Costantino G, Cardamone G, Foti G, Aneli S, Kurihara H, ElAbd H, My I, Galván-Femenia I, Martín J, Erdmann J, Ferrusquía-Acosta J, Garcia-Etxebarria K, Izquierdo-Sanchez L, Bettini LR, Sumoy L, Terranova L, Moreira L, Santoro L, Scudeller L, Mesonero F, Roade L, Rühlemann MC, Schaefer M, Carrabba M, Riveiro-Barciela M, Figuera Basso ME, Valsecchi MG, Hernandez-Tejero M, Acosta-Herrera M, D'Angiò M, Baldini M, Cazzaniga M, Schulzky M, Cecconi M, Wittig M, Ciccarelli M, Rodríguez-Gandía M, Bocciolone M, Miozzo M, Montano N, Braun N, Sacchi N, Martínez N, Özer O, Palmieri O, Faverio P, Preatoni P, Bonfanti P, Omodei P, Tentorio P, Castro P, Rodrigues PM, Blandino Ortiz A, de Cid R, Ferrer R, Gualtierotti R, Nieto R, Goerg S, Badalamenti S, Marsal S, Matullo G, Pelusi S, Juzenas S, Aliberti S, Monzani V, Moreno V, Wesse T, Lenz TL, Pumarola T, Rimoldi V, Bosari S, Albrecht W, Peter W, Romero-Gómez M, D'Amato M, Duga S, Banales JM, Hov JR, Folseraas T, Valenti L, Franke A, Karlsen TH. Genomewide Association Study of Severe Covid-19 with Respiratory Failure. *N Engl J Med* 2020; **383**: 1522-1534 [PMID: 32558485 DOI: 10.1056/NEJMoa2020283]

35 **Mills MC**, Rahal C. A scientometric review of genome-wide association studies. *Commun Biol* 2019; **2**: 9 [PMID: 30623105 DOI: 10.1038/s42003-018-0261-x]

36 **Battle A**, Montgomery SB. Determining causality and consequence of expression quantitative trait loci. *Hum Genet* 2014; **133**: 727-735 [PMID: 24770875 DOI: 10.1007/s00439-014-1446-0]

37 **Glubb DM**, Dholakia N, Innocenti F. Liver expression quantitative trait loci: a foundation for pharmacogenomic research. *Front Genet* 2012; **3**: 153 [PMID: 22912647 DOI: 10.3389/fgene.2012.00153]

38 **Nica AC**, Dermitzakis ET. Expression quantitative trait loci: present and future. *Philos Trans R Soc Lond B Biol Sci* 2013; **368**: 20120362 [PMID: 23650636 DOI: 10.1098/rstb.2012.0362]

39 **Ku CS**, Naidoo N, Pawitan Y. Revisiting Mendelian disorders through exome sequencing. *Hum Genet* 2011; **129**: 351-370 [PMID: 21331778 DOI: 10.1007/s00439-011-0964-2]

40 **Köser CU**, Bryant JM, Becq J, Török ME, Ellington MJ, Marti-Renom MA, Carmichael AJ, Parkhill J, Smith GP, Peacock SJ. Whole-genome sequencing for rapid susceptibility testing of M. tuberculosis. *N Engl J Med* 2013; **369**: 290-292 [PMID: 23863072 DOI: 10.1056/NEJMc1215305]

41 **Zhang Y**, Li S, Abyzov A, Gerstein MB. Landscape and variation of novel retroduplications in 26 human populations. *PLoS Comput Biol* 2017; **13**: e1005567 [PMID: 28662076 DOI: 10.1371/journal.pcbi.1005567]

42 **Rabbani B**, Tekin M, Mahdieh N. The promise of whole-exome sequencing in medical genetics. *J Hum Genet* 2014; **59**: 5-15 [PMID: 24196381 DOI: 10.1038/jhg.2013.114]

43 **Tang F**, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A, Lao K, Surani MA. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods* 2009; **6**: 377-382 [PMID: 19349980 DOI: 10.1038/nmeth.1315]

44 **Kukurba KR**, Montgomery SB. RNA Sequencing and Analysis. *Cold Spring Harb Protoc* 2015; **2015**: 951-969 [PMID: 25870306 DOI: 10.1101/pdb.top084970]

45 **Gasperskaja E**, Kučinskas V. The most common technologies and tools for functional genome analysis. *Acta Med Litu* 2017; **24**: 1-11 [PMID: 28630587 DOI: 10.6001/actamedica.v24i1.3457]

46 **Pillai S**, Chellappan SP. ChIP on chip and ChIP-Seq assays: genome-wide analysis of transcription factor binding and histone modifications. *Methods Mol Biol* 2015; **1288**: 447-472 [PMID: 25827896 DOI: 10.1007/978-1-4939-2474-5_26]

47 **Rhee HS**, Pugh BF. ChIP-exo method for identifying genomic location of DNA-binding proteins with near-single-nucleotide accuracy. *Curr Protoc Mol Biol* 2012; **Chapter** 21: Unit 21.24 [PMID: 23026909 DOI: 10.1002/0471142727.mb2124s100]

48 **Jensen E**. Technical review: In situ hybridization. *Anat Rec (Hoboken)* 2014; **297**: 1349-1353 [PMID: 24810158 DOI: 10.1002/ar.22944]

49 **He SL**, Green R. Northern blotting. *Methods Enzymol* 2013; **530**: 75-87 [PMID: 24034315 DOI: 10.1016/B978-0-12-420037-1.00003-8]

50 **Deepak S**, Kottapalli K, Rakwal R, Oros G, Rangappa K, Iwahashi H, Masuo Y, Agrawal G. Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. *Curr Genomics* 2007; **8**: 234-251 [PMID: 18645596 DOI: 10.2174/138920207781386960]

51 **Bumgarner R**. Overview of DNA microarrays: types, applications, and their future. *Curr Protoc Mol Biol* 2013; **Chapter** 22: Unit 22.1. [PMID: 23288464 DOI: 10.1002/0471142727.mb2201s101]

52 **Qu Y**, Boutjdir M. RNase protection assay for quantifying gene expression levels. *Methods Mol Biol* 2007; **366**: 145-158 [PMID: 17568123 DOI: 10.1007/978-1-59745-030-0_8]

53 **Bustin SA**, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* 2004; **15**: 155-166 [PMID: 15331581]

54 **Xu L**, Wang J, Liu Y, Xie L, Su B, Mou D, Wang L, Liu T, Wang X, Zhang B, Zhao L, Hu L, Ning H, Zhang Y, Deng K, Liu L, Lu X, Zhang T, Xu J, Li C, Wu H, Deng H, Chen H. CRISPR-Edited Stem Cells in a Patient with HIV and Acute Lymphocytic Leukemia. *N Engl J Med* 2019; **381**: 1240-1247 [PMID: 31509667 DOI: 10.1056/NEJMoa1817426]

55 **Wu SS**, Li QC, Yin CQ, Xue W, Song CQ. Advances in CRISPR/Cas-based Gene Therapy in Human Genetic Diseases. *Theranostics* 2020; **10**: 4374-4382 [PMID: 32292501 DOI: 10.7150/thno.43360]

56 **Tian X**, Gu T, Patel S, Bode AM, Lee MH, Dong Z. CRISPR/Cas9 - An evolving biological tool kit for cancer biology and oncology. *NPJ Precis Oncol* 2019; **3**: 8 [PMID: 30911676 DOI: 10.1038/s41698-019-0080-7]

57 **Lu XJ**, Xue HY, Ke ZP, Chen JL, Ji LJ. CRISPR-Cas9: a new and promising player in gene therapy. *J Med Genet* 2015; **52**: 289-296 [PMID: 25713109 DOI: 10.1136/jmedgenet-2014-102968]

58 **Cho GY**, Schaefer KA, Bassuk AG, Tsang SH, Mahajan VB. CRISPR GENOME SURGERY IN THE RETINA IN LIGHT OF OFF-TARGETING. *Retina* 2018; **38**: 1443-1455 [PMID: 29746416 DOI: 10.1097/IAE.0000000000002197]

59 **Kang SH**, Lee WJ, An JH, Lee JH, Kim YH, Kim H, Oh Y, Park YH, Jin YB, Jun BH, Hur JK, Kim SU, Lee SH. Prediction-based highly sensitive CRISPR off-target validation using target-specific DNA enrichment. *Nat Commun* 2020; **11**: 3596 [PMID: 32681048 DOI: 10.1038/s41467-020-17418-8]

60 **English MA**, Soenksen LR, Gayet RV, de Puig H, Angenent-Mari NM, Mao AS, Nguyen PQ, Collins JJ. Programmable CRISPR-responsive smart materials. *Science* 2019; **365**: 780-785 [PMID: 31439791 DOI: 10.1126/science.aaw5122]

61 **Broughton JP**, Deng X, Yu G, Fasching CL, Servellita V, Singh J, Miao X, Streithorst JA, Granados A, Sotomayor-Gonzalez A, Zorn K, Gopez A, Hsu E, Gu W, Miller S, Pan CY, Guevara H, Wadford DA, Chen JS, Chiu CY. CRISPR-Cas12-based detection of SARS-CoV-2. *Nat Biotechnol* 2020; **38**: 870-874 [PMID: 32300245 DOI: 10.1038/s41587-020-0513-4]

62 **U. S. Food and Drug Administration**. SARS-CoV-2 RNA DETECTR Assay Accelerated Emergency Use Authorization (EUA) Summary SARS-COV-2 RNA Detectr Assay (UCSF Health Clinical Laboratories, UCSF Clinical Labs at China Basin). [cited 10 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/media/139937/download

63 **Joung J**, Ladha A, Saito M, Segel M, Bruneau R, Huang MW, Kim NG, Yu X, Li J, Walker BD, Greninger AL, Jerome KR, Gootenberg JS, Abudayyeh OO, Zhang F. Point-of-care testing for COVID-19 using SHERLOCK diagnostics. *medRxiv* 2020 [PMID: 32511521 DOI: 10.1101/2020.05.04.20091231]

64 **U. S. Food and Drug Administration**. Instructions For Use Sherlock Tm Crispr SARS-CoV-2 kit.[cited 10 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from:

https://www.fda.gov/media/137746/download

65 **Matsoukas IG**. Prime Editing: Genome Editing for Rare Genetic Diseases Without Double-Strand Breaks or Donor DNA. *Front Genet* 2020; **11**: 528 [PMID: 32582281 DOI: 10.3389/fgene.2020.00528]

66 **Anzalone AV**, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 2019; **576**: 149-157 [PMID: 31634902 DOI: 10.1038/s41586-019-1711-4]

67 **Davies JP**, Kumar S, Sastry-Dent L. Use of Zinc-Finger Nucleases for Crop Improvement. *Prog Mol Biol Transl Sci* 2017; **149**: 47-63 [PMID: 28712500 DOI: 10.1016/bs.pmbts.2017.03.006]

68 **Kim MS**, Kini AG. Engineering and Application of Zinc Finger Proteins and TALEs for Biomedical Research. *Mol Cells* 2017; **40**: 533-541 [PMID: 28835021 DOI: 10.14348/molcells.2017.0139]

69 **Epstein BE**, Schaffer DV. Combining Engineered Nucleases with Adeno-associated Viral Vectors for Therapeutic Gene Editing. *Adv Exp Med Biol* 2017; **1016**: 29-42 [PMID: 29130152 DOI: 10.1007/978-3-319-63904-8_2]

70 **U. S. Food and Drug Administration**. Fact Sheet for Healthcare Providers Administering Vaccine (Vaccination Providers): emergency use authorization (EUA) of the Janssen COVID-19 vaccine. [cited 10 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.cdc.gov/vaccines/covid-19/clinical-considerations/managing-anaphylaxis.html

71 **U. S. Food and Drug Administration**. Joint CDC and FDA Statement on Johnson & Johnson COVID-19 Vaccine. [cited 11 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/news-events/press-announcements/joint-cdc-and-fda-statement-johnson-johnson-covid-19-vaccine

72 **U. S. Food and Drug Administration**. FDA and CDC Lift Recommended Pause on Johnson & Johnson (Janssen) COVID-19 Vaccine Use Following Thorough Safety Review. [cited 11 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/news-events/press-announcements/fda-and-cdc-lift-recommended-pause-johnson-johnson-janssen-covid-19-vaccine-use-following-thorough

73 **Polack FP**, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, Perez JL, Pérez Marc G, Moreira ED, Zerbini C, Bailey R, Swanson KA, Roychoudhury S, Koury K, Li P, Kalina WV, Cooper D, Frenck RW Jr, Hammitt LL, Türeci Ö, Nell H, Schaefer A, Ünal S, Tresnan DB, Mather S, Dormitzer PR, Şahin U, Jansen KU, Gruber WC; C4591001 Clinical Trial Group. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med* 2020; **383**: 2603-2615 [PMID: 33301246 DOI: 10.1056/NEJMoa2034577]

74 **U. S. Food and Drug Administration**. Fact Sheet for Healthcare Providers Administering Vaccine (Vaccination Providers): Emergency use authorization (EUA) of the Pfizer-BioNTech COVID-19 vaccine to prevent coronavirus disease 2019 (COVID-19). [cited 11 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/media/144413/download

75 **U. S. Food and Drug Administration**. Pfizer-BioNTech COVID-19 Vaccine. [cited 11 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/pfizer-biontech-covid-19-vaccine

76 **Jackson LA**, Anderson EJ, Rouphael NG, Roberts PC, Makhene M, Coler RN, McCullough MP, Chappell JD, Denison MR, Stevens LJ, Pruijssers AJ, McDermott A, Flach B, Doria-Rose NA, Corbett KS, Morabito KM, O'Dell S, Schmidt SD, Swanson PA 2nd, Padilla M, Mascola JR, Neuzil KM, Bennett H, Sun W, Peters E, Makowski M, Albert J, Cross K, Buchanan W, Pikaart-Tautges R, Ledgerwood JE, Graham BS, Beigel JH; mRNA-1273 Study Group. An mRNA Vaccine against SARS-CoV-2 - Preliminary Report. *N Engl J Med* 2020; **383**: 1920-1931 [PMID: 32663912 DOI: 10.1056/NEJMoa2022483]

77 **U. S. Food and Drug Administration**. Fact Sheet for Healthcare Providers Administering Vaccine (Vaccination Providers): Emergency use authorization (EUA) of the moderna COVID-19 vaccine to prevent coronavirus disease 2019 (COVID-19). [cited 11 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/media/144637/download

78 **U. S. Food and Drug Administration**. Moderna COVID-19 Vaccine. [cited 11 December 2020]. In: U.S. Food and Drug Administration [Internet]. Available from: https://www.fda.gov/emergency-preparedness-and-response/coronavirus-disease-2019-covid-19/moderna-covid-19-vaccine

79 **Liebow A**, Li X, Racie T, Hettinger J, Bettencourt BR, Najafian N, Haslett P, Fitzgerald K, Holmes RP, Erbe D, Querbes W, Knight J. An Investigational RNAi Therapeutic Targeting Glycolate Oxidase Reduces Oxalate Production in Models of Primary Hyperoxaluria. *J Am Soc Nephrol* 2017; **28**: 494-503 [PMID: 27432743 DOI: 10.1681/ASN.2016030338]

80 **Roshmi RR**, Yokota T. Viltolarsen for the treatment of Duchenne muscular dystrophy. *Drugs Today (Barc)* 2019; **55**: 627-639 [PMID: 31720560 DOI: 10.1358/dot.2019.55.10.3045038]

81 **Beyar-Katz O**, Gill S. Advances in chimeric antigen receptor T cells. *Curr Opin Hematol* 2020; **27**: 368-377 [PMID: 32925186 DOI: 10.1097/MOH.0000000000000614]

82 **Heo YA**. Golodirsen: First Approval. *Drugs* 2020; **80**: 329-333 [PMID: 32026421 DOI: 10.1007/s40265-020-01267-2]

83 **Scott LJ**. Givosiran: First Approval. *Drugs* 2020; **80**: 335-339 [PMID: 32034693 DOI: 10.1007/s40265-020-01269-0]

84 **Hoy SM**. Onasemnogene Abeparvovec: First Global Approval. *Drugs* 2019; **79**: 1255-1262 [PMID: 31270752 DOI: 10.1007/s40265-019-01162-5]

85 **Benson MD**, Waddington-Cruz M, Berk JL, Polydefkis M, Dyck PJ, Wang AK, Planté-Bordeneuve V, Barroso FA, Merlini G, Obici L, Scheinberg M, Brannagan TH 3rd, Litchy WJ, Whelan C, Drachman BM, Adams D, Heitner SB, Conceição I, Schmidt HH, Vita G, Campistol JM, Gamez J, Gorevic PD, Gane E, Shah AM, Solomon SD, Monia BP, Hughes SG, Kwoh TJ, McEvoy BW, Jung SW, Baker BF, Ackermann EJ, Gertz MA, Coelho T. Inotersen Treatment for Patients with Hereditary Transthyretin Amyloidosis. *N Engl J Med* 2018; **379**: 22-31 [PMID: 29972757 DOI: 10.1056/NEJMoa1716793]

86 **Riedell PA**, Bishop MR. Safety and efficacy of axicabtagene ciloleucel in refractory large B-cell lymphomas. *Ther Adv Hematol* 2020; **11**: 2040620720902899 [PMID: 32064069 DOI: 10.1177/2040620720902899]

87 **Maude SL**, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, Qayed M, De Moerloose B, Hiramatsu H, Schlis K, Davis KL, Martin PL, Nemecek ER, Yanik GA, Peters C, Baruchel A, Boissel N, Mechinaud F, Balduzzi A, Krueger J, June CH, Levine BL, Wood P, Taran T, Leung M, Mueller KT, Zhang Y, Sen K, Lebwohl D, Pulsipher MA, Grupp SA. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med* 2018; **378**: 439-448 [PMID: 29385370 DOI: 10.1056/NEJMoa1709866]

88 **Chiriboga CA**. Nusinersen for the treatment of spinal muscular atrophy. *Expert Rev Neurother* 2017; **17**: 955-962 [PMID: 28884620 DOI: 10.1080/14737175.2017.1364159]

89 **Lim KR**, Maruyama R, Yokota T. Eteplirsen in the treatment of Duchenne muscular dystrophy. *Drug Des Devel Ther* 2017; **11**: 533-545 [PMID: 28280301 DOI: 10.2147/DDDT.S97635]

90 **Bommareddy PK**, Patel A, Hossain S, Kaufman HL. Talimogene Laherparepvec (T-VEC) and Other Oncolytic Viruses for the Treatment of Melanoma. *Am J Clin Dermatol* 2017; **18**: 1-15 [PMID: 27988837 DOI: 10.1007/s40257-016-0238-9]

91 **Business Wire**. Pfizer and Sangamo Announce Updated Phase 1/2 Results Showing Sustained Factor VIII Activity Levels and No Bleeding Events or Factor Usage in 3e13 vg/kg Cohort Following giroctocogene fitelparvovec (SB-525) Gene Therapy. [cited 10 December 2020]. In: Business Wire: Berkshire Hathaway Company. Available from: https://www.businesswire.com/news/home/20200618005091/en/

92 **Brandts J**, Ray KK. Clinical implications and outcomes of the ORION Phase III trials. *Future Cardiol* 2020 [PMID: 33345605 DOI: 10.2217/fca-2020-0150]

93 **Witztum JL**, Gaudet D, Freedman SD, Alexander VJ, Digenio A, Williams KR, Yang Q, Hughes SG, Geary RS, Arca M, Stroes ESG, Bergeron J, Soran H, Civeira F, Hemphill L, Tsimikas S, Blom DJ, O'Dea L, Bruckert E. Volanesorsen and Triglyceride Levels in Familial Chylomicronemia Syndrome. *N Engl J Med* 2019; **381**: 531-542 [PMID: 31390500 DOI: 10.1056/NEJMoa1715944]

94 **Frangoul H**, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK, Foell J, de la Fuente J, Grupp S, Handgretinger R, Ho TW, Kattamis A, Kernytsky A, Lekstrom-Himes J, Li AM, Locatelli F, Mapara MY, de Montalembert M, Rondelli D, Sharma A, Sheth S, Soni S, Steinberg MH, Wall D, Yen A, Corbacioglu S. CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. *N Engl J Med* 2021; **384**: 252-260 [PMID: 33283989 DOI: 10.1056/NEJMoa2031054]

World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 September 20; 11(5): 243-262

DOI: 10.5662/wjm.v11.i5.243

ISSN 2222-0682 (online)

REVIEW

IgY technology: Methods for developing and evaluating avian immunoglobulins for the *in vitro* detection of biomolecules

Chrysoula-Evangelia Karachaliou, Vyronia Vassilakopoulou, Evangelia Livaniou

ORCID number: Chrysoula-Evangelia Karachaliou 0000-0002-0939-9009; Vyronia Vassilakopoulou 0000-0003-4843-5604; Evangelia Livaniou 0000-0003-0165-9029.

Author contributions: Karachaliou CE collected the literature articles and drafted the manuscript; Vassilakopoulou V contributed to the literature search and draft writing; Livaniou E designed the draft and edited the final paper; All authors have read and approved the final manuscript.

Conflict-of-interest statement: Authors declare no conflict of interest for this article.

Manuscript source: Invited manuscript

Chrysoula-Evangelia Karachaliou, Vyronia Vassilakopoulou, Evangelia Livaniou, Institute of Nuclear & Radiological Sciences & Technology, Energy & Safety, National Centre for Scientific Research "Demokritos", Athens 15310, Greece

Corresponding author: Evangelia Livaniou, PhD, Academic Research, Institute of Nuclear & Radiological Sciences & Technology, Energy & Safety, National Centre for Scientific Research "Demokritos," Agia Paraskevi, Athens 15310, Greece. livanlts@rrp.demokritos.gr

Abstract

The term "IgY technology" was introduced in the literature in the mid 1990s to describe a procedure involving immunization of avian species, mainly laying hens and consequent isolation of the polyclonal IgYs from the "immune" egg yolk (thus avoiding bleeding and animal stress). IgYs have been applied to various fields of medicine and biotechnology. The present article will deal with specific aspects of IgY technology, focusing on the currently reported methods for developing, isolating, evaluating and storing polyclonal IgYs. Other topics such as current information on isolation protocols or evaluation of IgYs from different avian species are also discussed. Specific advantages of IgY technology (*e.g.*, novel antibody specificities that may emerge *via* the avian immune system) will also be discussed. Recent *in vitro* applications of polyclonal egg yolk-derived IgYs to the field of disease diagnosis in human and veterinary medicine through *in vitro* immunodetection of target biomolecules will be presented. Moreover, ethical aspects associated with animal well-being as well as new promising approaches that are relevant to the original IgY technology (*e.g.*, development of monoclonal IgYs and IgY-like antibodies through the phage display technique or in transgenic chickens) and future prospects in the area will also be mentioned.

Key Words: Animal welfare; Polyclonal IgYs; Egg yolk; IgY technology; Relevant-to-IgY-technology approaches; *In vitro* immunodetection techniques

Core Tip: IgY technology has been widely used during the last decades, especially as a means for the efficient *in vitro* immunodetection of biomolecules in various fields of research and disease diagnosis. Despite the very promising relevant new approaches,

Specialty type: Immunology

Country/Territory of origin: Greece

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 4, 2021
Peer-review started: February 4, 2021
First decision: June 7, 2021
Revised: June 10, 2021
Accepted: July 13, 2021
Article in press: July 13, 2021
Published online: September 20, 2021

P-Reviewer: Neagu M
S-Editor: Zhang H
L-Editor: Filipodia
P-Editor: Guo X

there is still space to further exploit the original IgY technology due to functional, practical, and ethical reasons/advantages associated with the unique features of IgYs, the highly efficient isolation of large amounts of IgYs from the immune egg yolk, and the avoidance of animal bleeding, respectively.

Citation: Karachaliou CE, Vassilakopoulou V, Livaniou E. IgY technology: Methods for developing and evaluating avian immunoglobulins for the *in vitro* detection of biomolecules. *World J Methodol* 2021; 11(5): 243-262
URL: https://www.wjgnet.com/2222-0682/full/v11/i5/243.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i5.243

INTRODUCTION

The term "IgY technology" was introduced in the 1990s to describe a procedure consisting of immunization of birds, especially laying hens, in order to produce polyclonal antibodies of the Y class (IgYs). IgYs can be isolated in large quantities from "immune" egg yolk (thus avoiding the animal bleeding procedure, which is stressful for an animal) and has been applied to various fields of biotechnology and biomedicine[1-3]. To date, IgYs developed in poultry and isolated from the egg yolk as aforementioned have been and are still being used as specific laboratory tools, especially for detecting biomolecules in biological specimens through various *in vitro* techniques (and also as *in vivo* immunotherapeutic agents).

The origins of the IgY technology can be traced back many years, *i.e.* at the end of the 19th century, when Klemperer observed that immunized hens (*Gallus domesticus*) generated antibodies that were present in the egg yolk[2-4]. Subsequently, a new type of immunoglobulin was found in the blood and egg yolk of birds (also in lungfish, amphibians and reptiles), which was called IgY[3,5]. Actually, birds, which do not produce colostrum like mammalian organisms do, use the yolk of their eggs as a very effective source of antibodies through which they can transfer humoral immunity to their offspring, until the latter develops fully mature immune system[6]. Transfer/accumulation of IgY from blood to/in the egg yolk, which is realized by a selective transport mechanism in avian mature oocytes and mediated by specific receptor(s)[7-9], enables the non-invasive isolation of antibodies and eliminates the need to bleed the animal. Isolation and subsequent application of egg yolk–derived antibodies minimize animal suffering and this meets at least one of the three main requirements for animal welfare, *i.e.* "Reduction," "Replacement," "Refinement," as they have been summarized in the "3Rs principle"[10]. As a consequence, in 1996 the European Centre for the Validation of Alternative Methods to animal testing (ECVAM) strongly recommended avian antibodies as alternative to mammalian ones[1]. In parallel, in the mid 1990s the term "IgY technology" was introduced in the literature, as already mentioned; in 1999, the IgY technology was approved as an alternative method for supporting animal welfare by the Veterinary Office of the Swiss Government[3].

Egg yolk is composed mainly of water, which accounts for approximately 50% of its weight, and contains many important nutrients and preservatives, since it serves the role of a protective chamber for the hen embryo. The dry weight of egg yolk is composed mostly by lipids (67%) and also proteins (33%). Egg yolk proteins are distributed between granules and plasma, in which granules are suspended. Granule proteins are divided into α- and β-lipovitellins (70%), phosvitin (16%), and low-density lipoproteins (12%), whereas plasma proteins include α-, β- and γ-livetins and low-density proteins[11]. A precursor of the major egg yolk proteins is vitellogenin, consisting of vitellogenin I (molecular weight [MW]: 260 kDa), vitellogenin II or major vitellogenin (MW: 246 kDa), and vitellogenin III (MW: 210 kDa)[12-14]. IgYs, which are the main constituent of γ-livetin, are among the most important and most abundant egg yolk proteins[11].

IgY is considered to be the functional equivalent and evolutionary precursor of mammalian IgG and probably of mammalian IgE[15]. Due to this functional and evolutionary relationship, some researchers use the term (avian) IgG instead of IgY; however, the first articles in the field have put emphasis on the distinct differences between IgG and IgY and strongly suggested use of the term IgY[5]. In addition to IgYs, there are two more avian immunoglobulin classes, avian IgM and IgA, which are

similar to mammalian IgM and IgA. Mammalian equivalents of IgE and IgD have not been found in hens[16].

Like mammalian IgG, IgY is composed of two heavy (H) and two light (L) polypeptide chains, which are organized in the Y-shaped characteristic "unit," and contains two identical binding sites for the antigen. However, the structure of IgY is actually different than that of IgG and this results in distinct properties, as well. The nucleotide sequence corresponding to the hen upsilon ("υ") heavy chain has revealed that the molecule contains four constant and one variable Ig heavy chain domains; the additional domain (Cυ2) has been conserved in mammalian IgE, but "transformed" into the flexible hinge region in mammalian IgG. As a consequence, the IgY molecule has higher molecular mass (approximately 180 kDa), than mammalian IgG (approximately 160 kDa). Moreover, the Fc part of IgY has a different carbohydrate content compared to the Fc part of IgG. An intact Fc part is necessary for the transfer of IgY from blood serum to egg yolk. In ducks an alternatively spliced form of IgY, the so-called IgY ΔFc, is also present. This variant lacks the Fc region and is mainly found in the blood serum. Hen as well as ostrich and pigeon express only the full-length version of IgY. In some birds, including hen, duck, zebra finch and ostrich, only a single κ light-chain locus has been found. The bursa of fabricius is the site in which immature B-cells are differentiated into mature and competent B-cells, while the spleen is the organ in which plasma cells, *i.e.* the antibody-producing cells, proliferate and memory cells are located. IgY's heavy and light chain loci consist of single functional V, D, and J genes; in addition to the single functional V genes, there are several pseudo-V genes that lack the usual transcription-regulatory and signal-recognition sequences and are not functional. The antibody diversity in avian organisms is mainly achieved by the so-called gene conversion, through which 10 to more than 120 base pairs from not functional pseudo-genes are transferred to the functional V gene[3,16,17].

The distinct structural features of IgY offer several functional advantages to this unique immunoglobulin type, rendering IgY a versatile and invaluable *in vitro* tool in biotechnology research and in disease diagnostics. Moreover, many reports have suggested *in vivo* application of IgYs in various fields of immunotherapy. The advantages of IgYs include: high potential for developing specific IgYs against conserved mammalian proteins due to the evolutionary distance between mammals and birds, avoidance of activating the mammalian (including human) complement system and reaction with mammalian Fc receptors, ability to isolate substantial amounts of IgYs from immune egg yolks, and avoidance of animal bleeding, which fulfills the "refinement" ethical requirement, as already mentioned[3,18,19].

In the last several decades, more complicated technologies associated with the original IgY technology have emerged, such as the development of avian monoclonal antibodies *via* hybridoma and recombinant techniques, mainly through the phage display technique[20]. Although the above antibodies are IgYs (or IgY-like) immunoglobulins and therefore have all (or part of) the consequent advantages, they are isolated from the supernatant of suitable cell cultures and are not egg yolk-derived. Thus, strictly speaking and at least in our opinion, the techniques leading to the development of monoclonal IgYs cannot be classified as a part of the original IgY technology. On the other hand, transgenic chickens[21] have been used for the production of recombinant proteins, including recombinant antibodies (mostly human/humanized ones), which can be isolated mainly from egg white and are recommended especially for *in vivo* therapeutic applications. Though the aforementioned antibodies have not gained wide application yet and their development and evaluation are considered outside the main scope of the present article, they are considered very promising and will be briefly presented.

The present review article will focus on specific aspects of the original IgY technology, such as immunization of laying hens, isolation of the IgYs developed from the immune egg yolk and consequent immunochemical evaluation. Various recent applications of polyclonal IgYs to the *in vitro* immunodetection of various biomolecules will be also presented and discussed.

DEVELOPMENT AND EVALUATION OF EGG YOLK-DERIVED POLYCLONAL IGYS

General aspects

IgY technology has produced a large number of valuable immunochemical tools for biotechnology and medicine since the 1990s. Various parameters that are associated

with and can affect the results of the IgY technology have been reported in the literature such as housing and breeding conditions, line, age, and stage of development of the immunized birds[2,3,18,22]. Laying hens are the avian organisms of choice (*e.g.*, White Leghorn and Rhode Island Red hens) and are used for immunization to produce polyclonal IgYs throughout their egg-laying period. Other types of poultry such as duck, goose, ostrich, and quail have been referred to in the literature, though to a lesser extent[23-26]. Normal hen lines and conventional housing, *e.g.*, in suitable cages[27], are usually adequate to produce IgYs for research purposes; however, when the IgYs are to be applied as human therapeutics, the use of specific pathogen-free hens is considered necessary[1,3]. Administration of specific food supplements during hens' breeding, *e.g.*, carnitine, has been proposed in the literature as a means to improve overall yield of IgY production, but the results are often contradictory[28].

Immunizing protocols

Parameters that may influence the immune response include antigen nature and dose, use of adjuvants, route of administration, and overall immunization schedule[3].

Both, complex antigens, *e.g.*, whole viruses, bacteria and parasites[29-33] and individual biomolecules, *e.g.*, large proteins[34,35], or small peptides conjugated to a suitable carrier protein, such as keyhole limpet hemocyanin (KLH)[36,37], have been used to stimulate development of specific IgYs in hens. Our team tried to develop IgYs against various antigens, including a recombinant protein of high molecular mass, *i.e.* human kallikrein-related peptidase 6[38] as well as peptides of the alpha- and beta-thymosin families isolated from mammalian tissues or synthetically prepared, either conjugated to KLH or not[39-41]. Moreover, we successfully developed IgYs against the olive fruit fly pheromone by using a KLH-conjugate of the synthetic hapten (±)-β-[3-(1,7-dioxaspiro[5.5]undecane)] propionic acid[27].

The antigen dose may be also critical, since too much or too little antigen can lead to an undesirable immune response[2]. Different antigen doses have been reported in the literature. In an early study, a good immune response in hens immunized with bovine serum albumin at doses as low as 0.1-1.0 μg was reported[3]; however, higher doses ranging from 10 to 1000 μg (most often 50-100 μg) have been also used. Information on the doses administered to immunize hens has been presented in a recent review[18].

The outcome of immunization is commonly enhanced by the addition of adjuvants, though successful immunization of hens without any adjuvant has been reported in the literature[3]. Among the adjuvant preparations that have been described till now, Freund's complete adjuvant (FCA) is still considered the gold standard for generating high levels of antibodies in animals, including birds. FCA is a suspension of heat-killed and dried mycobacteria (*Mycobacterium spp.*) in mineral oil, which forms a depot at the injection site and slows down release of the antigen in the host organism, so that long-lasting exposure and a non-specific immune stimulation is achieved. The main problem of FCA is the severe tissue damage it causes at the injection sites, which is usually attributed to the mycobacteria it contains. Although a few studies have reported that hens can better tolerate FCA, in comparison with mammals, other studies have reported contradictory data. For this reason, Freund's incomplete adjuvant, *i.e.* Freund's adjuvant without mycobacteria, is commonly used for booster injections as an alternative to FCA, which is used only in the first immunization[18]. Use of other adjuvants has been also reported in IgY technology, such as the so-called, mineral-oil based Montanide adjuvant, along with oligodeoxynucleotides containing C-phosphate guanosine motifs, which are promising immunoenhancing agents[28]. Research in the area of developing new adjuvants, both highly efficient and animal welfare-friendly, is being continued.

Regarding the route of administration, several approaches have been tested. The most recommended one is the intramuscular injection (i.m.) into the breast tissues[3,29,34,42] in multiple sites; i.m. administration in the thigh muscle has been also used but according to some reports it may cause lameness and has to be avoided[18]. Subcutaneous (s.c.) immunization in the neck has also been used by several research teams including our team[27,38,39]. As reported, i.m. immunization in breast muscle is most suited especially for young hens[18]. The intravenous (i.v.) route has been very rarely used, without adjuvants and at a very slow injection rate. The intraperitoneal (i.p.) route, which Klemperer has followed in his pioneer work, is hardly used these days. Efforts to immunize hens orally have been also reported[3,30,43].

The interval between the first and second (*i.e.* first booster) immunization is considered a critical parameter in hen immunization protocols. Age of hens when first immunized might also be an issue. However, literature information on these specific parameters substantially varies. A general recommendation is to administer a booster immunization when the IgY titer reaches a plateau or begins to decrease[44]. If a

substantial decrease in the antibody titer has been observed, further immunizations can be performed during the entire laying period, which lasts about 72 wk[22], to keep the antibody titer adequately high for as long as possible, in many cases for more than 150 d[18]. As presented in a previous review[3], some immunization protocols have recommended antigen administration at days 0, 14 and 28, or once a week for 7 consecutive weeks, or at day 0, week 10, and week 15. Other protocols propose hen immunization at 10-d intervals, but in most cases, the interval between the first and second immunization is at least 4 wk, while another protocol has reported achievement of a high antibody titer by prolonging the boost interval from 14 to 42 d. Intervals among booster injections also vary, averaging 2 wk[3]. Our team has mainly used 3-mo-old hens for immunization; the first booster was administered 2 wk after first immunization, while several further injections were given, mostly at 4 wk-intervals[27].

In general, eggs are collected weekly, starting 1 wk prior to the first immunization (pre-immune eggs), eggshells are washed or sanitized with 70% ethanol, and stored at 4°C until further processed for IgY isolation. Lyophilization of egg yolk has also been reported, resulting in an easy-to-mix egg yolk powder with an extended shelf-life[45].

Immunization with plasmids: "DNA-designed" IgYs

Apart from the conventional administration of antigen along with adjuvant, the so-called genetic immunization has also been applied to the production of polyclonal IgYs in avian species[46]. In this context, avian organisms have been immunized with plasmid vectors encoding target eukaryotic antigens, *e.g.*, bovine interferon gamma protein[47], prokaryotic antigens, *e.g.*, *Botulinum* toxin A1[48], as well as viral ones, *e.g.*, antigens from Andes virus[23]; in almost all cases, antibodies Y of desired immunochemical characteristics have been developed. A great deal of effort has been put forth to improve DNA-vaccine delivery, and consequently, immunogenicity. The "gene gun" method has garnered much attention, since low doses of DNA applied *via* a gene gun can efficiently induce high antibody titers against the antigen encoded[49]. Although DNA immunization is a promising approach, which prevents costly and tedious preparation of purified antigens or presence of adjuvants in the immunization mixture, it has not yet gained wide application.

Isolation of IgYs from the egg yolk

Hen eggs are an excellent source of high amounts of antibodies[19]. An average hen can lay roughly 325 eggs a year. Given that according to the literature an egg can produce 60-150 mg[50], or 40-80 mg total IgY per egg yolk depending on the hen's age [22], one hen can roughly produce 20-40 g of antibodies a year, with 1%-2% up to 10% of the antibodies being antigen-specific[18,51], which is much higher than that obtained from mammalian sources[11].

Isolation of IgYs from the "immune" egg yolk in pure form is a challenging task. Several protocols have been described, with different characteristics in terms of total yield, purity, duration, convenience, and cost[42]. IgYs account for about 3%-5% of the egg yolk proteins, which are dispersed in a lipid emulsion combined with lipoproteins and glycoproteins. Consequently, in most cases, IgY isolation involves, first, removal of lipids to form a water-soluble fraction ("de-lipidation" step), and then precipitation of the antibodies that are present in the water- soluble fraction with various approaches[3,18].

The most commonly used de-lipidation technique is the "acidified water dilution method"[52], using 6- to 10-fold dilution of egg yolk in water at pH ~5, incubation for several hours at 4°C and then centrifugation, at the end of which the lipid portion is precipitated and the water-soluble portion is collected in the supernatant. Alternatively, lipid removal has been successfully performed by means of organic solvents (chloroform, acetone, isopropanol)[53,54], acids (caprylic acid, trichloroacetic acid)[55] or natural gums (polyanionic polysaccharides, *e.g.*, xanthans)[56]. A de-lipidation solution containing polysaccharides (such as pectin, λ-carrageenan, carboxymethylcellulose, methylcellulose, and dextran sulfate) has been also reported[57].

After de-lipidation, various IgY extraction methods that can be applied either to laboratory- or to large-scale production have appeared in the literature; these methods can be divided into three main groups, *i.e.* precipitation, chromatographic and filtration methods.

Precipitation methods, involving precipitation of IgYs with saturated salt solutions, such as ammonium sulfate, sodium sulfate or sodium chloride[58,59], polyethylene glycol (PEG)[60], caprylic acid[61,62] and carrageenan[63]. PEG precipitation usually involves, first, dilution of egg yolk in phosphate-buffered saline (PBS) containing PEG 6000 at low concentration (3.5%), to facilitate de-lipidation. After centrifugation, the

supernatant is treated with 8.5% and then with 12% PEG 6000 to precipitate IgYs[30]. Among the above methods, ammonium sulfate precipitation is considered one of the best choices for the scale-up purification of IgY[11], with most suitable concentration of ammonium sulfate being 20%[55]. Extracted IgY samples usually undergo a final dialysis step, usually against PBS, to eliminate residual salts from the extraction procedure.

Chromatographic methods include low-pressure chromatography[30], ion exchange chromatography[52,59], highresolution chromatography through multicolumn systems[64] and affinity chromatography[65]. Conventional affinity chromatographic methods using protein A or protein G columns cannot be performed for IgY purification, since IgYs, contrary to IgGs, do not bind to protein A or G[66]. Other types of ligands are therefore required, such as the elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G[67]. Still other ligands, such as IgY-binding peptides screened from a random peptide library, have been also proposed as a means of IgY purification[68]. IgY can also be purified with thiophilic adsorption chromatography, usually through commercially available IgY-extraction columns[18,69]. However, chromatographic techniques are generally expensive and impractical for the large-scale production of antibodies, while they have not proven to substantially increase purity of the final product when compared with simple precipitation methods, such as ammonium sulfate precipitation.

Filtration methods, such as ultrafiltration[52,70], have also been used as IgY extraction methods.

As reported, a combination of the aforementioned methods, *e.g.*, a combination of PEG precipitation with affinity chromatography[22] or ammonium sulfate precipitation with ion exchange chromatography[59], can further increase the purity of the IgY preparation. Moreover, sequential precipitation with 31% ammonium sulfate and 12% PEG resulted in IgY antibodies of more than 95% purity without any loss in immunoreactivity[64].

Despite the numerous protocols described in the literature, the most popular isolation strategy of IgYs from immune eggs involves a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or PEG, at suitable concentrations[30].

Storage

According to the literature, after their isolation, IgYs can be stored for long periods (from a few months to a few years), preferably at -20 °C[22,71], since they are considered reasonably stable biomolecules, like mammalian IgGs[72]. IgY is stable at pH 4-9 and up to 65 °C in aqueous solutions. The addition of stabilizing reagents or high concentrations of salts can further increase resistance of the IgY molecule; *e.g.*, heat stability could be increased up to 70 °C by the addition of sugars, such as 30% sucrose, trehalose or lactose[3]. Useful information concerning earlier findings on the stability and storage conditions of IgYs has appeared in recent review articles[73]. Freeze-drying has been used to facilitate long storage of IgYs[74], though some researchers have reported that freeze-drying may lead to some loss of antigen-binding activity of IgY[45]. Lyophilization of proteins, including IgYs, induces freezing and dehydration stresses, which may result in protein structural changes or even unfolding [75]. Therefore, the addition of cryoprotectants and lyoprotectants has been recommended to protect IgYs during lyophilization[45]. Our team has recently evaluated IgYs that were developed against a KLH-conjugate of the polypeptide prothymosin alpha many years ago and kept as lyophilized powder at -30 °C. As revealed, the IgYs have kept immunoreactivity and were successfully applied to a specific enzyme-linked immunosorbent assay (ELISA) for prothymosin alpha[76].

Evaluation of egg yolk IgYs

Protein concentration: Determination of protein concentration in IgY extracts is usually performed before proceeding to further IgY evaluation. Total protein concentration in IgY extracts has been determined mainly with the Bradford method (indicative references[30,34,35,38,42]), the Lowry method[58] and the bicinchoninic acid protein assay[77]. In addition, protein concentration was assessed with ultraviolet absorption at 280 nm, according to the Lambert-Beer law (indicative references[29,32,33,57,76]).

Purity: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is considered the gold standard technique and has been widely used to assess the purity of the egg yolk-isolated IgYs (indicative references[30,33,34,62,78]). SDS-PAGE

separation under non-reducing or reducing conditions would reveal one or two protein bands, the latter corresponding to heavy and light IgY chains.

Western blotting has been used complementarily with SDS-PAGE to confirm the presence and assess purity of IgYs isolated from immune egg yolks (indicative references[31,33,62,78]). Visualization of the specific protein bands is performed mainly through a color or chemiluminescence development.

In a few cases, additional analytical methods such as high-performance liquid chromatography[57] have also been used to evaluate the purity of IgYs.

Immunoreactivity: The immunoreactivity of egg yolk-derived IgYs is evaluated with well-established immunochemical methods such as dot-blot and ELISA. Dot-blot can be actually considered as a simplified form of ELISA offering mostly qualitative results. Nevertheless, it is a fast, easy, and low-cost technique that may provide useful information and has, therefore, been used by several researchers to evaluate immunoreactivity of IgYs[32,34,36,39,76]. In most cases, however, evaluation of IgY immunoreactivity involves determination of titer against the target antigen through non-competitive ELISAs (indicative references[32,35,45,76,78]). Moreover, other immunochemical characteristics of the isolated IgYs are assessed, such as putative cross-reactivity with various substances through competitive ELISAs (indicative references[31,36,39]). It should be noted that till now and despite the numerous new technologies introduced in the field, ELISA remains the gold standard method for evaluating the basic immunological characteristics of any antibody developed, independently of the antibody class or the production method.

IN VITRO APPLICATION OF EGG YOLK-DERIVED POLYCLONAL IGYS TO THE DETECTION OF BIOMOLECULES

IgY is considered an excellent tool especially for developing *in vitro* methods to detect biomolecules of interest in biological specimens for a series of reasons. First, the evolutionary distance between mammals and birds may facilitate generation of specific IgYs against conserved mammalian proteins, since avian organisms possess a different antibody repertoire than that of mammals and the epitope spectrum of avian antibodies is potentially larger/different than that of mammalian IgGs including novel specificities[19,64]. Second, IgY does not activate the mammalian (including human) complement system and does not react with mammalian Fc receptors; this feature has rendered IgYs an ideal *in vitro* reagent, especially for immunoassays designed to detect biomolecules in human blood serum[64]. Third, substantial amounts of IgY can be isolated from egg yolks; as already mentioned (isolation of IgYs from the egg yolks), one hen can produce 20-40 g of IgY in 1 year, 1%-10% of which is antigen-specific. This advantage of egg yolk IgY is accompanied by other practical superiorities, such as low animal care cost, ease of isolation of antibodies from the egg yolk with simple biochemical methods and overall low production cost[73]. These advantages along with the large-scale facilities currently available render production of egg yolk-derived IgYs, a technically feasible and efficient procedure at industrial level. Some other positive characteristics of IgYs have been reported in the literature, *e.g.*, they can be developed even when hens are immunized with very small amounts of the corresponding antigens[64,71] or that they show higher specificity, binding affinity, and avidity for their targets in comparison with mammalian IgGs[38,73], although other reports have shown controversial data[3]. Last but not least, in the list of IgY advantages is that use of egg yolk IgYs is especially desirable from an ethical aspect of view, concerning refinement of animal experimentation, as already mentioned. Some recent indicative applications of IgYs to the *in vitro* detection of biomolecules (as well as whole viruses/microorganisms) have been summarized and presented in Table 1. Lately, specific IgYs have been developed and used for the immunodiagnosis of pandemic coronavirus disease-2019 (COVID-19)[79], while non-specific IgY has been used to form/visualize the "control line" in point-of-care *in vitro* tests that detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens[80].

Table 1 *In vitro* applications of polyclonal IgYs

Target biomolecule(s)	*In vitro* immunochemical technique	Proposed field of application	Ref.
Major surface antigen of *Toxoplasma gondii* (SAG1)	Latex agglutination assay	Diagnosis of Toxoplasmosis	Cakir-Koc *et al* [132], 2020
Protein A of *Staphylococcus aureus*	Immunocapture PCR assay	Detection of *Staphylococcus aureus* in food samples, skin and nasal swabs	Kota *et al*[133], 2020
Peptides/proteins present in detoxified western Russell's viper venom	Paper-based microfluidic immunochromatographic test	Differential diagnosis of Russell's viper envenomation	Lin *et al*[134], 2020
SARS-CoV-2 antigen	Fluorescence immunochromatographic rapid-antigen test	Diagnosis of COVID-19	Porte *et al*[79], 2020
Antigens present in total saline extract of *Taenia crassiceps* metacestodes	ELISA	Detection of neurocysticercosis	daSilva *et al* [32], 2020
Antigens present in total saline extract of *Ancylostoma ceylanicum*	ELISA	Diagnosis of Hookworm infection	Souza *et al*[135], 2020
Non-glycosylated synthetic oligopeptides of *Dermatophagoides* group I allergens	Immuno-dot blot assay (with the use of IgY-colloidal gold nanoparticles conjugates)	Detection of indoor dust mite allergens	Egea *et al*[136], 2019
Antigens present in whole bacterial suspension of formalin- and heat-inactivated*Salmonella typhimurium* and *Salmonella enteritidis*	*In vitro* immunochemicaltechniques	Diagnosis of infection with *Salmonella typhimurium* and *Salmonella enteritidis*	Esmailnejad *et al*[26], 2019
Antigenic extracts of *Strongyloides venezuelensis* infectious filariform larvae and parthenogenetic females	ELISA	Diagnosis of human strongyloidiasis	deFaria *et al* [33], 2019
Antigens present in total saline extract of *Ascaris suum* adult life forms	Tissue indirect immunofluorescence assay & ELISA	Diagnosis of human ascariasis	Lopes *et al*[31], 2019
Free prostate specific antigen	ELISA	Diagnosis of human prostate cancer	Łupicka-Słowic *et al*[137], 2019
Antigens (capsid proteins VP2 & VP3) present in beta-propiolactone-inactivated enterovirus 71	Fluorescence sensor assay	Diagnosis of hand-foot-and-mouth disease caused by enterovirus 71 infection	Nie *et al*[138], 2019
Fusarium verticillioides 97K exoantigen	ELISA	Detection of *Fusarium verticillioides* (and prediction of fumonisin contamination) in poultry feed	Omori *et al* [139], 2019
Recombinant purified catalytic domain of Karilysin	ELISA	Evaluation of karilysin (*i.e.* an enzyme secreted by the periontopathogen *Tannerella forsythia*) as a biomarker for the diagnosis of periodontitis	Skottrup *et al* [34], 2019
Fumonisin B1	Lateral flow immunoassay	Detection of fumonisin B1 and fumonisin B2 in maize	Tran *et al*[140], 2019
Synthetic extracellular peptide of matrix-2 protein of influenza A virus, conserved in all strains	Latex agglutination assay	Diagnosis of infection with Influenza A virus	Budama-Kilinc *et al*[141], 2018
Sulfamethazine (SMZ)	ELISA, FPIA	Detection of veterinary drug residues (SMZ) in milk	Liang *et al*[142], 2018
Native calf adenosine deaminase (ADA)	ELISA	Evaluation of ADA as a cancer biomarker	Łupicka-Słowic *et al*[143], 2018
Nucleoprotein of influenza A virus	Immunocytochemistry, Immunohistochemistry	Diagnosis of infection with influenza A virus	da Silva *et al* [144], 2018

ADA: Adenosine deaminase; COVID-19: Coronavirus disease-2019; ELISA: Enzyme-linked immunosorbent assay; FPIA: Fluorescence polarization immunoassay; PCR: Polymerase chain reaction; SAG1: Surface antigen 1 of *Toxoplasma gondii*; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SMZ: Sulfamethazine.

RELEVANT APPROACHES AND FUTURE PROSPECTS

Monoclonal IgYs

Since the late 1980s many efforts have been directed toward development and use of avian monoclonal antibodies (mAbs) for research, diagnostic, and therapeutic purposes, because avian mAbs may combine the advantages of avian immuno-

globulins with those of monoclonality, *i.e.* precise characterization and continuous production. Initially, several technical difficulties have emerged; even after technical problems have been addressed and avian mAbs have been produced by hybridomas [81,82], the hybridoma technology has not gained wide application, because it is considered a complex, time-consuming and low-yield process by many researchers. By contrast, antibody-engineering methods proved to be the most frequently techniques used for the production of chicken mAbs. Actually, chicken provides an ideal basis for generating large immune antibody fragment libraries as compared to most mammalian species. In chickens, the large and diverse antibody repertoire is generated by gene conversion, in which segments from non-functional V pseudogenes located upstream are inserted into the rearranged gene, and somatic hypermutation. Since gene conversion has not been observed at the 5′- and 3′-ends of the rearranged gene, it is possible to perform real-time reverse transcription polymerase chain reaction (PCR) of the V-region repertoire with a single pair of primers[20,72]. Of the various recombinant antibody fragments, the full-length single-chain variable fragment (scFv) is the most commonly used. For construction of the scFv antibody library, total RNA is isolated from the spleen cells of immunized or non-immunized chicken and reverse-transcribed into cDNA. Then the variable heavy and light chain domain genes of immunoglobulin antibody cDNA are amplified by PCR and properly assembled to form the full-length scFv fragments, which resemble a functional Fv region. Then the scFv genes are cloned into suitable vectors to construct an antibody-expressing library [83]. Currently, phage display systems are the most often applied recombinant methods for generation and isolation of chicken mAbs[83,84]. In phage display methods, genetically-engineered phages that are capable of displaying recombinant fragments of antibodies on their coat surface can undergo several rounds of biopanning and re-propagation in *Escherichia coli* to enrich for clones exhibiting specific binding. Many IgY-scFv were produced with the phage display method combined with *in vitro* selection technologies, either by research groups[84-87] or companies that provide custom services for the development of monoclonal antibodies Y[88,89]. Among recent technologies reported for producing and isolating monoclonal IgYs is the gel encapsulated microenvironment assay, which is capable of "cross-examining" the entire population of splenic B cells from immunized chickens[90]. In an effort to produce mAbs suitable for *in vivo* administration in immunotherapy, the highly immunogenic constant region of chicken IgYs has been replaced with that of human to generate chicken-human chimeric antibodies[91]. Moreover, humanization of chicken scFvs has been successfully performed using the complementarity-determining region (CDR)-grafting strategy, which replaces human CDRs with chicken CDRs while retaining the human framework region residues, and followed by further optimization when necessary[92,93]. On the other hand, chimeric chicken-mouse or mouse-chicken recombinant mAbs have been produced and their characteristics have been studied[94,95].

Antibodies produced by genetically modified chickens (transgenic chickens)

Over the last decades, significant progress was made in generating recombinant proteins, including mAbs for therapeutic applications, in genetically modified chickens[21,96]. Difficulties in generating modified chickens are mainly attributed to the complex structure of the chicken zygote and the different organization of the chick embryo in comparison to mammals. To successfully generate genetically modified chickens, different methods have been used to achieve stable genomic integration of transgenes and the highest efficiency of germline transmission[97], including direct DNA microinjection into the chicken zygote[98] and use of viral vectors for gene transfer, which is the first applied and considered one of the most successful methods. Thus, the first genetically modified chicken was generated by the insertion of retroviral foreign DNA delivered by avian leukosis virus successfully integrated to the germline[99]. Since then, various viral vectors have been used to generate transgenic chickens for the production of recombinant proteins[100-102] including mAbs[103]. Among these, lentiviral vectors have been reported to offer specific advantages, including ability to transduce dividing and non-dividing cells, a relatively large transgene capacity and the apparent resistance of transduced cells to gene silencing [104]. Lentiviral vectors have been used to introduce transgene constructs comprising suitable sequences from the ovalbumin gene to direct synthesis of associated proteins to oviduct[105]. Despite the fact that the use of viral vectors improves germline transmission, the size limitation of the transgene and the lack of possibility of precise edits remain as drawbacks. One of the most effective approaches to produce transgenic chickens is the *in vitro* transfection of avian cell lines, such as primordial germ cells (PGCs) and embryonic stem cells (ES), the clonal selection and reinsertion

into the embryo leading to fully transgenic progeny in the next generation[106-108]. Following this approach, production of human mAbs in the egg white of chimeric transgenic chickens with the use of genetically modified ES cells carrying ovalbumin expression vectors was successfully performed for the first time; however, although a high amount of functional mAb was produced in the egg white, no transgenic offspring were initially obtained[107]. Heritable transgenic chickens capable of producing mAbs in their egg whites were generated using transfected PGCs with a gene construct designed to express the mAb in chicken oviduct magnum[108]. Specific gene editing of PGCs could be improved using genome-editing tools, such as transcriptional activator-like effector nucleases[109] and the clustered regularly interspaced short palindromic repeats–associated protein 9 system (CRISPR/Cas9 system)[110,111]. CRISPR/Cas9 has been used to generate transgenic chickens for the production of recombinant proteins in the white egg[111], including mAbs[112], or exhibiting resistance to pathogens[113]. Another recent promising approach is the replacement of the chicken immunoglobulin variable regions by human V regions and use of synthetic pseudogene arrays in order to produce affinity matured antibodies in transgenic chickens, called OmniChickens; OmniChicken can thus generate antibodies of basically human sequence, which retain the epitope repertoire of chicken immunoglobulins[114].

DISCUSSION

IgY technology has produced a great number of valuable immunochemical tools for biotechnology and medicine since 1990's. Various parameters that are associated with and can affect the results of the IgY technology have been reported in the literature, such as the immunization procedure. One of the most important parameters is the extraction/purification protocol used for isolating the IgYs from the egg yolk. Several methods of isolation and purification of IgYs from "immune" egg yolks have been reported, as already mentioned; the choice of a specific method depends on several criteria, such as desired yield, purity and final application of the IgYs along with cost and scale of extraction. The most popular isolation strategy consists in a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water, and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or with PEG, at suitable concentrations[30].

Our team have used the acidified water dilution method followed by precipitation with 19% sodium sulfate[39] or with 8.5% and 12% PEG 6000[27] for the isolation of IgYs from immune egg yolks. SDS-PAGE and western blot analysis of IgYs isolated with sodium sulfate precipitation has revealed a protein impurity with MW of ~35 kDa, which underwent liquid chromatography tandem mass spectrometry analysis and was proposed to be identical with the C-terminal fragment of vitellogenin II precursor protein[39]. The same impurity was also observed by other researchers, who had followed a different isolation protocol involving precipitation with PEG 6000[22]. As later shown[115,116], IgY from hen egg yolk occurs as a complex with peptides, named yolkin, which exhibit immunoregulatory and other biological activity. Yolkin contains several peptides with an apparent molecular weight ranging between 1 and 35 kDa. As reported, purified yolkin constituents are homologous with some fragments of the C-terminal region of vitellogenin II; more specifically, yolkin fractions of MW > 16 kDa are glycoproteins corresponding to the amino acid sequence of vitellogenin II starting at position 1572 aa[12,117]. In our hands, presence of the above impurity did not seem to interfere with the efficiency of IgYs as specific *in vitro* immune reagents.

As already mentioned, egg yolk IgYs have been thought to be superior to mammalian IgGs for *in vitro* applications. The *in vitro* efficiency of IgYs may be questioned only under rare conditions, *e.g.*, due to the putative presence of anti-hen antibodies in biological samples of specific individuals who have been sensitized to hen egg yolk[72]; however, to what extent IgY-specific antibodies may occur in human individuals remains to be clarified. Exempt from the aforementioned few concerns, IgYs are considered ideal and are being continuously developed and used as invaluable *in vitro* laboratory tools up to now (Table 1).

One of the great advantages of the IgY technology is the enhanced probability of generating specific IgYs against conserved mammalian proteins, since hens may exhibit a different antibody repertoire than that of mammalian organisms. With this in mind, our team has immunized hens against the poorly immunogenic, highly conserved polypeptide prothymosin alpha (ProTα, MW: approximately 12 kDa,

isolated from bovine thymus). The anti-ProTα antibodies Y were isolated from the egg yolk and evaluated through dot-blot and ELISA experiments in parallel with antibodies G isolated from the antiserum of rabbits immunized against the same immunogen. As revealed, not only antibodies G, but also antibodies Y showed hardly detectable titer/affinity for ProTα[39]. The above negative result may be attributed to the fact that ProTα is thought to be highly conserved during evolution and ProTα-homologues have been reported in non-mammalian organisms as well[76,118]. Similarly, hens were immunized against the highly conserved polypeptide thymosin beta4 (Tβ4, MW: ~5 kDa, synthetic), either conjugated to KLH (Tβ4/KLH) or non-conjugated, leading to IgYs of either relatively high titer or, on the contrary, not-detectable titer, respectively[41]. Interestingly, antibodies Y that we developed against a KLH-conjugate of ProTα (anti-ProTα/KLH IgYs) showed high titer and practically no cross-reactivity with a series of ProTα-fragments, including the N-terminal fragment ProTα[1-28] (also known as Tα1), being therefore highly specific for whole-length ProTα, while the corresponding anti-ProTα/KLH rabbit IgGs did cross-react with Tα1[76]. Moreover, when various synthetic fragments of ProTα or Tβ4 were conjugated to KLH and used for immunizing hens and rabbits, the results revealed that specific antibodies Y of hardly detectable titer were obtained; on the contrary, rabbit immunization with the same immunogens led to high-titer antibodies G, specific for ProTα or Tβ4, respectively[39,41]. The above results support the assumption that novel antibody specificities may emerge *via* the avian immune system and can be obtained through the IgY technology.

Although IgYs for research applications are mainly produced in hens, other birds have also served this purpose, as already mentioned, including duck[23,119], goose [24], quail[26] and ostrich[25], following immunization and isolation protocols similar to those used for hens[18]. Quail, ostrich and other avian species may provide further advantages in the field of IgY technology, such as convenient housing and breeding conditions (quail[26]) or exceptionally high amounts of IgYs obtained (ostrich[25]). Previously, our team has isolated immunoglobulins Y from the egg yolk of several avian species, including ostrich (Struthio camelus) and quail (Coturnix japonica); the isolation protocol has been developed in-house and based on the acidified water dilution and the PEG precipitation method. Ostrich and quail immunoglobulins Y were characterized in terms of their molecular weight (SDS-PAGE and western-blotting) and their ability to recognize and bind to a commercially available horseradish peroxidase (HRP)-labeled rabbit anti-hen IgY antibody in an ELISA system[120]. As revealed, the ostrich IgYs could be hardly recognized by the HRP-labeled anti-hen antibody we used, though other researchers reported successful use of commercially available secondary anti-hen antibodies to assess the immuno-chemical efficiency of specific ostrich IgYs[121]. On the other hand, HRP-labeled secondary anti-ostrich-IgY antibodies have been specially developed and used to evaluate ostrich IgYs with ELISA[25]. According to experimental results of ours[120] and others'[26,122], the quail IgYs could be recognized by the HRP-labeled secondary anti-hen antibody, which indicates that quail and hen IgYs may share at least some homology in immunochemically important structural features[123,124]. Wide availability of secondary antibodies for IgYs originated from avian species other than hens will support further expansion of the IgY technology.

In addition to their unequivocal usefulness as in *vitro* immunodetection reagents, IgYs have been proposed as promising *in vivo* therapeutics, *e.g.*, as an alternative to antibiotics treatment against multi-drug resistant or difficult-to-treat pathogens, since they exhibit *in vivo* pathogen-neutralizing activity, especially in mouth, throat, the respiratory tract and lungs[73]. Moreover, since IgYs are not absorbed by the gastro-intestinal tube, they have been proposed as perorally administered immunotherapeutics against various viral, bacterial, and fungal infections of the gastro-intestinal tract, especially in veterinary medicine and fish-cultivation[3]; a limitation in wide therapeutic application of perorally administered IgYs is their reduced stability at low pH[72] and several efforts have been made to address this shortcoming. IgYs have been also proposed as locally administered immunotherapeutics for treating skin and other local infections[3]. Lately, specific IgYs have been developed and used for treatment of the pandemic COVID-19[35,64,66,125]. Overall, despite the new promising technologies emerged, literature on the IgY technology continues to expand, encompassing various applications ranging from *in vitro* immunodetection of biomolecules and *in vitro* immunodiagnostics to *in vivo* immunotherapeutics[18,126].

Though development of monoclonal IgYs cannot be considered as a part of the original "IgY technology", it seems very attractive and will probably be the next big step in the area, since it combines the advantages of mAbs with those of avian IgYs. At the initial phase, production of chicken mAbs had to overcome several technical

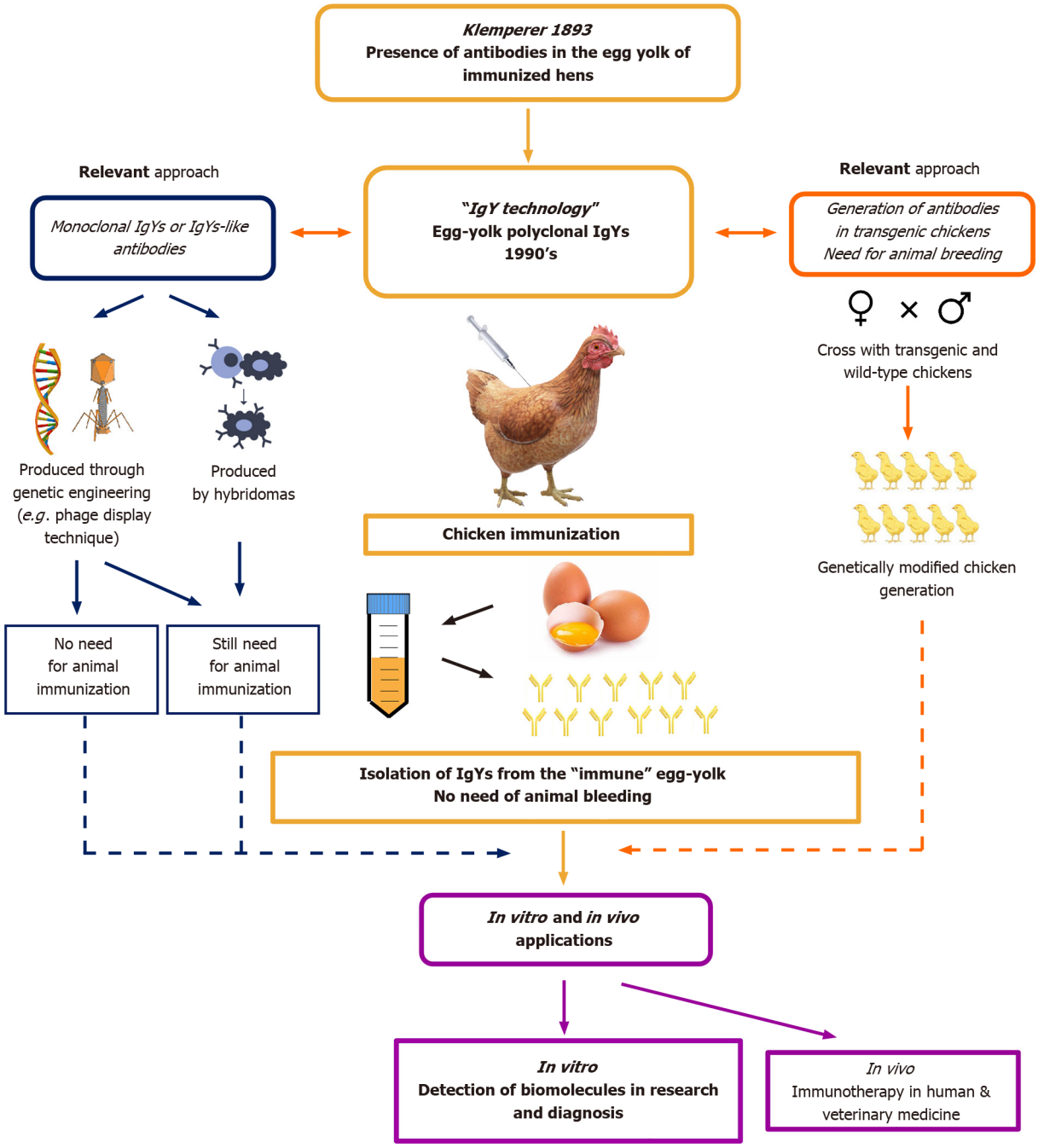

Figure 1 Schematic representation of the main parts comprising the original immunoglobulin Y technology (central axis); promising relevant approaches are also shown (periphery, left and right).

difficulties, including lack of appropriate fusion partners and loss of antibody secreting ability by the hybridoma cells over time[81]; this has been successfully addressed when monoclonal IgYs were generated through combinatorial antibody libraries *via* the phage display methodology[127]. Thus, over the past years, avian libraries have been constructed and several reports on the isolation of avian-derived antibody fragments have been published[20]. The different spectrum of epitopes recognized by the avian immune system may facilitate the development of novel diagnostics, *e.g.*, through targeting highly conserved mammalian proteins, while monoclonality may especially facilitate the development of novel therapeutics for human use, provided that the technology of chimeric avian/human fusions could be fully exploited. One should also keep in mind that recombinant technologies can lead to the generation of monoclonal IgY or IgY-like antibodies circumventing the need for animal immunization[72,83], which is desirable from an ethical point of view concerning the animal welfare.

It is important to remind that the IgY technology was introduced in 1990's as an alternative that could at least partly fulfil the ethics requirements set by the 3Rs principle[1,3]. Recently, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) has recommended that "animals should not be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications any longer", taking into account the Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on the scientific validity of replacements for animal-derived antibodies[128]. As referred to by the ESAC, the 2018 Nobel Prize in Chemistry was awarded "for the phage display of peptides and antibodies"[129,130], which, according to the Committee, proves maturity and supports wide application and full exploitation of the phage display technology in the area of antibody production. The EURL ECVAM recommendation may accelerate transformation/switch of the original IgY technology toward development of monoclonal IgYs through phage display techniques that totally avoid the animal immunization step. Total avoidance of animal immunization will further minimize the risk of zoonotic diseases, which is very low but still present when antibodies are produced in chickens, both wild and transgenic.

HIGHLIGHTS

The avian polyclonal antibodies/IgYs have unique and highly desirable functional features.

The term "IgY technology" describes the procedure involving immunization of avian species, consequent isolation of the polyclonal IgYs from the "immune" egg yolk (thus avoiding bleeding and animal stress) and application of the IgYs to various areas of medicine and biotechnology.

During the last decades the IgY technology has been widely used, especially as a means for the efficient *in vitro* immunodetection of biomolecules in many fields of research and disease diagnosis.

Despite the very promising relevant new approaches, there is still space for further exploiting the original IgY technology, due to specific functional, practical and ethical reasons and/or advantages.

CONCLUSION

Until now, development of polyclonal IgYs through the IgY technology has been widely used as a low cost and highly efficient tool, offering a lot of advantages and thus gaining wide application mainly in the *in vitro* immunodetection of biomolecules in biological specimens. Since polyclonal antibodies exhibit some unique functional qualities[131], there is still space for performing research to improve different aspects of the IgY technology. On the other hand, the original IgY technology may "merge" with relevant highly promising approaches, eventually leading, *e.g.*, to worldwide application of non-animal-derived recombinant IgYs or IgY-like immunoglobulins, which, among other benefits, will fulfil strict ethical requirements concerning animal welfare (Figure 1). However, until the practical problems associated with the above-mentioned approaches, *e.g.*, high-cost and/or limited availability of necessary reagents and protocols, have been fully addressed, the original IgY technology still remains a feasible, well-established procedure, in particular for low- and middle-income countries and research laboratories and especially in the field of *in vitro* immunodetection of biomolecules.

REFERENCES

1 **Schade R**, Staak C, Hendriksen C, Erhard M, Hugl H, Koch G, Larsson A, Pollmann W, van Regenmortel M, Rijke E, Spielmann H, Steinbusch H, Straughan D. The production of avian (egg yolk) antibodies: IgY. The report and recommendations of ECVAM workshop 21. *Altern Lab Anim* 1996; **24**: 925-934 [DOI: 10.1177/026119299602400607]

2 **Schade R**, Behn I, Erhard M, Hlinak A, C Staak C. Chicken egg yolk antibodies, production and application – IgY technology. Berlin: Springer Verlag, 2001

3 **Schade R**, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and

human and veterinary medicine. *Altern Lab Anim* 2005; **33**: 129-154 [PMID: 16180988 DOI: 10.1177/026119290503300208]
4 **Klemperer F**. Ÿber natörliche Immunität und ihre Verwerthung för die Immunisirungstherapie. *Arch Exp Pathol Pharmakol* 1893; **31**: 356-382
5 **Leslie GA**, Clem LW. Phylogen of immunoglobulin structure and function. 3. Immunoglobulins of the chicken. *J Exp Med* 1969; **130**: 1337-1352 [PMID: 5352783 DOI: 10.1084/jem.130.6.1337]
6 **Ulmer-Franco AM**, Cherian G, Quezada N, Fasenko GM, McMullen LM. Hatching egg and newly hatched chick yolk sac total IgY content at 3 broiler breeder flock ages. *Poult Sci* 2012; **91**: 758-764 [PMID: 22334753 DOI: 10.3382/ps.2011-01757]
7 **Murai A**, Hamano T, Kakiuchi M, Kobayashi M, Horio F. Evaluation of a receptor gene responsible for maternal blood IgY transfer into egg yolks using bursectomized IgY-depleted chickens. *Poult Sci* 2020; **99**: 1914-1920 [PMID: 32241471 DOI: 10.1016/j.psj.2019.11.045]
8 **Murai A**, Kakiuchi M, Hamano T, Kobayashi M, Tsudzuki M, Nakano M, Matsuda Y, Horio F. An ELISA for quantifying quail IgY and characterizing maternal IgY transfer to egg yolk in several quail strains. *Vet Immunol Immunopathol* 2016; **175**: 16-23 [PMID: 27269788 DOI: 10.1016/j.vetimm.2016.04.013]
9 **Hamal KR**, Burgess SC, Pevzner IY, Erf GF. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult Sci* 2006; **85**: 1364-1372 [PMID: 16903465 DOI: 10.1093/ps/85.8.1364]
10 **Russell WMS**, Burch RL. The principles of humane experimental technique. London: Methuen & Co., 1959
11 **Huang X**, Ahn DU. How Can the Value and Use of Egg Yolk Be Increased? *J Food Sci* 2019; **84**: 205-212 [PMID: 30620779 DOI: 10.1111/1750-3841.14430]
12 **Kazana W**, Mitkiewicz M, Ochnik M, Sochocka M, Zambrowicz A, Piechowiak G, Macała J, Miernikiewicz P, Zabłocka A. Yolkin Isolated from Hen Egg Yolk as a Natural Immunoregulator, Activating Innate Immune Response in BMDM Macrophages. *Oxid Med Cell Longev* 2020; **2020**: 5731021 [PMID: 32509146 DOI: 10.1155/2020/5731021]
13 **Zambrowicz A**, Dąbrowska A, Bobak Ł, Szołtysik M. Egg yolk proteins and peptides with biological activity. *Postepy Hig Med Dosw (Online)* 2014; **68**: 1524-1529 [PMID: 25834095 DOI: 10.5604/17322693.1133600]
14 **Yamamura J**, Adachi T, Aoki N, Nakajima H, Nakamura R, Matsuda T. Precursor-product relationship between chicken vitellogenin and the yolk proteins: the 40 kDa yolk plasma glycoprotein is derived from the C-terminal cysteine-rich domain of vitellogenin II. *Biochim Biophys Acta* 1995; **1244**: 384-394 [PMID: 7599159 DOI: 10.1016/0304-4165(95)00033-8]
15 **Taylor AI**, Fabiane SM, Sutton BJ, Calvert RA. The crystal structure of an avian IgY-Fc fragment reveals conservation with both mammalian IgG and IgE. *Biochemistry* 2009; **48**: 558-562 [PMID: 19115948 DOI: 10.1021/bi8019993]
16 **Härtle S**, Magor KE, Göbel TW, Davison F, Kaspers B. Structure and evolution of avian immunoglobulins. In: Schat KA, Kaspers B, Kaiser P, editors. Avian immunology. 2nd ed. Boston: Academic Press, 2014: 103-120
17 **Sun Y**, Wei Z, Li N, Zhao Y. A comparative overview of immunoglobulin genes and the generation of their diversity in tetrapods. *Dev Comp Immunol* 2013; **39**: 103-109 [PMID: 22366185 DOI: 10.1016/j.dci.2012.02.008]
18 **Pereira EPV**, van Tilburg MF, Florean EOPT, Guedes MIF. Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. *Int Immunopharmacol* 2019; **73**: 293-303 [PMID: 31128529 DOI: 10.1016/j.intimp.2019.05.015]
19 **Larsson A**, Sjöquist J. Chicken IgY: utilizing the evolutionary difference. *Comp Immunol Microbiol Infect Dis* 1990; **13**: 199-201 [PMID: 2076606 DOI: 10.1016/0147-9571(90)90088-B]
20 **Lee W**, Syed Atif A, Tan SC, Leow CH. Insights into the chicken IgY with emphasis on the generation and applications of chicken recombinant monoclonal antibodies. *J Immunol Methods* 2017; **447**: 71-85 [PMID: 28502720 DOI: 10.1016/j.jim.2017.05.001]
21 **Bahrami S**, Amiri-Yekta A, Daneshipour A, Jazayeri SH, Mozdziak PE, Sanati MH, Gourabi H. Designing A Transgenic Chicken: Applying New Approaches toward A Promising Bioreactor. *Cell J* 2020; **22**: 133-139 [PMID: 31721526 DOI: 10.22074/cellj.2020.6738]
22 **Pauly D**, Chacana PA, Calzado EG, Brembs B, Schade R. IgY technology: extraction of chicken antibodies from egg yolk by polyethylene glycol (PEG) precipitation. *J Vis Exp* 2011 [PMID: 21559009 DOI: 10.3791/3084]
23 **Brocato R**, Josleyn M, Ballantyne J, Vial P, Hooper JW. DNA vaccine-generated duck polyclonal antibodies as a postexposure prophylactic to prevent hantavirus pulmonary syndrome (HPS). *PLoS One* 2012; **7**: e35996 [PMID: 22558299 DOI: 10.1371/journal.pone.0035996]
24 **Haese N**, Brocato RL, Henderson T, Nilles ML, Kwilas SA, Josleyn MD, Hammerbeck CD, Schiltz J, Royals M, Ballantyne J, Hooper JW, Bradley DS. Antiviral Biologic Produced in DNA Vaccine/Goose Platform Protects Hamsters Against Hantavirus Pulmonary Syndrome When Administered Post-exposure. *PLoS Negl Trop Dis* 2015; **9**: e0003803 [PMID: 26046641 DOI: 10.1371/journal.pntd.0003803]
25 **Adachi K**, Handharyani E, Sari DK, Takama K, Fukuda K, Endo I, Yamamoto R, Sawa M, Tanaka M, Konishi I, Tsukamoto Y. Development of neutralization antibodies against highly pathogenic H5N1 avian influenza virus using ostrich (Struthio camelus) yolk. *Mol Med Rep* 2008; **1**: 203-209 [PMID: 21479398 DOI: 10.3892/mmr.1.2.203]

26 **Esmailnejad A**, Abdi-Hachesoo B, Hosseini Nasab E, Shakoori M. Production, purification, and evaluation of quail immunoglobulin Y against Salmonella typhimurium and Salmonella enteritidis. *Mol Immunol* 2019; **107**: 79-83 [PMID: 30665061 DOI: 10.1016/j.molimm.2019.01.012]

27 **Neokosmidi A**, Ragoussis V, Zikos C, Paravatou-Petsotas M, Livaniou E, Ragoussis N, Evangelatos G. Determination of natural olive fruit fly pheromone in insect samples by enzyme linked immunoassays. *Talanta* 2008; **74**: 539-546 [PMID: 18371673 DOI: 10.1016/j.talanta.2007.06.015]

28 **Marcq C**, Marlier D, Beckers Y. Improving adjuvant systems for polyclonal egg yolk antibody (IgY) production in laying hens in terms of productivity and animal welfare. *Vet Immunol Immunopathol* 2015; **165**: 54-63 [PMID: 25813905 DOI: 10.1016/j.vetimm.2015.02.012]

29 **Grando TH**, Baldissera MD, de Sá MF, do Carmo GM, Porto BCZ, Aguirre GSV, Azevedo MI, de Jesus FPK, Santurio JM, Sagrillo MR, Stefani LM, Monteiro SG. Avian antibodies (IgY) against Trypanosoma cruzi: Purification and characterization studies. *J Immunol Methods* 2017; **449**: 56-61 [PMID: 28697990 DOI: 10.1016/j.jim.2017.07.002]

30 **Amro WA**, Al-Qaisi W, Al-Razem F. Production and purification of IgY antibodies from chicken egg yolk. *J Genet Eng Biotechnol* 2018; **16**: 99-103 [PMID: 30647711 DOI: 10.1016/j.jgeb.2017.10.003]

31 **Lopes CA**, de Faria LS, de Sousa JEN, Borges IP, Ribeiro RP, Bueno LL, Rodrigues Ávila VM, Ferreira Júnior Á, Costa-Cruz JM. Anti-Ascaris suum immunoglobulin Y as a novel biotechnological tool for the diagnosis of human ascariasis. *J Helminthol* 2019; **94**: e71 [PMID: 31409433 DOI: 10.1017/S0022149X19000701]

32 **Silva GBD**, Faria LSD, Lopes CA, Nunes DS, Ribeiro VS, de Sousa JEN, Paiva GCM, Gonçalves-Pires MRF, Borges IP, Santos MM, Ávila VMR, Júnior ÁF, Costa-Cruz JM. Egg yolk immunoglobulin Y as a promising tool to detect immune complexes in neurocysticercosis serum samples. *Trans R Soc Trop Med Hyg* 2020; **114**: 585-592 [PMID: 32484880 DOI: 10.1093/trstmh/traa028]

33 **de Faria LS**, de Souza DLN, Ribeiro RP, de Sousa JEN, Borges IP, Ávila VMR, Ferreira-Júnior Á, Goulart LR, Costa-Cruz JM. Highly specific and sensitive anti-Strongyloides venezuelensis IgY antibodies applied to the human strongyloidiasis immunodiagnosis. *Parasitol Int* 2019; **72**: 101933 [PMID: 31128257 DOI: 10.1016/j.parint.2019.101933]

34 **Skottrup PD**, López R, Ksiazek M, Højrup P, Baelum V, Potempa J, Kaczmarek JZ. An IgY-based immunoassay to evaluate the biomarker potential of the Tannerella forsythia virulence factor karilysin in human saliva. *J Immunol Methods* 2019; **469**: 26-32 [PMID: 30880264 DOI: 10.1016/j.jim.2019.03.003]

35 **Lu Y**, Wang Y, Zhang Z, Huang J, Yao M, Huang G, Ge Y, Zhang P, Huang H, Li H, Wang W. Generation of Chicken IgY against SARS-COV-2 Spike Protein and Epitope Mapping. *J Immunol Res* 2020; **2020**: 9465398 [PMID: 33134398 DOI: 10.1155/2020/9465398]

36 **Grzywa R**, Łupicka-Słowik A, Walczak M, Idzi M, Bobrek K, Boivin S, Gaweł A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Highly sensitive detection of cancer antigen 15-3 using novel avian IgY antibodies. *ALTEX* 2014; **31**: 43-52 [PMID: 24270753 DOI: 10.14573/altex.1309181]

37 **Łupicka-Słowik A**, Walczak M, Grzywa R, Bobrek K, Łęcka M, Boivin S, Gaweł A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Generation and application of polyclonal IgY antibodies specific for full-length and nicked prostate-specific antigen. *Bioanalysis* 2014; **6**: 3197-3213 [PMID: 25529887 DOI: 10.4155/bio.14.172]

38 **Sotiropoulou G**, Pampalakis G, Prosnikli E, Evangelatos GP, Livaniou E. Development and immunochemical evaluation of a novel chicken IgY antibody specific for KLK6. *Chem Cent J* 2012; **6**: 148 [PMID: 23216878 DOI: 10.1186/1752-153X-6-148]

39 **Klimentzou P**, Paravatou-Petsotas M, Zikos C, Beck A, Skopeliti M, Czarnecki J, Tsitsilonis O, Voelter W, Livaniou E, Evangelatos GP. Development and immunochemical evaluation of antibodies Y for the poorly immunogenic polypeptide prothymosin alpha. *Peptides* 2006; **27**: 183-193 [PMID: 16150512 DOI: 10.1016/j.peptides.2005.07.002]

40 **Klimentzou P**, Drougou A, Fehrenbacher B, Schaller M, Voelter W, Barbatis C, Paravatou-Petsotas M, Livaniou E. Immunocytological and preliminary immunohistochemical studies of prothymosin alpha, a human cancer-associated polypeptide, with a well-characterized polyclonal antibody. *J Histochem Cytochem* 2008; **56**: 1023-1031 [PMID: 18711212 DOI: 10.1369/jhc.2008.950956]

41 **Livaniou E**, Paravatou-Petsotas M, Bourkoula A, Klimentzou P, Zikos C, Evangelatos GP. Development of anti-thymosin beta-4 IgY-antibodies and their preliminary application to the immunostaining of human breast cancer cells. In: Flegel M, Fridkin M, Gilon C, Slaninova J, editors. Proceedings of the 28th European Peptide Symposium; 2004 Sep 5-10; Prague, Czech Republic. Geneva: Kenes International, 2005: 1125-1126

42 **Ren H**, Yang W, Thirumalai D, Zhang X, Schade R. A comparative evaluation of six principal IgY antibody extraction methods. *Altern Lab Anim* 2016; **44**: 11-20 [PMID: 27031600 DOI: 10.1177/026119291604400111]

43 **Thibodeau A**, Fravalo P, Perron A, Lewandowski SL, Letellier A. Production and characterization of anti-Campylobacter jejuni IgY derived from egg yolks. *Acta Vet Scand* 2017; **59**: 80 [PMID: 29208016 DOI: 10.1186/s13028-017-0346-4]

44 **Leenaars M**, Hendriksen CF. Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. *ILAR J* 2005; **46**: 269-279 [PMID: 15953834 DOI: 10.1093/ilar.46.3.269]

45 **Vandeputte J**, Martel A, Antonissen G, Verlinden M, De Zutter L, Heyndrickx M, Haesebrouck F,

Pasmans F, Garmyn A. Research Note: Lyophilization of hyperimmune egg yolk: effect on antibody titer and protection of broilers against Campylobacter colonization. *Poult Sci* 2020; **99**: 2157-2161 [PMID: 32241501 DOI: 10.1016/j.psj.2019.11.054]

46 **Cova L**. DNA-designed avian IgY antibodies: novel tools for research, diagnostics and therapy. *J Clin Virol* 2005; **34** Suppl 1: S70-S74 [PMID: 16461227 DOI: 10.1016/s1386-6532(05)80013-7]

47 **Nikbakht Brujeni G**, Jalali SA, Koohi MK. Development of DNA-designed avian IgY antibodies for quantitative determination of bovine interferon-gamma. *Appl Biochem Biotechnol* 2011; **163**: 338-345 [PMID: 20652441 DOI: 10.1007/s12010-010-9042-9]

48 **Niederstadt L**, Hohn O, Dorner BG, Schade R, Bannert N. Stimulation of IgY responses in gene gun immunized laying hens by combined administration of vector DNA coding for the target antigen Botulinum toxin A1 and for avian cytokine adjuvants. *J Immunol Methods* 2012; **382**: 58-67 [PMID: 22580181 DOI: 10.1016/j.jim.2012.05.005]

49 **Witkowski PT**, Bourquain DR, Hohn O, Schade R, Nitsche A. Gene gun-supported DNA immunisation of chicken for straightforward production of poxvirus-specific IgY antibodies. *J Immunol Methods* 2009; **341**: 146-153 [PMID: 19100269 DOI: 10.1016/j.jim.2008.11.008]

50 **Pauly D**, Dorner M, Zhang X, Hlinak A, Dorner B, Schade R. Monitoring of laying capacity, immunoglobulin Y concentration, and antibody titer development in chickens immunized with ricin and botulinum toxins over a two-year period. *Poult Sci* 2009; **88**: 281-290 [PMID: 19151341 DOI: 10.3382/ps.2008-00323]

51 **Tini M**, Jewell UR, Camenisch G, Chilov D, Gassmann M. Generation and application of chicken egg-yolk antibodies. *Comp Biochem Physiol A Mol Integr Physiol* 2002; **131**: 569-574 [PMID: 11867282 DOI: 10.1016/s1095-6433(01)00508-6]

52 **Akita EM**, Nakai S. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic E. coli strain. *J Immunol Methods* 1993; **160**: 207-214 [PMID: 8459107 DOI: 10.1016/0022-1759(93)90179-b]

53 **Bade H**, Stegemann H. Rapid method of extraction of antibodies from hen egg yolk. *J Immunol Methods* 1984; **72**: 421-426 [PMID: 6432912 DOI: 10.1016/0022-1759(84)90010-3]

54 **Bizhanov G**, Vyshniauskis G. A comparison of three methods for extracting IgY from the egg yolk of hens immunized with Sendai virus. *Vet Res Commun* 2000; **24**: 103-113 [PMID: 10720096 DOI: 10.1023/a:1006460506303]

55 **Araújo AS**, Lobato ZI, Chávez-Olórtegui C, Velarde DT. Brazilian IgY-Bothrops antivenom: Studies on the development of a process in chicken egg yolk. *Toxicon* 2010; **55**: 739-744 [PMID: 19925817 DOI: 10.1016/j.toxicon.2009.11.004]

56 **Hatta H**, Kim M, Yamamoto T. A novel isolation method for hen egg yolk antibody, "IgY". *Agric Biol Chem* 1990; **54**: 2531-2535 [PMID: 1368596 DOI: 10.1271/bbb1961.54.2531]

57 **Tong C**, Geng F, He Z, Cai Z, Ma M. A simple method for isolating chicken egg yolk immunoglobulin using effective delipidation solution and ammonium sulfate. *Poult Sci* 2015; **94**: 104-110 [PMID: 25542196 DOI: 10.3382/ps/peu005]

58 **Lee HY**, Abeyrathne ED, Choi I, Suh JW, Ahn DU. Sequential separation of immunoglobulin Y and phosvitin from chicken egg yolk without using organic solvents. *Poult Sci* 2014; **93**: 2668-2677 [PMID: 25085938 DOI: 10.3382/ps.2014-04093]

59 **Ko KY**, Ahn DU. Preparation of immunoglobulin Y from egg yolk using ammonium sulfate precipitation and ion exchange chromatography. *Poult Sci* 2007; **86**: 400-407 [PMID: 17234857 DOI: 10.1093/ps/86.2.400]

60 **Polson A**, Coetzer T, Kruger J, von Maltzahn E, van der Merwe KJ. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Invest* 1985; **14**: 323-327 [PMID: 4065934 DOI: 10.3109/08820138509022667]

61 **McLaren RD**, Prosser CG, Grieve RC, Borissenko M. The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine alpha-lactalbumin. *J Immunol Methods* 1994; **177**: 175-184 [PMID: 7822824 DOI: 10.1016/0022-1759(94)90154-6]

62 **Redwan EM**, Aljadawi AA, Uversky VN. Simple and efficient protocol for immunoglobulin Y purification from chicken egg yolk. *Poult Sci* 2021; **100**: 100956 [PMID: 33652537 DOI: 10.1016/j.psj.2020.12.053]

63 **Tan SH**, Mohamedali A, Kapur A, Lukjanenko L, Baker MS. A novel, cost-effective and efficient chicken egg IgY purification procedure. *J Immunol Methods* 2012; **380**: 73-76 [PMID: 22484081 DOI: 10.1016/j.jim.2012.03.003]

64 **Constantin C**, Neagu M, Diana Supeanu T, Chiurciu V, A Spandidos D. IgY - turning the page toward passive immunization in COVID-19 infection (Review). *Exp Ther Med* 2020; **20**: 151-158 [PMID: 32536989 DOI: 10.3892/etm.2020.8704]

65 **Ntakarutimana V**, Demedts P, van Sande M, Scharpé S. A simple and economical strategy for downstream processing of specific antibodies to human transferrin from egg yolk. *J Immunol Methods* 1992; **153**: 133-140 [PMID: 1517583 DOI: 10.1016/0022-1759(92)90315-k]

66 **Pérez de la Lastra JM**, Baca-González V, Asensio-Calavia P, González-Acosta S, Morales-delaNuez A. Can Immunization of Hens Provide Oral-Based Therapeutics against COVID-19? *Vaccines (Basel)* 2020; **8** [PMID: 32872186 DOI: 10.3390/vaccines8030486]

67 **Xia W**, Lu H, Li Y, Cao J, Zhou X, Zhang X, Xia X, Sun H. Purification of chicken IgY by binding capture using elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G. *Vet Immunol Immunopathol* 2017; **192**: 13-19 [PMID: 29042010 DOI: 10.1016/j.vetimm.2017.09.002]

68 **Khan KH**, Himeno A, Kosugi S, Nakashima Y, Rafique A, Imamura A, Hatanaka T, Kato DI, Ito Y. IgY-binding peptide screened from a random peptide library as a ligand for IgY purification. *J Pept Sci* 2017; **23**: 790-797 [PMID: 28758361 DOI: 10.1002/psc.3027]

69 **Hansen P**, Scoble JA, Hanson B, Hoogenraad NJ. Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. *J Immunol Methods* 1998; **215**: 1-7 [PMID: 9744742 DOI: 10.1016/s0022-1759(98)00050-7]

70 **Hernández-Campos FJ**, Brito-De la Fuente E, Torrestiana-Sánchez B. Purification of egg yolk immunoglobulin (IgY) by ultrafiltration: effect of pH, ionic strength, and membrane properties. *J Agric Food Chem* 2010; **58**: 187-193 [PMID: 19994898 DOI: 10.1021/jf902964s]

71 **Larsson A**, Bålöw RM, Lindahl TL, Forsberg PO. Chicken antibodies: taking advantage of evolution--a review. *Poult Sci* 1993; **72**: 1807-1812 [PMID: 8415358 DOI: 10.3382/ps.0721807]

72 **Spillner E**, Braren I, Greunke K, Seismann H, Blank S, du Plessis D. Avian IgY antibodies and their recombinant equivalents in research, diagnostics and therapy. *Biologicals* 2012; **40**: 313-322 [PMID: 22748514 DOI: 10.1016/j.biologicals.2012.05.003]

73 **Abbas AT**, El-Kafrawy SA, Sohrab SS, Azhar EIA. IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. *Hum Vaccin Immunother* 2019; **15**: 264-275 [PMID: 30230944 DOI: 10.1080/21645515.2018.1514224]

74 **Fu CY**, Huang H, Wang XM, Liu YG, Wang ZG, Cui SJ, Gao HL, Li Z, Li JP, Kong XG. Preparation and evaluation of anti-SARS coronavirus IgY from yolks of immunized SPF chickens. *J Virol Methods* 2006; **133**: 112-115 [PMID: 16325277 DOI: 10.1016/j.jviromet.2005.10.027]

75 **Emami F**, Vatanara A, Park EJ, Na DH. Drying Technologies for the Stability and Bioavailability of Biopharmaceuticals. *Pharmaceutics* 2018; **10** [PMID: 30126135 DOI: 10.3390/pharmaceutics10030131]

76 **Karachaliou CE**, Kostopoulos IV, Vassilakopoulou V, Klimentzou P, Paravatou-Petsotas M, Voelter W, Kalbacher H, Zikos C, Tsitsilonis O, Livaniou E. Development of a specific IgY-based ELISA for prothymosin alpha, a bioactive polypeptide with diagnostic and therapeutic potential. *Heliyon* 2019; **5**: e02616 [PMID: 31720448 DOI: 10.1016/j.heliyon.2019.e02616]

77 **Yi L**, Qin Z, Lin H, Zhou Y, Li J, Xu Z, Babu V S, Lin L. Features of chicken egg yolk immunoglobulin (IgY) against the infection of red-spotted grouper nervous necrosis virus. *Fish Shellfish Immunol* 2018; **80**: 534-539 [PMID: 29906624 DOI: 10.1016/j.fsi.2018.06.024]

78 **Zhu Y**, Ma Y, Lu M, Zhang Y, Li A, Liang X, Li J. Efficient Production of Human Norovirus-Specific IgY in Egg Yolks by Vaccination of Hens with a Recombinant Vesicular Stomatitis Virus Expressing VP1 Protein. *Viruses* 2019; **11** [PMID: 31100802 DOI: 10.3390/v11050444]

79 **Porte L**, Legarraga P, Vollrath V, Aguilera X, Munita JM, Araos R, Pizarro G, Vial P, Iruretagoyena M, Dittrich S, Weitzel T. Evaluation of a novel antigen-based rapid detection test for the diagnosis of SARS-CoV-2 in respiratory samples. *Int J Infect Dis* 2020; **99**: 328-333 [PMID: 32497809 DOI: 10.1016/j.ijid.2020.05.098]

80 **Grant BD**, Anderson CE, Williford JR, Alonzo LF, Glukhova VA, Boyle DS, Weigl BH, Nichols KP. SARS-CoV-2 Coronavirus Nucleocapsid Antigen-Detecting Half-Strip Lateral Flow Assay Toward the Development of Point of Care Tests Using Commercially Available Reagents. *Anal Chem* 2020; **92**: 11305-11309 [PMID: 32605363 DOI: 10.1021/acs.analchem.0c01975]

81 **Nishinaka S**, Suzuki T, Matsuda H, Murata M. A new cell line for the production of chicken monoclonal antibody by hybridoma technology. *J Immunol Methods* 1991; **139**: 217-222 [PMID: 2045661 DOI: 10.1016/0022-1759(91)90191-h]

82 **Matsuda H**, Mitsuda H, Nakamura N, Furusawa S, Mohri S, Kitamoto T. A chicken monoclonal antibody with specificity for the N-terminal of human prion protein. *FEMS Immunol Med Microbiol* 1999; **23**: 189-194 [PMID: 10219590 DOI: 10.1111/j.1574-695X.1999.tb01238.x]

83 **Davies EL**, Smith JS, Birkett CR, Manser JM, Anderson-Dear DV, Young JR. Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *J Immunol Methods* 1995; **186**: 125-135 [PMID: 7561141 DOI: 10.1016/0022-1759(95)00143-x]

84 **Nakamura N**, Shimokawa M, Miyamoto K, Hojyo S, Horiuchi H, Furusawa S, Matsuda H. Two expression vectors for the phage-displayed chicken monoclonal antibody. *J Immunol Methods* 2003; **280**: 157-164 [PMID: 12972196 DOI: 10.1016/s0022-1759(03)00204-7]

85 **van Wyngaardt W**, Malatji T, Mashau C, Fehrsen J, Jordaan F, Miltiadou D, du Plessis DH. A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnol* 2004; **4**: 6 [PMID: 15059288 DOI: 10.1186/1472-6750-4-6]

86 **Ge S**, Xu L, Li B, Zhong F, Liu X, Zhang X. Canine Parvovirus is diagnosed and neutralized by chicken IgY-scFv generated against the virus capsid protein. *Vet Res* 2020; **51**: 110 [PMID: 32883344 DOI: 10.1186/s13567-020-00832-7]

87 **Lee YC**, Leu SJ, Hung HC, Wu HH, Huang IJ, Hsieh WS, Chiu WT, Hsieh MS, Cheng TF, Yang YY. A dominant antigenic epitope on SARS-CoV spike protein identified by an avian single-chain variable fragment (scFv)-expressing phage. *Vet Immunol Immunopathol* 2007; **117**: 75-85 [PMID: 17360045 DOI: 10.1016/j.vetimm.2007.02.001]

88 **Genwaybiotech**. IgY Monoclonal Antibodies. [cited 4 February 2021]. Available from: https://www.genwaybio.com/technologies/igy-monoclonal-antibodies

89 **Creative-biolabs**. Chicken IgY Antibody Production. [cited 4 February 2021]. Available from: https://www.creative-biolabs.com/igY-antibody-generation.html

90 **Mettler Izquierdo S**, Varela S, Park M, Collarini EJ, Lu D, Pramanick S, Rucker J, Lopalco L,

Etches R, Harriman W. High-efficiency antibody discovery achieved with multiplexed microscopy. *Microscopy (Oxf)* 2016; **65**: 341-352 [PMID: 27107009 DOI: 10.1093/jmicro/dfw014]

91 **Nishibori N**, Shimamoto T, Nakamura N, Shimokawa M, Horiuchi H, Furusawa S, Matsuda H. Expression vectors for chicken-human chimeric antibodies. *Biologicals* 2004; **32**: 213-218 [PMID: 15572103 DOI: 10.1016/j.biologicals.2004.09.002]

92 **Tsurushita N**, Park M, Pakabunto K, Ong K, Avdalovic A, Fu H, Jia A, Vásquez M, Kumar S. Humanization of a chicken anti-IL-12 monoclonal antibody. *J Immunol Methods* 2004; **295**: 9-19 [PMID: 15627607 DOI: 10.1016/j.jim.2004.08.018]

93 **Nishibori N**, Horiuchi H, Furusawa S, Matsuda H. Humanization of chicken monoclonal antibody using phage-display system. *Mol Immunol* 2006; **43**: 634-642 [PMID: 16360012 DOI: 10.1016/j.molimm.2005.04.002]

94 **Tateishi Y**, Nishimichi N, Horiuchi H, Furusawa S, Matsuda H. Construction of chicken-mouse chimeric antibody and immunogenicity in mice. *J Vet Med Sci* 2008; **70**: 397-400 [PMID: 18460836 DOI: 10.1292/jvms.70.397]

95 **Choi J**, Kim M, Lee J, Seo Y, Ham Y, Kim JK, Kwon MH. Antigen-binding affinity and thermostability of chimeric mouse-chicken IgY and mouse-human IgG antibodies with identical variable domains. *Sci Rep* 2019; **9**: 19242 [PMID: 31848417 DOI: 10.1038/s41598-019-55805-4]

96 **Park JS**, Lee KY, Han JY. Precise Genome Editing in Poultry and Its Application to Industries. *Genes (Basel)* 2020; **11** [PMID: 33053652 DOI: 10.3390/genes11101182]

97 **Sid H**, Schusser B. Applications of Gene Editing in Chickens: A New Era Is on the Horizon. *Front Genet* 2018; **9**: 456 [PMID: 30356667 DOI: 10.3389/fgene.2018.00456]

98 **Love J**, Gribbin C, Mather C, Sang H. Transgenic birds by DNA microinjection. *Biotechnology (N Y)* 1994; **12**: 60-63 [PMID: 7764327 DOI: 10.1038/nbt0194-60]

99 **Salter DW**, Smith EJ, Hughes SH, Wright SE, Crittenden LB. Transgenic chickens: insertion of retroviral genes into the chicken germ line. *Virology* 1987; **157**: 236-240 [PMID: 3029962 DOI: 10.1016/0042-6822(87)90334-5]

100 **Salter DW**, Crittenden LB. Artificial insertion of a dominant gene for resistance to avian leukosis virus into the germ line of the chicken. *Theor Appl Genet* 1989; **77**: 457-461 [PMID: 24232709 DOI: 10.1007/BF00274263]

101 **Kamihira M**, Ono K, Esaka K, Nishijima K, Kigaku R, Komatsu H, Yamashita T, Kyogoku K, Iijima S. High-level expression of single-chain Fv-Fc fusion protein in serum and egg white of genetically manipulated chickens by using a retroviral vector. *J Virol* 2005; **79**: 10864-10874 [PMID: 16103139 DOI: 10.1128/jvi.79.17.10864-10874.2005]

102 **Harvey AJ**, Speksnijder G, Baugh LR, Morris JA, Ivarie R. Consistent production of transgenic chickens using replication-deficient retroviral vectors and high-throughput screening procedures. *Poult Sci* 2002; **81**: 202-212 [PMID: 11873828 DOI: 10.1093/ps/81.2.202]

103 **Kamihira M**, Kawabe Y, Shindo T, Ono K, Esaka K, Yamashita T, Nishijima K, Iijima S. Production of chimeric monoclonal antibodies by genetically manipulated chickens. *J Biotechnol* 2009; **141**: 18-25 [PMID: 19428726 DOI: 10.1016/j.jbiotec.2009.02.022]

104 **McGrew MJ**, Sherman A, Ellard FM, Lillico SG, Gilhooley HJ, Kingsman AJ, Mitrophanous KA, Sang H. Efficient production of germline transgenic chickens using lentiviral vectors. *EMBO Rep* 2004; **5**: 728-733 [PMID: 15192698 DOI: 10.1038/sj.embor.7400171]

105 **Lillico SG**, Sherman A, McGrew MJ, Robertson CD, Smith J, Haslam C, Barnard P, Radcliffe PA, Mitrophanous KA, Elliot EA, Sang HM. Oviduct-specific expression of two therapeutic proteins in transgenic hens. *Proc Natl Acad Sci U S A* 2007; **104**: 1771-1776 [PMID: 17259305 DOI: 10.1073/pnas.0610401104]

106 **van de Lavoir MC**, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, Bradshaw R, Kerchner A, Hooi LT, Gessaro TM, Swanberg SE, Delany ME, Etches RJ. Germline transmission of genetically modified primordial germ cells. *Nature* 2006; **441**: 766-769 [PMID: 16760981 DOI: 10.1038/nature04831]

107 **Zhu L**, van de Lavoir MC, Albanese J, Beenhouwer DO, Cardarelli PM, Cuison S, Deng DF, Deshpande S, Diamond JH, Green L, Halk EL, Heyer BS, Kay RM, Kerchner A, Leighton PA, Mather CM, Morrison SL, Nikolov ZL, Passmore DB, Pradas-Monne A, Preston BT, Rangan VS, Shi M, Srinivasan M, White SG, Winters-Digiacinto P, Wong S, Zhou W, Etches RJ. Production of human monoclonal antibody in eggs of chimeric chickens. *Nat Biotechnol* 2005; **23**: 1159-1169 [PMID: 16127450 DOI: 10.1038/nbt1132]

108 **Kim YM**, Park JS, Kim SK, Jung KM, Hwang YS, Han M, Lee HJ, Seo HW, Suh JY, Han BK, Han JY. The transgenic chicken derived anti-CD20 monoclonal antibodies exhibits greater anti-cancer therapeutic potential with enhanced Fc effector functions. *Biomaterials* 2018; **167**: 58-68 [PMID: 29554481 DOI: 10.1016/j.biomaterials.2018.03.021]

109 **Taylor L**, Carlson DF, Nandi S, Sherman A, Fahrenkrug SC, McGrew MJ. Efficient TALEN-mediated gene targeting of chicken primordial germ cells. *Development* 2017; **144**: 928-934 [PMID: 28174243 DOI: 10.1242/dev.145367]

110 **Dimitrov L**, Pedersen D, Ching KH, Yi H, Collarini EJ, Izquierdo S, van de Lavoir MC, Leighton PA. Germline Gene Editing in Chickens by Efficient CRISPR-Mediated Homologous Recombination in Primordial Germ Cells. *PLoS One* 2016; **11**: e0154303 [PMID: 27099923 DOI: 10.1371/journal.pone.0154303]

111 **Oishi I**, Yoshii K, Miyahara D, Kagami H, Tagami T. Targeted mutagenesis in chicken using CRISPR/Cas9 system. *Sci Rep* 2016; **6**: 23980 [PMID: 27050479 DOI: 10.1038/srep23980]

112 **Mukae T**, Okumura S, Watanobe T, Yoshii K, Tagami T, Oishi I. Production of Recombinant Monoclonal Antibodies in the Egg White of Gene-Targeted Transgenic Chickens. *Genes (Basel)* 2020; **12** [PMID: 33396657 DOI: 10.3390/genes12010038]

113 **Lee HJ**, Lee KY, Jung KM, Park KJ, Lee KO, Suh JY, Yao Y, Nair V, Han JY. Precise gene editing of chicken Na+/H+ exchange type 1 (chNHE1) confers resistance to avian leukosis virus subgroup J (ALV-J). *Dev Comp Immunol* 2017; **77**: 340-349 [PMID: 28899753 DOI: 10.1016/j.dci.2017.09.006]

114 **Ching KH**, Collarini EJ, Abdiche YN, Bedinger D, Pedersen D, Izquierdo S, Harriman R, Zhu L, Etches RJ, van de Lavoir MC, Harriman WD, Leighton PA. Chickens with humanized immunoglobulin genes generate antibodies with high affinity and broad epitope coverage to conserved targets. *MAbs* 2018; **10**: 71-80 [PMID: 29035625 DOI: 10.1080/19420862.2017.1386825]

115 **Polanowski A**, Zabłocka A, Sosnowska A, Janusz M, Trziszka T. Immunomodulatory activity accompanying chicken egg yolk immunoglobulin Y. *Poult Sci* 2012; **91**: 3091-3096 [PMID: 23155018 DOI: 10.3382/ps.2012-02546]

116 **Polanowski A**, Sosnowska A, Zabłocka A, Janusz M, Trziszka T. Immunologically active peptides that accompany hen egg yolk immunoglobulin Y: separation and identification. *Biol Chem* 2013; **394**: 879-887 [PMID: 23492558 DOI: 10.1515/hsz-2012-0337]

117 **Zambrowicz A**, Zabłocka A, Bobak Ł, Macała J, Janusz M, Polanowski A, Trziszka T. A simple and rapid method of isolation of active polypeptide complex, yolkin, from chicken egg yolk. *Food Chem* 2017; **230**: 705-711 [PMID: 28407970 DOI: 10.1016/j.foodchem.2017.03.101]

118 **Hannappel E**, Huff T. The thymosins. Prothymosin alpha, parathymosin, and beta-thymosins: structure and function. *Vitam Horm* 2003; **66**: 257-296 [PMID: 12852257 DOI: 10.1016/s0083-6729(03)01007-0]

119 **Chiou VY**. The development of IgY(DeltaFc) antibody based neuro toxin antivenoms and the study on their neutralization efficacies. *Clin Toxicol (Phila)* 2008; **46**: 539-544 [PMID: 18584367 DOI: 10.1080/15563650701771973]

120 **Gouliaris K**. Preliminary immunochemical evaluation of immunoglobulins Y isolated from twelve avian species. M.Sc. Thesis, NCSR "Demokritos" & Birmingham University, 2003

121 **Tobias FL**, Garcia LN, Kanashiro MM, Medina-Acosta E, Brom-de-Luna JG, de Almeida CM, Azevedo Junior RR, Lemos M, Vieira-da-Motta O. Growth inhibition of Staphylococcus aureus and escherichia coli strains by neutralizing IgY antibodies from ostrich egg yolk. *Braz J Microbiol* 2012; **43**: 544-551 [PMID: 24031862 DOI: 10.1590/S1517-83822012000200015]

122 **Najdi S**, Nikbakht Brujeni G, Sheikhi N, Chakhkar S. Development of anti-*Helicobacter pylori* immunoglobulins Y (IgYs) in quail. *Iran J Vet Res* 2016; **17**: 106-110 [PMID: 27822235]

123 **Bae HD**, Kitaguchi K, Horio F, Murai A. Higher incorporation of heterologous chicken immunoglobulin Y compared with homologous quail immunoglobulin Y into egg yolks of Japanese quail (Coturnix japonica). *Poult Sci* 2009; **88**: 1703-1711 [PMID: 19590086 DOI: 10.3382/ps.2008-00238]

124 **Losonczy S**, Szabó C, Kiss Z, Bárdos L. Application of an anti-HQIgY antibody for the measurement of IgY concentrations of hen's and quail's serum and yolk. *Acta Physiol Hung* 1999; **86**: 253-258 [PMID: 10943656]

125 **IGY Life Sciences**. IgY antibodies—sustainable and efficacious therapeutics for human and animal health. [cited 4 February 2021]. Available from: https://www.nature.com/articles/d43747-020-01049-5

126 **Leiva CL**, Gallardo MJ, Casanova N, Terzolo H, Chacana P. IgY-technology (egg yolk antibodies) in human medicine: A review of patents and clinical trials. *Int Immunopharmacol* 2020; **81**: 106269 [PMID: 32036273 DOI: 10.1016/j.intimp.2020.106269]

127 **McCafferty J**, Griffiths AD, Winter G, Chiswell DJ. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 1990; **348**: 552-554 [PMID: 2247164 DOI: 10.1038/348552a0]

128 **JRC Publications Repository**. EURL ECVAM Recommendation on Non-Animal-Derived Antibodies. [cited 4 February 2021]. Available from: https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eurl-ecvam-recommendation-non-animal-derived-antibodies

129 **Nature**. Nobel Prize in Chemistry 2018. [cited 4 February 2021]. Available from: https://www.nature.com/collections/mnphfjdrdf

130 **Barderas R**, Benito-Peña E. The 2018 Nobel Prize in Chemistry: phage display of peptides and antibodies. *Anal Bioanal Chem* 2019; **411**: 2475-2479 [PMID: 30888467 DOI: 10.1007/s00216-019-01714-4]

131 **Ascoli CA**, Aggeler B. Overlooked benefits of using polyclonal antibodies. *Biotechniques* 2018; **65**: 127-136 [PMID: 30089399 DOI: 10.2144/btn-2018-0065]

132 **Cakir-Koc R**, Budama-Kilinc Y, Ustun E, Babur C. Conjugation and Characterization of Latex Particles with Toxoplasma gondii-specific Immunoglobulin Y Antibodies for Diagnostic Aim and Evaluation Efficiency in In Vitro Culture. *J Equine Vet Sci* 2020; **92**: 103145 [PMID: 32797775 DOI: 10.1016/j.jevs.2020.103145]

133 **Kota RK**, Reddy PN, Sreerama K. Application of IgY antibodies against staphylococcal protein A (SpA) of Staphylococcus aureus for detection and prophylactic functions. *Appl Microbiol Biotechnol* 2020; **104**: 9387-9398 [PMID: 32960294 DOI: 10.1007/s00253-020-10912-5]

134 **Lin JH**, Lo CM, Chuang SH, Chiang CH, Wang SD, Lin TY, Liao JW, Hung DZ. Collocation of avian and mammal antibodies to develop a rapid and sensitive diagnostic tool for Russell's Vipers Snakebite. *PLoS Negl Trop Dis* 2020; **14**: e0008701 [PMID: 32956365 DOI: 10.1371/journal.pntd.0008701]

135 **Souza DC**, de Faria LS, Sousa JEN, Lopes CA, Ribeiro VDS, da Silva VJ, Ribeiro RP, Rabelo ÉML, Rodrigues Ávila VM, Ferreira Júnior Á, Costa-Cruz JM. Use of polyclonal IgY antibodies to detect serum immune complexes in patients with active hookworm infection. *Parasitology* 2020; **147**: 715-720 [PMID: 32051048 DOI: 10.1017/S0031182020000220]

136 **Egea E**, Mendoza D, Garavito G, Saavedra S, Gómez H, Sanjuan M. Nanogold - IgY antibodies. An immunoconjugated for the detection of house dust mite (Dermatophagoides) allergens. *J Immunol Methods* 2019; **464**: 15-21 [PMID: 30165063 DOI: 10.1016/j.jim.2018.08.013]

137 **Łupicka-Słowik A**, Grzywa R, Leporowska E, Procyk D, Oleksyszyn J, Sieńczyk M. Development and Evaluation of an Immunoglobulin Y-Based ELISA for Measuring Prostate Specific Antigen in Human Serum. *Ann Lab Med* 2019; **39**: 373-380 [PMID: 30809983 DOI: 10.3343/alm.2019.39.4.373]

138 **Nie W**, Zhao C, Guo X, Sun L, Meng T, Liu Y, Song X, Xu K, Wang J, Li J. Preparation and identification of chicken egg yolk immunoglobulins against human enterovirus 71 for diagnosis of hand-foot-and-mouth disease. *Anal Biochem* 2019; **573**: 44-50 [PMID: 30831098 DOI: 10.1016/j.ab.2019.02.029]

139 **Omori AM**, Ono EYS, Hirozawa MT, de Souza Suguiura IM, Hirooka EY, Pelegrinelli Fungaro MH, Ono MA. Development of Indirect Competitive Enzyme-Linked Immunosorbent Assay to Detect *Fusarium verticillioides* in Poultry Feed Samples. *Toxins (Basel)* 2019; **11** [PMID: 30658385 DOI: 10.3390/toxins11010048]

140 **Tran TV**, Do BN, Nguyen TPT, Tran TT, Tran SC, Nguyen BV, Nguyen CV, Le HQ. Development of an IgY-based lateral flow immunoassay for detection of fumonisin B in maize. *F1000Res* 2019; **8**: 1042 [PMID: 31956398 DOI: 10.12688/f1000research.19643.2]

141 **Budama-Kilinc Y**, Cakir-Koc R, Ozdemir B, Kaya Z, Badur S. Production and characterization of a conserved M2e peptide-based specific IgY antibody: evaluation of the diagnostic potential *via* conjugation with latex nanoparticles. *Prep Biochem Biotechnol* 2018; **48**: 930-939 [PMID: 30388960 DOI: 10.1080/10826068.2018.1525564]

142 **Liang X**, Sheng Y, Yu W, Zhao S, Shan H, Zhang Q, Wang Z. Comparison of chicken IgY and mammalian IgG in three immunoassays for detection of sulfamethazine in milk. *Food Anal Methods* 2018; **11**: 3452-3463 [DOI: 10.1007/s12161-018-1316-9]

143 **Łupicka-Słowik A**, Psurski M, Grzywa R, Bobrek K, Smok P, Walczak M, Gaweł A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Development of Adenosine Deaminase-Specific IgY Antibodies: Diagnostic and Inhibitory Application. *Appl Biochem Biotechnol* 2018; **184**: 1358-1374 [PMID: 29043661 DOI: 10.1007/s12010-017-2626-x]

144 **da Silva MC**, Schaefer R, Gava D, Souza CK, da Silva Vaz I Jr, Bastos AP, Venancio EJ. Production and application of anti-nucleoprotein IgY antibodies for influenza A virus detection in swine. *J Immunol Methods* 2018; **461**: 100-105 [PMID: 30158073 DOI: 10.1016/j.jim.2018.06.023]

World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 September 20; 11(5): 263-277

DOI: 10.5662/wjm.v11.i5.263

ISSN 2222-0682 (online)

SYSTEMATIC REVIEWS

Evaluation of the red reflex: An overview for the pediatrician

Aspasia Toli, Asli Perente, Georgios Labiris

ORCID number: Aspasia Toli 0000-0002-9091-2921; Asli Perente 0000-0001-9219-8496; Georgios Labiris 0000-0003-4364-1943.

Author contributions: Toli A designed the research, performed the literature research and wrote the article; Perente A performed the literature research and wrote the article; Labiris G supervised the paper; all authors read and approved the final manuscript.

Conflict-of-interest statement: All the authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Aspasia Toli, Asli Perente, Georgios Labiris, Department of Ophthalmology, University Hospital of Alexandroupolis, Alexandroupolis 68100, Greece

Corresponding author: Georgios Labiris, MD, PhD, Associate Professor, Department of Ophthalmology, University Hospital of Alexandroupolis, Dragana Alexandroupolis 68100, Greece. labiris@usa.net

Abstract

BACKGROUND

Red reflex test (RRT) is a simple, non-invasive method that can be performed easily by pediatricians during the clinical examination in neonatal period, infancy and childhood. Abnormal reflexes can lead to prompt diagnosis of several ocular disorders, with potentially severe consequences on patient's vision, cognitive function and even life.

AIM

To underline the contribution of pediatricians to early detection of vision and life threatening diseases by using RRT effectively.

METHODS

For the present systematic review, PubMed searches were performed using the key words "red reflex and newborn"; "red reflex and neonate"; "red reflex and complications"; "red reflex and necessity"; "red reflex and retinoblastoma"; "red reflex and congenital cataract"; "red reflex and glaucoma"; "red reflex and prematurity"; "red reflex and leukocoria"; "red reflex and blindness"; "red reflex sensitivity and specificity"; "red reflex and differential diagnosis"; "red reflex and guidelines". The relevant articles were selected without language restrictions. When a full-text publication was not available, their English abstracts were used. In some cases, studies from the reference lists of the selected articles provided useful information. The research took place in September 2020, in the Ophthalmology Department of University Hospital of Alexandroupolis.

RESULTS

A total of 45 articles were selected according to the used key words. After reviewing data from these articles, it is supported that red reflex remains an effective tool of undeniable importance for early detection of severe eye conditions, such as cataract, retinoblastoma, retinopathy of prematurity and glaucoma. Although literature reports some limitations of RRT, including a notable percentage of false positive tests, the inability to detect small, peripheral retinoblastomas and the lower sensitivity for posterior segment pathology, it is widely accepted that the benefits from the regular evaluation of the test on public

Manuscript source: Invited manuscript

Specialty type: Pediatrics

Country/Territory of origin: Greece

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 27, 2021
Peer-review started: January 27, 2021
First decision: May 6, 2021
Revised: May 19, 2021
Accepted: August 3, 2021
Article in press: August 3, 2021
Published online: September 20, 2021

P-Reviewer: Marickar F
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Guo X

health are significant. Therefore, RRT has been established by international guidelines and should be an essential component of pediatricians clinical practice. Red reflex implementation should be incorporated in pediatricians educational programs, so that they would be able to provide quality services and safe diagnoses.

CONCLUSION
The implementation of RRT should be encouraged in all neonatal/pediatric departments. Prompt education of pediatricians should be empowered in order to achieve careful vision screening, according to current guidelines.

Key Words: Red eye reflex; Leukocoria; Visual screening; Newborn; Prevention; Pediatric examination

Core Tip: Red reflex test (RRT) is an easy, non-invasive examination that enables detection of vision- and life-threatening eye disorders. Various studies have dealt with the effectiveness, sensitivity/specificity and abnormalities of the RRT. The aim of the present review is to emphasize the advantages of RRT implementation from neonatal period to childhood and to underline the pediatricians' role in early diagnosis and treatment of the aforementioned diseases. This study presents a practical guide for the evaluation of the RRT, based on literature data. With appropriate education and compliance to the vision screening protocols, the pediatric society could reduce the incidence of preventable pediatric blindness.

Citation: Toli A, Perente A, Labiris G. Evaluation of the red reflex: An overview for the pediatrician. *World J Methodol* 2021; 11(5): 263-277
URL: https://www.wjgnet.com/2222-0682/full/v11/i5/263.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i5.263

INTRODUCTION

The first few weeks of life are of paramount importance for the development of visual function, thus the assessment of newborns' visual system should be part of the routine clinical examination. Numerous eye disorders of the neonatal and childhood period can lead to permanent visual impairment, even to blindness and loss of life[1].

It is worth mentioning that about 75% of blindness cases are preventable[2], a fact that emphasizes the need for early detection of the sight threatening conditions. Congenital infections, metabolic and chromosomal disorders and inheritance are among the most common causes of childhood blindness[3]. Therefore, eye examination should begin in newborn infancy and be continued as a part of the routine pediatric examination in order to achieve early diagnosis, prompt treatment and better prognosis.

The red reflex test (RRT), which was firstly introduced by Bruckner in 1962[3], is an effective, non-invasive examination that contributes to the diagnosis of various eye diseases, such as cataract, glaucoma, retinoblastoma and retinal disorders[3,4]. The RRT is easily performed in a darkened room by holding a direct ophthalmoscope, focused on the patient's pupil, with a lens power at ''0'', from a distance of approximately 30-45 cm (12-18 in)[4,5]. The normal RRT should be symmetric in both eyes, round, bright reddish-yellow or light grey in darkly colored eyes. Any asymmetry or lack of a red reflex, white reflexes or dark spots are abnormal and require referral to ophthalmologists[4,6]. The main causes of red reflex abnormality include congenital cataract, opacity of the cornea, iris abnormalities, vitreous opacities, tumors or chorioretinal malformations[6].

According to the latest suggestions of the American Academy of Pediatrics (AAP) and the American Association for Pediatric Ophthalmology and Strabismus (2016) the RRT should be performed at every well-baby visit from newborn to 6 mo age, afterwards, at 12 mo, 1-3 years, 4-5 years and 6 years and older[7,8].

The primary objective of this article is to provide an updated review on RRT published literature and highlight its importance in the detection of potentially sight threatening or even life threatening ocular diseases.

MATERIALS AND METHODS

Study design and selection criteria

This systematic review met the statements checklist of the Preferred Reporting Items for Systematic Reviews (PRISMA)[9]. The selection criteria were defined by applying the Problem/Population, Intervention, Comparison and Outcome framework. Articles were screened by title and abstract, according to the following inclusion criteria: Articles focused on the contribution of the RRT in diagnosing pathologic conditions, articles on RRT sensitivity and specificity, articles on guidelines about RRT and articles focused on compliance with RRT recommendations. Articles referring to adult patients were excluded. All of the eligible articles provided valuable information about the usage of the RRT in pediatric clinical practice.

Literature research strategy

A systematic search on PubMed databases was performed by two reviewers (A.T. and A.P.) in September 2020. Search terms used in this review are presented in Table 1. The initial search was performed without search filters and language restrictions. When the eligible articles were not available in full text in English, abstracts were used as a source of information. The date of publication was not an exclusion criterion. Additionally, the reference lists of the eligible articles were checked, and articles that met the inclusion criteria and provided useful information were also selected.

Study selection and quality assessment

A total of 45 articles that were relevant to the topic of interest and exclusively referring to the pediatric population were finally selected. Afterwards, the eligible articles were scanned diligently and independently by the two reviewers and the following data were extracted: Correct evaluation of RRT, correlation between RRT and certain ocular diseases (such as retinoblastoma and congenital cataract) and compliance and limitations of RRT. Any conflict was dissolved by a third reviewer (G.L.). Risk of bias of the eligible articles was conducted with "Quality Assessment Tool for Quantitative Studies" by Effective Public Health Practices[10]. Again, the same two individual reviewers assessed the articles, blinded to each other's decisions, and a third reviewer resolved any conflict. The results are demonstrated in Table 2.

RESULTS

Literature review returned 45 articles that met our inclusion criteria. They covered the whole spectrum of the topic in interest. The final selection of the eligible papers are presented in Table 1. Detailed data on the articles are shown in Table 3.

Diagnostic procedure of RRT

The evaluation of RRT is an easy, low-cost method that can be performed by pediatricians and other first care physicians in order to provide early diagnosis of severe pathologies. A necessary precondition that allows valid diagnoses is the appropriate training of medical students and trainee pediatricians during the years of specialty. For ideal performance of the test, it is essential to keep the room completely darkened (to maximize pupil dilation) and the direct ophthalmoscope fully charged. The lens power should be set at "0", unless there is a refractive error of the clinician's eyes. In this case, she/he could examine without wearing spectacles, by holding the ophthalmoscope closely to the examiner's eye and dialing the spectacle corrective power into the instrument. A practical way is to look through the peephole and dial the lenses till a pure image is viewed. The clinician should sit at a distance of approximately 0.5 m, but it could be increased in case of a nervous or uncooperative child. The co-axial position of the examiner is appropriate for estimating the RRT, however, it may not reveal ocular pathologies of small dimensions in peripheral areas. Therefore, the pediatrician needs to perform the RRT by using different angles along the horizontal meridian of the retina in order to assess the nasal and temporal retina by oblique viewing[4,5,11]. It is quite easy to perform, even in younger patients, as after

Table 1 Search terms

RRT and neonate/newborn	RRT congenital cataract
RRT and complications	RRT retinoblastoma
RRT and necessity	RRT and glaucoma
RRT and sensitivity and specificity	RRT and blindness
RRT and differential diagnosis	RRT and leukocoria
RRT guidelines	RRT and prematurity

RRT: Red reflex test

evaluating the red reflex from a co-axial position, the pediatrician should make the patient look in different directions. It may be helpful for the examiner to make the child focus behind the examiner's back by using a light or a toy. In case of infants or newborns, parents should hold the baby with a "chair hold" manner. This may keep the baby calm and able to focus the gaze straight forward. In this way, the examiner could perform the RRT without opening the baby's eyes with her/his hands. Taking into account that in the first days of life it is hard for the neonates to fixate or even to open their eyes, pediatricians should have plenty of patience and time in order to evaluate RRT safely.

A normal RRT consists of symmetrical bright red reflexes of both eyes, indicating that the ocular media (cornea, aqueous humor, lens, vitreous body) are transparent. A reduced or absent red reflex indicates an obstacle to the anatomical path to and from the retina (Table 4)[12].

Despite its name, the "red" reflex is often normally yellow, orange, red or any combination of these colors. In some patients with darker complexion, an increased pigmentation of the eye could be the cause for less bright reflex. Therefore, many variations among different racial or ethnic groups may be observed. Thus, examination of the parents would set the normal baseline[4,12].

In any case of atypical coloration of the red reflex, pediatricians should take into account many parameters and risk factors, such as gestational age, birth weight, use of oxygen therapy, phototherapy, blood transfusion and conjunctivitis, that could significantly affect the development of vision problems and subsequently the result of RRT[2]. Black reflex, which is suggestive of corneal scar, cataract or intraocular hemorrhage, and asymmetrical or non-homogenous reflexes require further investigation[13]. Asymmetry in the refractive power of the eye may cause asymmetrical red reflexes and should be checked, because any delay could lead to amblyopia and loss of vision[7]. Refractive errors may also give a yellow-white edge to a red reflex[14].

A white pupillary reflex is characterized as leukocoria, from the Greek words "leucos" (white) and "kóre" (pupil). White pupils are often noted by parents and described as something white, shiny, jello-like or a discoloration of the eye. This finding is pathological. Therefore, its presence is always concerning and requires urgent referral to an ophthalmologist. The most common cause of leukocoria in newborns is congenital cataract[15], while the most ominous pathology is retinoblastoma[16]. Other causes of leukocoria are presented in Table 3[15].

Pharmaceutical pupil dilation before performing RRT remains a controversial issue. Some infants and young children may have small pupils and restricted fixation, making the ophthalmoscopy a difficult examination. Moreover, patients with risk factors, such as family history of retinoblastoma or cataract, need a thorough evaluation of RRT in order to exclude any possibility of pathological lesions. In these cases, dilation of pupils could enhance the evaluation of RRT. In a survey by Ozkurt *et al*[17], RRT without pupillary dilation presented a positive predictive value of 70%; as without dilation, 2.2% of newborns presented an abnormal RRT. After dilation, ocular pathology that caused an abnormal RRT was detected in 1.5% of these neonates. For many years, pupil dilation has been used by pediatric ophthalmologists on infants over 2 wk on a routine basis. However, the pharmaceutical agents used for dilation (phenylephrine, anticholinergic agents such as cyclopentolate hydrochloride, tropicamide) were occasionally associated with significant complications. The reported adverse effects include elevated blood pressure and heart rate, urticaria, cardiac arrhythmias, and contact dermatitis. It is worth noting that extra caution is needed in cases of preterm infants, as they presented increased sensitivity to the aforementioned dilating eye drops[4]. Thus, the last policy statement of the AAP in 2016 clarified that if

Table 2 Quality assessment

Ref.	Year	Selection bias	Study design	Confounders	Blinding	Data collection methods	Withdrawals and drop-outs	Global rating
Nye[1]	2014	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
De Aguiar *et al*[2]	2011	Strong	Weak	Strong	Moderate	Strong	NA	Moderate
Eventov-Friedman *et al*[3]	2010	Moderate	Weak	Strong	Moderate	Strong	NA	Moderate
AAP[4]	2008	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Litmanovitz *et al*[5]	2010	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Cagini *et al*[6]	2017	Strong	Weak	Strong	Moderate	Strong	NA	Moderate
Loh *et al*[7]	2018	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Donahue *et al*[8]	2016	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Li *et al*[11]	2010	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Gurney *et al*[12]	2018	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Levin[13]	2015	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Sloot *et al*[14]	2015	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Wan *et al*[15]	2014	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Tuli *et al*[16]	2011	Moderate	Weak	Weak	Moderate	Moderate	NA	Weak
Ozkurt *et al*[17]	2018	Strong	Weak	Strong	Moderate	Strong	NA	Moderate
Bell *et al*[19]	2014	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Balmer *et al*[20]	2007	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Mansoor *et al*[21]	2016	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Popoola *et al*[22]	2019	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Abramson *et al*[23]	2003	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
AAP *et al*[24]	2002	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Li *et al*[25]	2013	Strong	Weak	Strong	Weak	Strong	NA	Weak
Butros *et al*[26]	2002	Moderate	Weak	Weak	Weak	Weak	NA	Weak
Sun *et al*[28]	2016	Strong	Weak	Strong	Weak	strong	NA	Weak
DerKinderen *et al*[29]	1989	Moderate	Weak	Strong	Weak	Strong	NA	Weak
Goddard *et al*[30]	1999	Moderate	Weak	Strong	Weak	Strong	NA	Weak
Bhatti *et al*[31]	2003	Moderate	Weak	Weak	Moderate	Moderate	NA	Weak
Rajavi *et al*[32]	2016	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Gogate *et al*[33]	2011	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Haargaard *et al*[34]	2015	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Atiq *et al*[35]	2004	Weak	Weak	Strong	Moderate	Weak	NA	Weak
Meier *et al*[36]	2006	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Donahue *et al*[37]	2016	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
Mndeme *et al*[38]	2010	Moderate	Weak	Strong	Moderate	Strong	NA	Moderate
Magnusson *et al*[39]	2013	Moderate	Weak	Strong	Moderate	Strong	NA	Moderate
Özkurt *et al*[40]	2019	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
Ulanovsky *et al*[41]	2015	Strong	Weak	Strong	Moderate	Moderate	NA	Moderate
Raoof *et al*[42]	2016	Weak	Weak	Weak	Moderate	Strong	NA	Weak
Wall *et al*[44]	2002	Weak	Weak	Weak	Moderate	Strong	NA	Weak

Gupta *et al*[45]	2019	Weak	Weak	Strong	Moderate	Weak	NA	Weak
Munson *et al*[46]	2019	Moderate	Weak	Weak	Moderate	Moderate	NA	Weak
Chen *et al*[52]	2019	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
AAP[53]	2003	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Anderson[54]	2019	Moderate	Weak	Weak	Moderate	Weak	NA	Weak

NA: Not applicable.

Table 3 Data extracted from the eligible articles

	Total number of articles	Ref.
Emphasis on the importance of RRT	44	[1-8,11-17,19-26,28-42,44,46,52-54]
Retinoblastoma	20	[3,6,11,12,15-17,19-23,26,28-30,36,38,40,54]
Congenital cataract	16	[3,5,6,12,15-17,19,21,31-35,38-40]
Retinopathy of prematurity	4	[2,3,16,20]
Specificity and/or Sensitivity of RRT	10	[3,5,6,11,12,22,25,28,32,38]
Compliance of health care providers with current screening protocols	12	[2,3,5,6,14,22,23,32,39,40,42,44]
Limitations of RRT	9	[1,4,6,11,12,23,26,28,41]
Comparison of RRT with other techniques	7	[17,25,28,34,38,45,46]

RRT: Red reflex test.

Table 4 Anatomical approach for an abnormal red reflex test[4,12]

Tear film	Mucus or other foreign bodies
Cornea	Dysgenesis of the anterior segment (Peters anomaly), congenital glaucoma, birth trauma
Lens	Cataract
Vitreous	Persistent fetal vasculature, vitreous hemorrhage or inflammation
Retina	Retinoblastoma, retinal detachment, Coat's disease, chorioretinalcoloboma, toxocariasis
Other	Anisometropia, strabismus

the pediatrician provides conditions of a fully darkened room, further pharmaceutical dilation is not necessary. Abnormal findings in RRT including dark spots, absent or significantly reduced reflex, leukocoria, or any asymmetry of the reflexes are indications for referral to an ophthalmologist with experience in children for thorough dilated fundus examination[8].

Retinoblastoma

Retinoblastoma is a neuroblastic tumor of the retina, with an incidence of approximately 1:20000 live births per year, which can lead to blindness, metastatic disease and loss of life[18,19]. The onset of the disease may occur *in utero* and up to 4 years of age[20]. It was estimated to be responsible for 17% of all neonatal cancers[15,20]. Sixty percent of retinoblastomas are non-heritable, usually unilateral[21,22]. Studies recorded positive family history in only 15%-25% of patients[19,22]. Typically, heritable retinoblastomas are bilateral, usually presented within the first year of life, with better visual potential in the eye with the smaller tumor size. According to literature data, the most common reason that concerned the family was the presence of leukocoria (initial sign in 50%-60%), often observed on flash photographs. Other manifestations included strabismus (initial sign in 20%-25%), inflammatory signs (initial sign in 6%-10%), *e.g.*, painful/red eye, tearing, heterochromia and hyphema[15,18,20,23]. In 50% of the cases worldwide there were extraocular signs and symptoms, which were associated with poorer survival rate (0%-50% *vs* 95%)[23].

Currently, the RRT is the main screening tool used by primary care physicians for the detection of retinoblastoma[24]. Li *et al*[25] reported a case of a 3 d old newborn with retinoblastoma, as the youngest patient with the disease. Notably, the median age of retinoblastoma diagnosis is 24 mo[8,26]. Therefore, the RRT should be performed by the pediatrician on a regular basis at every age, from birth to childhood. During the clinical examination, leukocoria seen on RRT is the primary sign that sets the suspicion for retinoblastoma[20]. A normal RRT is not equal with the absence of retinoblastoma [27]. According to some studies[26,28], peripheral or small tumors could give falsely normal RRT, while larger tumors were generally detected by RRT. The studies underlined that early diagnosis *via* RRT implementation and prompt treatment of retinoblastoma were associated with better prognosis and higher cure rate (95%)[18, 19].

Delayed detection increases the possibility for larger tumors and metastases, rendering the treatment significantly more aggressive and costly, with no certain outcome[20]. More specifically, an 8-wk-delay after the onset of signs and symptoms led to elevated risk of local invasion[29] and a 6-mo-delay highly increased the extraocular extension risk[30]. Untreated retinoblastomas are fatal[18,19]. A recent study reported that in most cases (80%), the parents firstly noticed the presenting signs of retinoblastoma and not pediatricians (8%) or ophthalmologists (10%)[23]. Possible explanations for these findings included (1) The difficulty to evaluate peripheral tumors with RRT performed by co-axial position *vs* more opportunities for the family members to view the eye from multiple angles; (2) Underutilization of well-child care visits; (3) A not dark enough pediatric office during RRT; (4) Uncooperative child; (5) Miotic pupils; (6) Inappropriate RRT technique, lack of education; and (7) Low clinical suspicion[23]. Another study mentioned delayed referral from the primary care physicians to specialists in ⅓ of the cases, due to justification of the presenting signs as normal findings or as part of other diagnosis. An additional reason for delayed therapy was the time spent by parents seeking treatment. The family unwittingly contributed to the delay in 77% of patients[26].

It is also emphasized that the pediatricians must have the education and skills to identify and refer to specialists a patient with the suspicion of retinoblastoma, in prompt time[26]. It has to be clear to all pediatricians that a positive family history of retinoblastoma, regardless of the RRT result, is an absolute indication for ophthalmologic examination in newborn nursery. Afterwards, regular ophthalmologist evaluations must be arranged, with aggressive surveillance and communication between the supervisor pediatrician and ophthalmologist until at least 28 mo of life. In case of revealed tumor, the follow-up should be continued until at least the age of 7 years. Additionally, the observation of eye abnormalities (leukocoria, strabismus) by the family, at any age, regardless of the RRT results, requires similar investigation to rule out any possibility for malignancy[23,26].

Congenital cataract

Congenital cataract is the opacification of the crystalline lens, which can be present at birth or develop within the first 3 mo of life. According to published literature[6,21,31-33], the incidence of congenital cataract ranges between 0.6 to 15 per 10000 live births, while it was estimated to be responsible for approximately 10% of childhood blindness [3]. In most cases, the etiology remains unknown. Inheritance is involved in 25% of congenital cataracts (autosomal dominant pattern). Other causes include chromosomal abnormalities (trisomy 21, trisomy 18), metabolic disorders (*e.g.*, galactosaemia) and congenital infection syndrome (toxoplasmosis, cytomegalovirus, syphilis, rubella, herpes simplex virus, varicella zoster virus)[21]. It is worth mentioning that most unilateral cataracts are isolated anomalies, however, 20% of cataracts attributed to congenital rubella are unilateral. Bilateral cataracts that are not correlated with genetic mutations need further investigation to exclude systemic disorders[15].

Early detection and treatment of congenital cataract have become a priority of the Global Vision 2020 initiatives of the World Health Organization[6]. The RRT is a highly sensitive screening test for congenital cataract. Cagini *et al*[6] performed RRT screening on neonates up to 3 d old, over a period of 3 years, indicating a congenital cataract rate of 0.009%. Haargaard *et al*[34] indicated the superior sensitivity of RRT compared to other diagnostic techniques in the detection of congenital cataract. The absence of red reflex during the routine neonatal eye screening could reveal early diagnosis of congenital cataract. This finding requires a thorough systemic clinical examination and the appropriate investigation to rule out every common or rare condition causing congenital cataract. Atiq *et al*[35] presented a case report of a 5-mo infant with bilateral congenital cataract, which at birth was investigated only for rubella and galactosemia. In the months that followed, the progressive clinical status

(including delayed motor milestones, irritability, sweating during feeding, generalized hypotonia, supraventricular tachycardia) and the family history of neonatal deaths, in combination with detected lactic acidosis and hypertrophic cardiomyopathy led to the diagnosis of Senger's disease. Other clinical findings that would empower the suspicion for cataract include nystagmus, absence of interest for surroundings and inability to fix and follow[21].

The time of detection of congenital cataract is crucial for the visual outcome following surgery, since early therapeutic intervention before the age of 6 wk for unilateral cases and 8 wk for bilateral cataracts was associated with best visual outcome[6,32]. Any delay on the detection and treatment of congenital cataract could give rise to severe consequences in visual evolution, even blindness or amblyopia, which has considerable impact on the neurobiological development of the children. In Bhatti *et al*[31] study, more than half of the infants with isolated cataract were diagnosed during the first 6 wk of life, but 38% of them were detected later, with a percentage of 15% after the age of 5 mo. The findings above underlined the importance of the method in detecting the cases of congenital cataract.

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a vasoproliferative disorder that affects premature babies, especially those with low weight of birth[2]. In Meier *et al*[36] study, ROP was responsible for 12% of leukocoria cases. Gestational age of less than 30 wk, birth weight less than 1500 g, history of oxygen therapy, septicemia and blood transfusion are among the most prevalent risk factors for the development of this potentially vision threatening disease[2,16]. The first clinical finding that could be observed in these newborns is the demarcation line, (Stage 1) which indicates the difference between avascular and vascularized retina. As the disease progresses, capillary growth begins at the edges of the demarcation line leading to the formation of ''ridge'' (Stage 2); the development of fibrovascular proliferation from the ridge into the vitreous body constitutes the third stage of ROP. From this stage, fibrovascular membrane may be grown posteriorly causing tractions and thus partial (Stage 4) or total retinal detachment (Stage 5)[20]. It becomes clear that advanced stages are equal with severe and permanent visual impairment. Therefore, careful examination of all preterm infants from pediatric ophthalmologists is of great clinical importance and has to be emphasized and encouraged from pediatricians[37]. It is worth noting that leukocoria may be detected after Stage 3 of ROP, however, the RRT performed by pediatricians or primary care physicians and the early referral of all suspicious cases may save the vision of a neonate as early implementation of laser or cryotherapy on peripheral retina protects the central retina from damage[16].

Sensitivity and specificity of RRT

Several studies have dealt with the sensitivity and specificity of the RRT. The estimated rates of sensitivity and specificity of RRT without pupillary dilation immediately after birth were 85% and 38.5% respectively for the diagnosis of any congenital ocular disorder[32]. Concerning the early diagnosis of congenital cataract and retinoblastoma, the RRT was proved to be a useful tool, with high rates of estimated respective sensitivity (100%) and specificity (97.9%). However, researchers identified a remarkable percentage of false positive tests (2%) and a positive predictive value of 0.7%[6]. On the contrary to these findings, another study revealed a much smaller percentage of false positives (0.0006%), with a positive predictive value of 42%[3]. Furthermore, Sun *et al*[28] evaluated the sensitivity and specificity of RRT in the detection of anterior (cornea, iris, aqueous humor or lens) and posterior (vitreous body, retina, choroid, optic nerve) ocular disorders. They considered the RRT as a useful diagnostic tool for diseases located on the anterior segment of the eye, since it detected 99.6% of the anterior pole anomalies (*vs* 4.1% of the posterior pole diseases). Therefore, RRT presented high rates of overall specificity (95%), but low overall sensitivity (13.9%). The rates of RRT sensitivity and specificity may range depending on the circumstances. Rajavi *et al*[32] found a significant increase of false positive RRT when newborns were examined by pediatricians in the first hours of life, under non-standard conditions (at the delivery room, beside mother and without pupil dilation), in comparison with the examination that took place by ophthalmologists, on the third day of life, under standard conditions (dark room and dilated eyes). In this study, the RRT sensitivity was 85% and specificity 38.5%, when performed under non-standard conditions. The results could be explained by the lack of experience of the pediatricians and by the ocular and tear film problems that usually disappear within a few days after birth. However, the researchers underlined that better conditions could make the evaluation of RRT more accurate.

Importance of the RRT

All of the eligible articles of the present review unanimously pointed out the value of the red reflex examination as an efficient tool for early diagnosis of pediatric ocular diseases, achieving prompt treatment with better outcomes. At this point, the literature highlights the unique role of pediatricians, who own the advantage not only to contribute to the improvement of the course of eye diseases and of the quality of patients' lives but also to save lives of children with severe diseases, such as retinoblastoma. The low incidence of retinoblastoma might reassure the general pediatrician. Nevertheless, the fact that plenty national newborn screening programs include tests for rare diseases, such as phenylketonuria (1/18000) and galactosemia (1/57000), indicates that early detection of severe diseases could prevent grave consequences for the economies, the health care systems and also for the quality and even the existence of the patient's life[26]. Similarly, applying RRT correctly could prevent the bad outcomes of delayed detection of retinoblastoma.

The performance of the RRT, from infancy and at every well child visit, following current pediatric guidelines could prevent childhood blindness[3,38]. Literature data correlated the RRT with early detection of congenital cataract. In Sweden[39] RRT seemed to be performed at the maternity ward at high rates (90%), a fact that was connected with an increase in the percentage of early detection and treatment of patients with congenital cataract. More specifically, 75% of the children who underwent surgery before the age of 1 year were cases with diagnosis and treatment before the sixth week of life. Another study[34] dealt with the 5-year experience of the different eye screening protocols of Sweden and Denmark. The results revealed a significantly higher rate of prompt diagnosis of pediatric cataract, when RRT was performed at maternity wards, in comparison with the absence of RRT. A survey in Tanzania concluded the same results[38]. The need for implementation of red reflex screening is even stronger in Turkey, the Middle East, Asia and Africa. Countries with high rates of consanguineous marriages showed significant correlation with red reflex abnormality (Turkey: 70.6% of neonates with abnormal RRT with a history of parental intermarriages *vs* 29.4% among normal reflexes), which was related with higher prevalence of genetic causes of common pediatric ocular diseases[17]. In a Turkish study[40], 72% of the pediatricians questioned considered that they should add RRT to follow-up charts, as they do for somatometric data. After all, pediatricians should be aware and well educated on including RRT in their routine clinical practice, as the importance of this method is to enable them to assess the quality of the transparent media of the eye, in an easy, non-invasive, low-cost manner[2].

Limitations of RRT

Although RRT remains a useful method for detecting severe pediatric ocular diseases, it has to be noticed that it also has some drawbacks. It has been emphasized that abnormalities of RRT have to be referred to ophthalmologists. However, a normal RRT does not exclude ocular pathology. First of all, the RRT enables the evaluation of only a small area and not the whole retina[11]. Thus, abnormal lesions, including retinoblastoma, could be missed when they are of small size or situated peripherally[12,26,28]. It is worth mentioning, that the detection of such cases was significantly improved while using additionally oblique viewing of the fundus and even more with pupil dilation[11]. Nevertheless, some tumors could still be missed. So, patients at high risk of retinoblastoma or other eye disorders leading to leukocoria (*e.g.*, family history of retinoblastoma, infantile or juvenile cataracts, retinal dysplasia, glaucoma) should be evaluated by specialists, regardless of the RRT result[4,11]. Moreover, RRT cannot diagnose some disorders of the retina or the optic nerve, which cause visual impairment, such as retinal dystrophy or optic atrophy. As a result, cases with impaired vision demand ophthalmologist's investigation, despite a normal RRT[12]. In another survey[28], RRT was unable to detect some fundus abnormalities, such as pigment, vascular, hemorrhage and subretinal exudative changes. Furthermore, the researchers proved that the accuracy of RRT in diagnosing disorders of the posterior pole of the eye was significantly lower than those of the anterior pole[12,28]. Literature data also recorded a limitation of the RRT in the neonatal period, enforcing the recommendations for repetition of the test routinely. Sun *et al*[28] reported some newborns with normal RRT in the first days of life who were diagnosed with familial exudative vitreoretinopathy after abnormal RRT or presence of nystagmus at the age of 1-6 mo. Performing RRT on a newborn or an infant's eyes could be a difficult challenge for inexperienced pediatricians. An infant's pupils are small and difficult to assess, so that physicians' complaints usually include "infant is uncooperative", "eyelids are closed tightly" or "unable to evaluate red reflex"[1]. However, these

statements are unacceptable and dangerous and underline the need for education to provide the RRT procedure effectively in order to avoid undesirable consequences. Ulanovsky *et al*[41] performed a retrospective observational study including 18872 neonates born from 2008 to 2011. During the years that RRT was performed, the researchers found a significantly higher incidence of clinical conjunctivitis with positive bacterial culture. This result was correlated with direct contact of the examiner's hands with newborns' eyes, hence the avoidance of direct contact with neonates during the RRT should be the general rule.

Despite the reported high levels of sensitivity for certain ocular diseases, false positive results (reduced red reflexes on eyes without abnormalities) were not unusual in the literature. Cagini *et al*[6] noticed high rates of false positives (only 3 of 461 patients with a positive or equivocal test were diagnosed with a congenital disease). On the contrary, another study recorded a much lower percentage of false positives (1 of 1643 tests was false positive)[6]. Inexperienced examiners, inappropriate equipment, small pupils, strongly pigmented fundus and conditions during the diagnostic procedure could explain the high rates of false positives[12,32]. However, the researchers considered the RRT essential part of the neonatal eye screening, as it provided early detections with high sensitivity rates[6].

Compliance with RRT guidelines

Although the necessity and effectiveness of RRT have been supported by international guidelines, data from the literature revealed insufficient implementation of the examination. Raoof *et al*[42] assessed the performance of RRT from health care professionals (general practitioners, midwives, pediatricians) *via* questionnaire. They found that 10% of responders admitted implementation of the test only when they had the time or when the parents were worried. Despite the fact that New Zealand's guidelines clearly define the appropriate time of red reflex examination, the majority of professionals (50.1%) seemed to perform the RRT at 6 wk age. Only 17.3% of the responders had received formal training for RRT, while 16.6% declared to feel underconfident during the examination[42]. Moreover, 46.1% of the neonatal units in Israel performed RRT during the years 2007-2008, while in Sao Paulo Brazil, the relative percentage was 81% during 2004[3]. Another study measured the lowest number of RRT evaluations at the high-risk neonatal units, and this finding was attributed to the unstable health status of the neonates[43]. Wall and colleagues[44] investigated the compliance of the pediatricians with vision screening guidelines. They found that a significant part of the pediatricians did not examine red reflexes beyond 6 and 24 mo of age (23% and 44%, respectively). The reasons for non-compliance seemed to be multifactorial and included the lack of time, patience, education, skills and also some worries about adequate reimbursement for vision screening[44]. Additionally, the limited staff and the perception of the pediatricians that the RRT was not their responsibility were also among the reported causes[3]. Moreover, several environmental factors could affect physician and patient compliance on RRT implementation. Nowadays, cataract remains one of the most usual reasons of preventable blindness in middle-low income countries, with very poor post-surgery visual outcomes. In these countries, late presentation of patients was very common[38]. A survey in East Turkey mentioned that 19% of pediatricians did not have an ophthalmoscope or did not know how to use it[40].

RRT and other techniques

Nowadays, RRT is a part of visual screening, supported by official guidelines for the pediatric community. However, the last recommendations published by the World Health Organization (WHO) did not include RRT but the pencil light examination. Literature provided controversial data regarding this method. Concerning the torchlight, Mndeme *et al*[38] strongly recommended that WHO guidelines should replace torchlight examination with RRT using direct ophthalmoscope due to the very low sensitivity rates (7.5%) of torchlight. Moreover, a Turkish study[17] emphasized that pencil light illumination should not be used by pediatricians and general practitioners, as this method missed most of the cases with congenital cataract. On the other hand, the researchers made it clear that RRT should become an essential part of the national pediatric eye screening protocol. Haargaard *et al*[34] studied the different protocols for congenital cataract screening that were used in Sweden and Denmark during 2008-2012. In 2011, Denmark introduced the pencil light as the screening tool for detection of congenital cataract at 5 wk of life, without any change in the age of diagnosis to be noted, in comparison with the absence of screening previously. On the other hand, the disease was detected significantly earlier in Sweden, where the RRT was a part of routine newborn examination.

Mndeme *et al*[38] dealt with three alternatives to the standard direct ophthalmoscopy for the evaluation of red reflex and also compared the standard RRT with the torchlight examination. The first tool was ArchLight, an easy and cheap device that uses a light emitting diode (LED). The second device was Peek Retina, an adaptor for smartphones, with prisms and LED that allows examination of the retina snaps and differentiation of normal and abnormal red reflexes *via* the coaxial light source. Thirdly, CatCam device, a prototype comprising a modified smartphone with a coaxial infra-red LED and infrared sensitive camera, provides evaluation of fundus reflex without causing miosis. All three methods had very high sensitivity (over 90%) and specificity (86.7%-100%), with the CatCam performing the best, followed by the Archlight and then the Peek Retina. The CatCam could improve the accuracy of pediatric cataract diagnosis; however, it still remains expensive and commercially unavailable. On the other hand, the Archlight is easily performed on infants, with much lower cost. These new technologies could strengthen the accuracy of RRT and make it easier and more feasible to the medical community.

Digital images analysis could provide an opportunity for telemedicine as well. However, it was underlined that more investigation of their efficacy on detecting pediatric ocular disorders is required. Another study[25] included advanced digital fundus imaging using RetCam during the eye screening of newborns. The digital examination revealed a significant number of well-being neonates with abnormal ocular findings, which RRT was unable to detect. The most common among them was retinal hemorrhages, a usually benign and transient condition after birth trauma. However, more studies need to be performed to investigate any correlation between early fundus findings and final visual impairment. Smartphone photography for the detection of amblyogenic conditions in children 5-8 years of age, through snaps of pupillary red reflexes, was the objective of Gupta *et al*[45] trial. All high refractive errors were detected with success, however, moderate errors revealed false negative results. The sensitivity of the photographs for all other ocular diseases was 100%. The usage of smartphones should be further investigated in all ages and for more eye disorders in order to provide a low-cost, effective screening tool for developing countries. It has been reported[23]for more than 90% of leukocoria cases that the family firstly noticed the presence of white pupils in photographs. Munson *et al*[46] dealt with a tool based on red reflex of the pupils that could provide to parents the detection of leukocoria earlier. They presented a novel application for smartphones, the CRADLE, which can be downloaded for free and allows early detection of leukocoria. More specifically, the application creates a private storage of photographs and after digital analysis, it can detect cases of leukocoria and automatically alert the parents for further investigation. In this study, the CRADLE detected leukocoria in the snaps captured before diagnosis by 1.3 years, for the majority of cases with ocular diseases (80%). The estimated sensitivity of the application was increased and the specificity was decreased with age. The CRADLE could provide to parents a useful tool for early detection of leukocoria. However, it was not able to differentiate the pathological for the physiological leukocoria due to specific conditions during photo shooting, and it definitely could not replace clinical evaluation with RRT and further examination. The researchers underlined that although pathological white pupils are usually signs of refractive errors or amblyopia and physiological leukocoria is a typical artifact of off-axis photography; recurrent white pupils in many photos are red flags. However, they made it clear that all cases of leukocoria in photographs should be investigated. More studies on these new technologies could empower the contribution of the RRT in diagnosing certain ocular pathologies of the pediatric population in the future.

DISCUSSION

RRT is a non-invasive, low-cost, essential diagnostic method, highly effective for the detection of several sight and life threatening ocular diseases. Its importance has been highlighted in 2016 by the AAP, American Association for Pediatric Ophthalmology and Strabismus, American Academy of Ophthalmology and American Association of Certified Orthoptists who published the revised policy statement on "Visual System Assessment in Infants, Children, and Young Adults by Pediatricians"[37]. The policy recommended that RRT should be performed at every pediatrician visit for newborns and infants 0-6 mo and again at 6 mo, 12 mo, 1-3 years, 4-5 years and 6 years. Furthermore, it has been emphasized that cases with abnormal findings or with history of prematurity or family history of congenital cataracts, retinoblastoma,

metabolic disease or systemic diseases with suspicion of severe ocular disorders must be referred to the ophthalmologist immediately[37].

Despite its clinical importance, literature research revealed that there is heterogeneity worldwide, regarding current recommendations for RRT implementation. Indicatively, some of the different strategies of European countries are hereby presented. In the United Kingdom, healthy infants should be examined for red reflexes during the first 3 d of life and again between 6-8 wk[47]. In Germany, the guidelines require repeated RRT at the ages of 4, 6, 12 and 24 mo. In Sweden, RRT is performed at birth and again at 4-6 wk[48]. In Greece, RRT is recommended at birth, at the age of 1-2 wk, 2 mo, 3-36 mo, and 36 mo-7 years[49]. Denmark and the recommendations by WHO do not include RRT in the pediatric vision screening but rather pencil light examination[38,50]. Remarkably, data from literature supported that RRT could never be replaced by pencil light examination, as the second tool seemed to be able to detect only advanced cases of cataract and retinoblastoma[17,38]. In Australia, each state and territory health department provides separate guidelines for pediatric vision screening. Most of the better quality guidelines recommended red reflex examination during newborn to 6 mo of age and later in the following years (from 18 mo to 3.5 years of age)[51]. The literature provides a little information about RRT recommendations in Asia and Africa. However, many countries, such as China and middle-low income countries, do not include RRT in the vision screening programs. The main reason may be the increased cost of eye examination from infancy[52].

Despite its clinical value, the RRT is not implemented by the entire pediatric community appropriately, as it is recommended. In order to improve the pediatricians' awareness and compliance with the current guidelines, prompt emphasis should be placed on the educational procedure of the physicians starting from university programs and during the years of pediatrics training. In this context, some researchers even suggested education videos on the internet[23,39]. The fact that many relevant articles are published only in ophthalmologic and not pediatric journals could be an obstacle for the pediatricians' awareness on RRT. Indicatively, 20 of the eligible articles for this review were available in pediatric journals, while the rest of them were published in non-pediatric journals.

The majority of the eligible articles of this review highlighted the importance of the RRT technique in the detection of certain pathologies (Table 3). On the other hand, Munson *et al*[46] considered the RRT as "dying art", since they focused on the limitations of the technique that have been described previously. However, the contribution of RRT in pediatric visual screening could be more promising in the future. New technologies based on red reflex may improve RRT's weak points[38,45,53]. Further investigation of these new devices and development of appropriate software is required in order to improve RRT accuracy on pediatric ocular diagnosis.

CONCLUSION

The present paper attempts to provide an updated review of the red reflex examination for the pediatrician. Published research indicates that the RRT should begin at birth and afterwards it should be an essential part of the routine clinical examination by pediatricians. Its simplicity and high sensitivity and specificity suggest that RRT should be part of the educational curriculum for every new pediatrician, since it will contribute to prompt diagnosis of many sight and life threatening conditions.

ARTICLE HIGHLIGHTS

Research background

Red eye reflex test (RRT) is a widely known examination that has been used by clinicians for the diagnosis of several ocular disorders. However, its implementation by pediatricians in the clinical practice still remains controversial. This study aims to highlight the importance of RRT and to provide a practical guide for its usage for pediatricians.

Research motivation

The literature data show insufficient implementation of the RRT. This result is in accordance with clinical observation in our country. Therefore, the present study could

contribute to raise pediatricians' awareness on this diagnostic tool.

Research objectives

The main objectives of the present study were the assessment of RRT value in specific disorders (such as retinoblastoma and congenital cataract) and the compliance of the clinicians with current guidelines. Moreover, this article investigated reported limitations of this diagnostic technique and motivated future research for the improvement of this method through new technological achievements.

Research methods

A thorough search on PubMed databases took place by two independent reviewers.

Research results

Eligible articles highlighted the significance of the RRT in the diagnosis of sight threatening or even life threatening eye pathologies. The implementation rates seemed to present a wide range among the countries. This fact, underlines the need for appropriate education and official guidelines from health systems.

Research conclusions

This study demonstrates why pediatricians should include the RRT in their clinical practice and provides a practical guide for the prompt implementation of this diagnostic examination. Further investigations are in progress in order to overcome the main limitations of the traditional red reflex examination.

Research perspectives

The rapid progress of technology achievements should improve the usage of the traditional red reflex method, making it easier and more efficient.

REFERENCES

1 **Nye C**. A child's vision. *Pediatr Clin North Am* 2014; **61**: 495-503 [PMID: 24852147 DOI: 10.1016/j.pcl.2014.03.001]

2 **de Aguiar AS**, Ximenes LB, Lúcio IM, Pagliuca LM, Cardoso MV. Association of the red reflex in newborns with neonatal variables. *Rev Lat Am Enfermagem* 2011; **19**: 309-316 [PMID: 21584377 DOI: 10.1590/s0104-11692011000200012]

3 **Eventov-Friedman S**, Leiba H, Flidel-Rimon O, Juster-Reicher A, Shinwell ES. The red reflex examination in neonates: an efficient tool for early diagnosis of congenital ocular diseases. *Isr Med Assoc J* 2010; **12**: 259-261 [PMID: 20929074]

4 **American Academy of Pediatrics**. Section on Ophthalmology; American Association for Pediatric Ophthalmology And Strabismus; American Academy of Ophthalmology; American Association of Certified Orthoptists. Red reflex examination in neonates, infants, and children. *Pediatrics* 2008; **122**: 1401-1404 [PMID: 19047263 DOI: 10.1542/peds.2008-2624]

5 **Litmanovitz I**, Dolfin T. Red reflex examination in neonates: the need for early screening. *Isr Med Assoc J* 2010; **12**: 301-302 [PMID: 20929085]

6 **Cagini C**, Tosi G, Stracci F, Rinaldi VE, Verrotti A. Red reflex examination in neonates: evaluation of 3 years of screening. *Int Ophthalmol* 2017; **37**: 1199-1204 [PMID: 27822637 DOI: 10.1007/s10792-016-0393-2]

7 **Loh AR**, Chiang MF. Pediatric Vision Screening. *Pediatr Rev* 2018; **39**: 225-234 [PMID: 29716965 DOI: 10.1542/pir.2016-0191]

8 **Donahue SP**, Baker CN; Committee on Practice and Ambulatory Medicine, American Academy of Pediatrics; Section on Ophthalmology, American Academy of Pediatrics; American Association of Certified Orthoptists; American Association for Pediatric Ophthalmology and Strabismus; American Academy of Ophthalmology. Procedures for the Evaluation of the Visual System by Pediatricians. *Pediatrics* 2016; **137** [PMID: 26644488 DOI: 10.1542/peds.2015-3597]

9 **Moher D**, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 2009; **6**: e1000097 [PMID: 19621072 DOI: 10.1371/journal.pmed.1000097]

10 Effective Public Health Practice Project. Quality assessment tool for quantitative studies. [cited 15 May 2021]. Available from: https://www.nccmt.ca/knowledge-repositories/search/14

11 **Li J**, Coats DK, Fung D, Smith EO, Paysse E. The detection of simulated retinoblastoma by using red-reflex testing. *Pediatrics* 2010; **126**: e202-e207 [PMID: 20587677 DOI: 10.1542/peds.2009-0882]

12 **Gurney SP**, Makanjuola T, Kutubi M, Parulekar M, Abbott J. How to use…the direct ophthalmoscope. *Arch Dis Child Educ Pract Ed* 2018; **103**: 102-109 [PMID: 28935833 DOI: 10.1136/archdischild-2016-312378]

13 **Levin AV**. Err on the side of caution if concerns arise during red reflex test. *AAP News* 2015; **36**: 12

14 **Sloot F**, Hoeve HL, de Kroon ML, Goedegebure A, Carlton J, Griffiths HJ, Simonsz HJ; EUSCREEN Study Group. Inventory of current EU paediatric vision and hearing screening programmes. *J Med Screen* 2015; **22**: 55-64 [PMID: 25742803 DOI: 10.1177/0969141315572403]

15 **Wan MJ**, VanderVeen DK. Eye disorders in newborn infants (excluding retinopathy of prematurity). *Arch Dis Child Fetal Neonatal Ed* 2015; **100**: F264-F269 [PMID: 25395469 DOI: 10.1136/archdischild-2014-306215]

16 **Tuli SY**, Giordano BP, Kelly M, Fillipps D, Tuli SS. Newborn with an absent red reflex. *J Pediatr Health Care* 2013; **27**: 51-55 [PMID: 22197583 DOI: 10.1016/j.pedhc.2011.10.010]

17 **Ozkurt Z**, Balsak S, Yildirim Y, Yuksel H, Caca I. Associations between red reflex abnormality, consanguinity and intensive care hospitalization of newborns in Turkey. *East Mediterr Health J* 2018; **24**: 631-636 [PMID: 30215472 DOI: 10.26719/2018.24.7.631]

18 **Kliegman**, Robert. Nelson Textbook of Pediatrics. Edition 21. Philadelphia, PA: Elsevier, 2020

19 **Bell AL**, Rodes ME, Collier Kellar L. Childhood eye examination. *Am Fam Physician* 2013; **88**: 241-248 [PMID: 23944727]

20 **Balmer A**, Munier F. Differential diagnosis of leukocoria and strabismus, first presenting signs of retinoblastoma. *Clin Ophthalmol* 2007; **1**: 431-439 [PMID: 19668520]

21 **Mansoor N**, Mansoor T, Ahmed M. Eye pathologies in neonates. *Int J Ophthalmol* 2016; **9**: 1832-1838 [PMID: 28003988 DOI: 10.18240/ijo.2016.12.22]

22 **Ademola-Popoola DS**, Opocher E, Reddy MA. Contemporary management of retinoblastoma in the context of a low-resource country. *Niger Postgrad Med J* 2019; **26**: 69-79 [PMID: 31187745 DOI: 10.4103/npmj.npmj_21_19]

23 **Abramson DH**, Beaverson K, Sangani P, Vora RA, Lee TC, Hochberg HM, Kirszrot J, Ranjithan M. Screening for retinoblastoma: presenting signs as prognosticators of patient and ocular survival. *Pediatrics* 2003; **112**: 1248-1255 [PMID: 14654593 DOI: 10.1542/peds.112.6.1248]

24 reflex examination in infants. Section on Ophthalmology. American Academy of Pediatrics. *Pediatrics* 2002; **109**: 980-981 [PMID: 11986467]

25 **Li LH**, Li N, Zhao JY, Fei P, Zhang GM, Mao JB, Rychwalski PJ. Findings of perinatal ocular examination performed on 3573, healthy full-term newborns. *Br J Ophthalmol* 2013; **97**: 588-591 [PMID: 23426739 DOI: 10.1136/bjophthalmol-2012-302539]

26 **Butros LJ**, Abramson DH, Dunkel IJ. Delayed diagnosis of retinoblastoma: analysis of degree, cause, and potential consequences. *Pediatrics* 2002; **109**: E45 [PMID: 11875173 DOI: 10.1542/peds.109.3.e45]

27 **Hay WW**, Levin MJ, Deterding RR, Abzug MJ. Current Diagnosis & Treatment Pediatrics, McGraw-Hill; 23rd Edition, Lange,2016

28 **Sun M**, Ma A, Li F, Cheng K, Zhang M, Yang H, Nie W, Zhao B. Sensitivity and Specificity of Red Reflex Test in Newborn Eye Screening. *J Pediatr* 2016; **179**: 192-196.e4 [PMID: 27640356 DOI: 10.1016/j.jpeds.2016.08.048]

29 **DerKinderen DJ**, Koten JW, Van Romunde LK, Nagelkerke NJ, Tan KE, Beemer FA, Den Otter W. Early diagnosis of bilateral retinoblastoma reduces death and blindness. *Int J Cancer* 1989; **44**: 35-39 [PMID: 2744895 DOI: 10.1002/ijc.2910440107]

30 **Goddard AG**, Kingston JE, Hungerford JL. Delay in diagnosis of retinoblastoma: risk factors and treatment outcome. *Br J Ophthalmol* 1999; **83**: 1320-1323 [PMID: 10574806 DOI: 10.1136/bjo.83.12.1320]

31 **Bhatti TR**, Dott M, Yoon PW, Moore CA, Gambrell D, Rasmussen SA. Descriptive epidemiology of infantile cataracts in metropolitan Atlanta, GA, 1968-1998. *Arch Pediatr Adolesc Med* 2003; **157**: 341-347 [PMID: 12695229 DOI: 10.1001/archpedi.157.4.341]

32 **Rajavi Z**, Sabbaghi H. Congenital Cataract Screening. *J Ophthalmic Vis Res* 2016; **11**: 310-312 [PMID: 27621790 DOI: 10.4103/2008-322X.188389]

33 **Gogate P**, Gilbert C, Zin A. Severe visual impairment and blindness in infants: causes and opportunities for control. *Middle East Afr J Ophthalmol* 2011; **18**: 109-114 [PMID: 21731320 DOI: 10.4103/0974-9233.80698]

34 **Haargaard B**, Nyström A, Rosensvärd A, Tornqvist K, Magnusson G. The Pediatric Cataract Register (PECARE): analysis of age at detection of congenital cataract. *Acta Ophthalmol* 2015; **93**: 24-26 [PMID: 25613125 DOI: 10.1111/aos.12445]

35 **Atiq M**, Iqbal S, Ibrahim S. Sengers disease: a rare association of hypertrophic cardiomyopathy and congenital cataracts. *Indian J Pediatr* 2004; **71**: 437-440 [PMID: 15163876 DOI: 10.1007/BF02725636]

36 **Meier P**, Sterker I, Tegetmeyer H. [Leucocoria in childhood]. *Klin Monbl Augenheilkd* 2006; **223**: 521-527 [PMID: 16804823 DOI: 10.1055/s-2005-859005]

37 **Donahue SP**, Nixon CN; Section on Opthamology, American Academy of Pediatrics; Committee on Practice and Ambulatory Medicine, American Academy of Pediatrics; American Academy of Ophthalmology; American Association for Pediatric Ophthalmology and Strabismus; American Association of Certified Orthoptists. Visual System Assessment in Infants, Children, and Young Adults by Pediatricians. *Pediatrics* 2016; **137**: 28-30 [PMID: 29756730 DOI: 10.1542/peds.2015-3596]

38 **Mndeme FG**, Mmbaga BT, Kim MJ, Sinke L, Allen L, Mgaya E, Bastawrous A, MacLeod D, Burton MJ, Gilbert C, Bowman R. Red reflex examination in reproductive and child health clinics for early detection of paediatric cataract and ocular media disorders: cross-sectional diagnostic accuracy and feasibility studies from Kilimanjaro, Tanzania. *Eye (Lond)* 2021; **35**: 1347-1353 [PMID: 32546747

DOI: 10.1038/s41433-020-1019-5]

39 **Magnusson G**, Bizjajeva S, Haargaard B, Lundström M, Nyström A, Tornqvist K. Congenital cataract screening in maternity wards is effective: evaluation of the Paediatric Cataract Register of Sweden. *Acta Paediatr* 2013; **102**: 263-267 [PMID: 23205674 DOI: 10.1111/apa.12111]

40 **Gürsel Özkurt Z**, Balsak S, Çamçi MS, Bilgen K, KatranİH, Aslan A, Han ÇÇ. Approach of Family Physicians to Pediatric Eye Screening in Diyarbakır. *Turk J Ophthalmol* 2019; **49**: 25-29 [PMID: 30829022 DOI: 10.4274/tjo.galenos.2018.10829]

41 **Ulanovsky I**, Shnaider M, Geffen Y, Smolkin T, Mashiach T, Blazer S, Makhoul IR. Performing red reflex eye examinations increases the rate of neonatal conjunctivitis. *Acta Paediatr* 2015; **104**: e541-e545 [PMID: 26362116 DOI: 10.1111/apa.13170]

42 **Raoof N**, Dai S. Red reflex screening in New Zealand: a large survey of practices and attitudes in the Auckland region. *N Z Med J* 2016; **129**: 38-43 [PMID: 27447134]

43 **Lúcio IML**. MétodoEducativo para a prática do teste do reflexovermelho no cuidadoaorecém-nascido. [tese de doutorado]. *Fortaleza (CE): Departamento de Enfermagem da Universidade Federal do Ceará* 2008; 137

44 **Wall TC**, Marsh-Tootle W, Evans HH, Fargason CA, Ashworth CS, Hardin JM. Compliance with vision-screening guidelines among a national sample of pediatricians. *Ambul Pediatr* 2002; **2**: 449-455 [PMID: 12437391 DOI: 10.1367/1539-4409(2002)002<0449:cwvsga>2.0.co;2]

45 **Gupta R**, Agrawal S, Srivastava RM, Singh V, Katiyar V. Smartphone photography for screening amblyogenic conditions in children. *Indian J Ophthalmol* 2019; **67**: 1560-1563 [PMID: 31546480 DOI: 10.4103/ijo.IJO_2090_18]

46 **Munson MC**, Plewman DL, Baumer KM, Henning R, Zahler CT, Kietzman AT, Beard AA, Mukai S, Diller L, Hamerly G, Shaw BF. Autonomous early detection of eye disease in childhood photographs. *Sci Adv* 2019; **5**: eaax6363 [PMID: 31616792 DOI: 10.1126/sciadv.aax6363]

47 **Mazzone P**, Carlton J, Griffiths H. Summary Vision Screening Data: England & Wales, 2018. Available from: https://www.euscreen.org/

48 **Mazzone P**, Carlton J, Griffiths H. Summary Vision Screening Data: Sweden. EU Screening vision & hearing. Available from: https://www.euscreen.org/

49 **Mazzone P**, Carlton J, Griffiths H. Summary Vision Screening Data: Greece. EU Screening vision & hearing. Available from: https://www.euscreen.org/

50 **Mazzone P**, Carlton J, Griffiths H. Summary Vision Screening Data: Denmark, 2018. Available from: https://www.euscreen.org/

51 **Murdoch Childrens Research Institute**. National children's vision screening project, Centre for Community Child Health (CCCH). Available from: https://www.rch.org.au/ccch/

52 **Chen AH**, Abu Bakar NF, Arthur P. Comparison of the pediatric vision screening program in 18 countries across five continents. *J Curr Ophthalmol* 2019; **31**: 357-365 [PMID: 31844783 DOI: 10.1016/j.joco.2019.07.006]

53 **Committee on Practice and Ambulatory Medicine, Section on Ophthalmology**; American Association for Pediatric Ophthalmology and Strabismus; American Academy of Ophthalmology. Eye examination in infants, children, and young adults by pediatricians. *Pediatrics* 2003; **111**: 902-907 [PMID: 12671132]

54 **Anderson J**. Don't Miss This! *J Binocul Vis Ocul Motil* 2019; **69**: 106-109 [PMID: 31329054 DOI: 10.1080/2576117X.2019.1607429]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com