Diabetes Mellitus Interagency Coordinating Committee (DMICC)

Lister Hill Auditorium, NIH Campus
Bethesda, Maryland
April 11, 2003

Summary Minutes

Dr. Judith E. Fradkin, Director, Division of Diabetes, Endocrinology, and Metabolic Diseases, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), opened the session by thanking the speakers and attendees for their participation in the 20th anniversary symposium of the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) Study to which the DMICC meeting was appended. Dr. Fradkin then reiterated the conference goals of the DCCT/EDIC symposium, held April 10–11 and entitled "Metabolic Imprinting and the Long-Term Complications of Diabetes Mellitus: Bench to Bedside and Back":

- To celebrate and commemorate the accomplishments of the DCCT/EDIC on its 20th anniversary;
- To explore the possible mechanistic basis for what has been tentatively termed "metabolic memory" or "imprinting"; and
- To generate plans for the fostering of research in developing new therapies for the complications of type 1 diabetes.

Dr. Fradkin explained to the guests present that the DMICC is a forum for the coordination of diabetes research and healthcare aspects across multiple institutes and centers at the National Institutes of Health (NIH), other agencies within the U.S. Department of Health and Human Services (DHHS), and beyond.

Special funding for type 1 diabetes research began in 1998, with a $30 million annual budget, and has increased to five times that amount, $150 million per year, for FY 2004-2008, providing a total funding of $1.14 billion over the course of its legislative history (Balanced Budget Act of 1997, P.L. 105–33, amended by FY 2001 Consolidated Appropriations Act, P.L. 106–554). Since its inception, this funding has been the source of a number of initiatives, such as the establishment of genetic consortia including a genetic collection being carried out by the EDIC study group. DCCT/EDIC has provided a very well-characterized group of patients in terms of metabolic control for examination of the potential genetic factors that might influence the risk of complications. Other initiatives relevant to complications pursued with the special funds include the Animal Models of Diabetic Complications Consortium (AMDCC), the macular edema clinical research consortia, initiatives for the development of surrogate markers for diabetes complications, and pilot studies for the development of new therapies.

Of particular emphasis has been the funding of studies fostering bench-to-bedside research. Dr. Fradkin stressed that development of partnerships between individuals working in type 1 diabetes with experts from outside the field, such as some of those who were very much a part of

the current DCCT/EDIC conference, is an area that will aid in the exploration and examination of new directions for diabetes research.

In May 2002, an Advisory Panel recommended expanding the areas of opportunity for type 1 diabetes research to include research in inflammation and vascular disease complications, development of improved animal models, expanded clinical research, and the application of new technologies. They further recognized that available resources and infrastructure can be enhanced by the development of consortia to examine multiple complications and the fostering of partnerships between researchers in academia, Government, and industry. Preclinical development of therapeutic applications and a central knowledge base of complications-related initiatives were also recommended. Dr. Fradkin said a Web site will be developed to identify opportunities using type 1 funds in response to these recommendations and to announce the availability of resources resulting from such initiatives.

To capitalize on what was presented during the DCCT/EDIC conference and to focus future fundamental research on potential opportunities and initiatives recommended by conference participants, Dr. Fradkin outlined several key questions concerning the pathogenesis, prevention, and therapy of complications and invited speakers to respond with specific recommendations. The following sections summarize their presentations and the attendees' comments.

What Are the Major Gaps in Our Knowledge of the Pathogenesis and Therapy of Vascular Complications?

Dr. David M. Nathan, Professor of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, summarized the following lessons learned from DCCT/EDIC that were presented during the symposium and outlined opportunities for future research:

- Glycemia is clearly the predominant mediator of the effects of intensive versus conventional therapy, explaining more than 95 percent of the effect of intensive therapy.

- Despite the subsequent narrowing of glycemia levels, the differences in outcomes between the original intensive and conventional therapy groups persist.

- The persistent difference in diabetic complications, potentially mediated by long-term beneficial effects of lower glycemia and/or persistent adverse effects of hyperglycemia, appears to be maintained for as long as 8 years after the separation in glycemia has dissipated, a phenomenon currently termed "imprinting" or "metabolic memory."

- One of the major and most interesting observations from DCCT was the demonstration that it is the original separation in glycemia level that accounts for most of the original effect.

- Glycemic levels and the changes mediated by intensive therapy may play a role in the development of macrovascular disease, as well as microvascular disease.

- Recent data with regard to calcification in the heart appear to demonstrate a difference between intensive and conventional groups.

During the DCCT/EDIC conference, several pathophysiologic mechanisms were presented to explain the effects of glycemic control and other currently used interventions on diabetic micro- and macrovascular complications, including glycation, inflammation, glycoxidation, apoptosis, lipoxidation, cellular issues, oxidation, and genetics/epigenetics. Investigators from diverse backgrounds explored several of these potential mechanisms that might explain the imprinting effects or metabolic memory from the early intensive glycemic control on long-term complications, including glycation/receptors for advanced glycation endproducts (RAGE), genetics/epigenetics, cellular/vascular/angiogenesis issues, and immunologic factors. Topics addressed during the conference included:

- Imprinting in DCCT/EDIC.
- Pathophysiology of diabetic complications.
- Potential mechanisms for long-term effects.
- Animal models and data regarding micro- and macrovascular disease.
- New methods of detecting and tracking complications that may be useful in clinical trials.
- Results of clinical trials directed at a number of factors that may be operant in diabetic complications.

Dr. Nathan also mentioned several topics that were not discussed, but which might have been considered within the scope of the conference, such as the limitations in achieving long-term control of hyperglycemia with currently available therapy; the ways of improving glucose control in type 1 diabetes, either by biological or mechanical approaches to maintain normal glycemia; and the prevention or cure of type 1 diabetes.

A significant outcome of the symposium was the identification of several areas for additional research. First, a consensus must be reached regarding reliable, practical biomarkers or surrogates for cardiovascular disease, so that meaningful comparisons can be made in clinical trials between the effects of different interventions. Doing so will ultimately result in clarification of what sometimes appear to be contradictory results in studies and will allow for greater efficiency in the performance of interventional studies. Second, a better understanding is needed of the differences and similarities in the effects of glycemic and other interventions on different end organs, as well as the influence of genetic factors in this regard.

Dr. Nathan emphasized that the DCCT/EDIC group is the most vigorously and thoroughly studied population of type 1 diabetic patients in history, with 95 percent retention of subjects over a span of 20 years (n=1385/1441), and with an average follow-up of approximately 16 years. The population has been extensively characterized and phenotyped over time with regard to complications, diabetes therapy and chronic glycemia, and established and potential risk factors, and it has provided researchers with an incredibly valuable resource of stored biological specimens, including DNA, which can be well utilized for the validation of biomarkers.

Dr. Nathan suggested that the DCCT/EDIC group continue to examine the relationship between the panoply of risk factors and macrovascular disease and the more severe stages of microvascular disease. As the DCCT/EDIC population evolves and develops more advanced eye, kidney, and macrovascular disease, investigators will be able to study the effects of established

and putative risk factors on these clinically onerous complications. Diabetes researchers should also continue to study and to define the imprinting phenomenon described during the DCCT/EDIC symposium, including expanding epidemiologic approaches currently in use and through case-control studies.

DCCT/EDIC data can be used to identify and define clinically relevant biomarkers of complications that may be used in future studies, using phenotypic data and stored samples, which may also be used to identify biochemical steps in the pathogenesis of complications. Finally, the current DCCT/EDIC genetic initiative that is looking at the genetic contribution to susceptibility for developing complications ought to be continued.

Dr. Saul Genuth, Professor of Medicine, Division of Clinical and Molecular Endocrinology, Case Western Reserve University, added that the DCCT/EDIC cohort is not only the most vigorously and consistently studied group of type 1 diabetics, but that it is also the most accurately studied group, producing high quality data as a result of good quality control measures. Dr. Genuth stressed the importance of and opportunity for lifelong follow-up by NIH, given the high level of commitment of the patient participants in the cohort, due in part to the research mindset of the patients and to the personal bonding between patients and study investigators. He recommended that researchers capitalize on the strong research motivation of the cohort patients in their consideration of future studies and initiatives.

During the discussion following Dr. Nathan's presentation, Dr. Michael Brownlee, Anita and Jack Saltz Professor of Diabetes Research, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, pointed out a further knowledge gap with regard to the adverse effects of acute hyperglycemia or stress hyperglycemia. Data suggest that coronary disease is largely a metabolic disease; in treatment of individuals with stress hyperglycemia, the outcome in the area of infarction in the brain is proportional to the level of hyperglycemia on admission. Seventy percent of those who have myocardial infarctions are either diagnosed diabetics or people with impaired glucose tolerance. A better understanding of the mechanisms of the disease will aid in the prevention of damaging effects on outcomes, especially since the events typically measured in the diabetic population are ultimately fatal.

During the discussion following Dr. Nathan's presentation, Dr. Michael Brownlee, Anita and Jack Saltz Professor of Diabetes Research, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, pointed out a further knowledge gap with regard to the adverse effects of acute hyperglycemia or stress hyperglycemia. In addition to recent data showing that the majority of patients with coronary artery disease are either diabetic or have impaired glucose tolerance, acute hyperglycemia has been shown to adversely affect the outcome of myocardial infarction and stroke. The area of infarction in the brain is proportional to the level of hyperglycemia on admission. Seventy percent of those who have myocardial infarctions are either diagnosed diabetics or people with impaired glucose tolerance.

Dr. Mark E. Cooper, Director, Baker Heart Research Institute, Melbourne, Australia, stressed that the development of macrovascular complications from diabetes will prove to be especially important over the next 10 years for the DCCT/EDIC population. Why some diabetic individuals are less able to withstand a given load of macrovascular disease than their non-diabetic

counterparts is an area of research that may be further examined with data from the DCCT/EDIC cohort, since baseline data such as echocardiography is available for these patients. Advanced echocardiography allows diastolic dysfunction—which may be linked to the mechanisms reviewed during the DCCT/EDIC symposium—to be more easily discernible and more accurately diagnosed. Dr. Genuth added that the DCCT/EDIC patient population is exceptionally receptive to further testing or exams, especially where heart function is concerned, stating that 85 percent have already had coronary calcium scans performed.

Dr. Peter Savage, Director, Division of Epidemiology and Clinical Applications, National Heart, Lung, and Blood Institute (NHLBI), offered three points that bear closer scrutiny: (1) the subclinical cardiac dysfunction known to occur in diabetics; (2) the amount of vascular disease prior to and following the onset of renal disease and the association of renal disease with the exacerbation or progression of atherosclerosis; and (3) the importance of more efficient clinical trials to examine the means for and to document the correlate between subclinical disease measures and events, particularly in light of the new and multiple interventions available. As an example, Dr. Savage suggested that abnormalities in the system might add substantially to the subclinical disease; if not, then the subclinical disease could be used as a predictor.

Dr. David R. Matthews, Professor of Diabetic Medicine, Oxford Centre for Diabetes Endocrinology and Metabolism, England, observed that perhaps part of the "imprinting" in the DCCT/EDIC cohort is due to the education of and attention given to the patient participants.

Dr. John W. Baynes, Carolina Distinguished Professor, Department of Chemistry and Biochemistry, University of South Carolina, Columbia, cautioned that the group not become too glucocentric. While glucose might turn out to be a statistically important mediator, understanding the downstream effects is also critical. Dr. Baynes suggested that a greater emphasis be placed on insulin resistance in pre-diabetic states, which often precede the development of type 2 diabetes, during which time substantial damage can occur. Dr. Helen Vlassara, Director, Division of Experimental Diabetes and Aging, Mount Sinai School of Medicine, New York, added that researchers ought not to ignore derivatives of glucose metabolism.

The area of implementation and dissemination research, also termed translational research, was an area not covered by the symposium, but one which Dr. Denise Simons-Morton, Acting Director, Clinical Applications and Prevention, Division of Epidemiology and Clinical Applications, NHLBI, brought to the attention of the group. It was suggested by Dr. Daniel Stryer, Acting Director, Center for Quality Improvement and Patient Safety, Agency for Healthcare Research and Quality (AHRQ), that banked clinical data studies could also be supported by R03 or hyper-accelerated grant applications, and that these data could provide information on general markers of inflammation.

Dr. John M. Lachin, Professor of Biostatistics and Epidemiology, The Biostatistics Center, George Washington University, Rockville, Maryland, offered the idea that a future challenge for researchers will be the characterization of lesions at the cellular level, which would represent the true factors that are determining the risk of further disease progression or the risk of complications.

How Can We Foster Development of Animal Models in Which Potential New Therapies Can be Explored?

Dr. Timothy S. Kern, Director, Center for Diabetes Research, Case Western Reserve University, Cleveland, addressed the issues of animal models in type 1 diabetes research. Although most purely diabetic animal models do not progress to advanced stages, they nonetheless provide valuable information, including biochemical abnormalities that seem to play a role in the development of various forms of pathology.

Areas that warrant further attention and research include:

- Establishment of the validity of animal models, given that they largely tend to develop the early lesions, but fail to progress.

- Use of animal models in the development and validation of surrogate markers.

- Examination of genetic contributions to complications, since animal models offer a unique opportunity in terms of cross-breeding.

- Understanding the clonal basis or "imprinting" basis of "metabolic memory."

Considering how long complications take to develop in humans, barriers exist in the use of animal models in diabetes research on complications because the animals have relatively short lifespans. A further obstacle is the lack of macular edema models and the inability of researchers to make specialized measurements. However, the latter difficulty might be overcome through the use of core facilities to provide measurement services.

Dr. Kern encouraged the establishment of a group that would evaluate therapies and decide methods for moving therapies into the clinical setting. He also suggested expansion of the consortium on animal models to provide an arena for discussion beyond the grant recipients and broaden the scope of researchers, a suggestion echoed by several participants at the symposium.

Dr. Cooper expressed concern that appropriate animal models be used. Since the consortium is trying to generate new animal models, they might consider starting with animals such as the db/db mice, which have fewer complications that will affect study results. Drs. Kern and Vlassara agreed with this comment, and Dr. Vlassara further challenged the definition of what constitutes a normal animal model or normal baseline. She suggested a new "hyperglycotoxemic" model be developed.

Dr. Eva L. Feldman, Professor of Neurology, University of Michigan, Ann Arbor, shared information from the AMDCC. The consortium has moved from having 2 animal models to 12 models and is now gathering interesting data on atherosclerotic and nephropathic models. The large bioinformation component of the consortium has allowed for a generous amount of shared data.

Dr. Fradkin suggested that further comments regarding expansion of the consortium be directed

to Dr. Robert Star, Senior Scientific Advisor, NIDDK, at Robert_Star@nih.gov.

How Can We Foster Development of Surrogate Markers Useful for Clinical Trials of Potential New Therapies?

Dr. Ann Marie Schmidt, Associate Professor and Chief, Division of Surgical Science, College of Physicians and Surgeons, Columbia University, New York, categorized cardiovascular disease and diabetes into three parts: (1) the innate cardiac dysfunction; (2) surrogate endpoints for long-term vascular disease, including stenting and the amount of neointimal expansion as a potential surrogate endpoint, given that diabetic individuals undergoing angioplasty and revascularization procedures do very poorly; and (3) macrovascular disease and atherosclerosis itself. (Dr. Schmidt served on the May 16, 2002, Advisory Panel.)

Dr. Schmidt proposed that intravascular ultrasonography (IVUS) might also be used as an endpoint. Since the increase in IVUS quantification of macrovascular disease has been demonstrated, it appears that the Food and Drug Administration (FDA) might be softening with respect to endpoints other than death and clinical events.

With regard to plaque and instability, examination of the inflammatory mediators and inflammatory markers produced by peripheral monocytes in humans following intervention may provide a useful surrogate marker. MMP9, antigen activity, and procoagulant response are also being investigated, as well as impaired endothelial independent relaxation, although the last is not an FDA-approved endpoint. The response to acetylcholine is very abnormal in diabetic individuals and can in and of itself be a surrogate marker.

Clearly, a very important surrogate marker is C-reactive protein (CRP), which might prove useful not only with regard to defining response to therapy, but also when examining quartiles of elevated CRP levels at baseline and their application to relative risk.

Dr. Schmidt identified the following methods for development of surrogate markers:

- Functional MRI is a promising study method, particularly because of its wide availability, but one which may require incentives to encourage study participation.

- Urine protocytes may be a potential marker of early injury, although albuminuria is not an FDA-approved endpoint.

- Degree of alveolar bone loss and periodontal disease are potential surrogate markers for inflammatory baseline and response, since epidemiological data suggest that periodontal disease, regardless of the presence or absence of diabetes, is a risk factor for the development of atherosclerosis.

- Erectile dysfunction, because it involves not only neurology but also vasculature, is a possible surrogate marker.

- Skin biopsies could be surrogate markers for levels of collagen abnormalities.

- Live oxidation products are possible surrogate markers for measurement.

Information presented during the DCCT/EDIC symposium suggested proteomics and genomics as possible surrogate markers, an idea Dr. Schmidt found attractive not only because of the availability of DCCT/EDIC samples, but also because research in these areas encourages basic researchers and clinical trialists to partner with biotech companies, thereby increasing the sample pool and fostering further multidisciplinary action.

Following Dr. Schmidt's presentation, Dr. Bruce Berkowitz, Professor, Department of Cell Biology and Opthalmology, Wayne State University School of Medicine, Detroit, cautioned researchers to use the most finely honed tools available, and as the MRI community possesses an extremely powerful set of tools for diabetic research, they ought to be enticed to form partnerships.

Dr. Matthews commented that better data would become available if researchers could get repeat measures where some specific change or threshold could be predefined. Surrogate markers for the process as an endpoint would reduce regulators' dependency on hard endpoints such as myocardial infarction and death.

Dr. Josephine Briggs, Director, Division of Kidney, Urologic, and Hematologic Diseases, NIDDK, offered a follow-up to Dr. Matthew's remarks on working with regulators, saying that she and Dr. Thomas Hostetter, Director, National Kidney Disease Education Program, NIDDK, have been in contact with FDA regarding the development of a research agenda that would lead to clarity in proteinuria as a process marker.

How Can We Foster Identification of New Therapeutic Targets and Agents?

Dr. Lloyd Paul Aiello, Assistant Director, Beetham Eye Institute and Associate Professor of Ophthalmology, Harvard Medical School, Joslin Diabetes Center, suggested that increased consortium or network approaches would prove useful in moving research findings into clinical trials more rapidly. Excellent characterization and uniformly standardized evaluation of consortia resources would speed evaluation, provide larger sample numbers, and improve comparability between studies. These repositories could also provide some fundamental analyses that are helpful or commonly utilized for this transition, either within the collected samples or perhaps within the repositories. Benefits would include improved comparability between studies, more efficient and consistent evaluation, and services for investigators who are in possession of samples but are unfamiliar with a particular evaluation technique.

Dr. Aiello pointed out the need to rapidly identify, evaluate, characterize, and implement new technologies that may become increasingly important both in the identification of new targets and the evaluation of potential surrogate markers. Such approaches, in addition to providing novel targets may provide cross-fertilization among different complication disciplines and characterize new mechanisms by which researchers could evaluate markers in clinical trials in an efficient and rigorous manner.

During subsequent discussion, Dr. Aiello emphasized that a functional genomics/proteomics approach, conducted with homogenous patients or animal models and identifying different targets, would aid in fostering identification of new therapeutic targets.

Dr. Feldman proposed that some type 1 funds might be directed toward discovery studies, which could lead to new mechanisms, particularly in the proteomics field. Dr. Brownlee commented that discussion seemed to center on two general topics: (1) a focus on optimizing what is currently available, and (2) the concept of discovery. Dr. Vlassara remarked that the DCCT was basically an era of intervention, focusing on the control and modification of blood sugar; perhaps now it was time to add another dimension to the DCCT.

How Can We Move Promising Therapeutic Agents From Bench to Bedside?

Dr. Nigel Calcutt, Associate Professor, Department of Pathology, University of California San Diego, used his experience with moving a molecule (prosaptide) from discovery to phase 2 clinical trials over a relatively short time as an analysis of the bench-to-bedside procedure. According to Dr. Calcutt, doing so involves correctly targeted funding. He identified several factors which contributed to the successful process:

- Personal drive and focus of the Principal Investigators, which included discovery of the molecule and raising money through private funds and venture capital.

- Availability of the STAR program, a fast-moving funding mechanism, where funding was provided in part by the State of California, part by the company of interest. A most important aspect of this funding program is the recognition of the academic as Principal Investigator. The funding is therapy-oriented and results-driven, protects company intellectual property, and provides for initial proof-of-concept studies, allowing investigators to produce the preliminary data necessary to qualify for NIH funding.

- Availability of an NIH Request for Application (RFA), an important aspect because it targeted money at therapy-driven research, rather than purely mechanistic-driven studies.

- Luck and opportunity for collaboration between researchers.

Dr. Calcutt noted that, while these conditions are admittedly unlikely to reoccur in the near future, there are steps NIH can take to create a similarly helpful environment. For example, NIH could fund exploratory research programs that provide money for 1-year rolling, results-driven projects, such as those provided by the Juvenile Diabetes Research Foundation (JDRF) International. An incentive for academics to participate could be initiated through the creation of modified STAR/SBIR (Small Business Innovation Research) funding to include both industry and academia, where both parties would receive recognition for participation. RFAs for R01s to support therapy-driven research should be made available, not to the exclusion of mechanistic-driven research, but to allow for quicker progression. Support systems, both informational banks and funding sources, to connect Principal Investigators having potential therapeutics with those skilled in phase 1 and 2 trials, might be made available through the use of paired grants. Further, NIH could provide assistance through both funding and information to small biotechnology

companies to aid them in moving potential therapeutic agents through phase 1 and 2 trials.

During discussion, the point was made that using surrogate markers and non-regulatory approved endpoints may speed up the process. Dr. Calcutt suggested the formation of a body to negotiate a compromise between NIH's scientific position and FDA's required position from a safety point of view.

Dr. Spiegel, Director, NIDDK, recommended Rapid Access to Interventional Development (RAID), a program used at the National Cancer Institute that provides, on a contract basis, some functions such as producing a sufficient quantity or quality of a product, by means that ordinarily would not be available to an investigator who has a patented therapeutic agent. Production issues might also be expanded through this program.

Dr. Fradkin pointed out the availability of the innovative partnerships RFA that pairs researchers working in diabetes with scientists who have expertise relative to diabetes but who are working in other fields, and proposed the notion that rather than a single grant, two paired grants might be a more attractive option for investigators, a suggestion that was met with general agreement.

Dr. Spiegel concluded with the observation that future research teams ought to embody the concept of a multidisciplinary approach, acknowledging that equal credit for more than a single Principal Investigator is a crucial aspect of team research.

What Are the Most Promising Opportunities To Advance Research To Develop New Therapies for Complications?

Dr. Brownlee posed several possible research questions for consideration by those in attendance:

- What are the mechanisms responsible for microvascular complications?
- What are the mechanisms responsible for macrovascular complications?
- What genetic issues determine the development and progression of diabetic complications?

He expressed the opinion that further investigation and definition of the issue of metabolic memory is certainly necessary, including expansion of the concept to include other areas such as insulin-resistance and fatty acid memory.

Dr. Brownlee recognized the importance of drugs with regard to the prevention of diabetic complications, but remarked that perhaps a greater focus ought to be placed on secondary prevention, since the mechanisms responsible for initiation may not be the same mechanisms responsible for progression of complications. Surrogate markers and a new clinical study paradigm are also areas that he believed warrant additional study, because current paradigms are too costly and require too many years to effectively screen treatments that show promise in animal models.

It is generally accepted that, when considering genetic susceptibility to complications, animal models such as those provided by the AMDCC provide investigators with the advantage of using animals with known genetic backgrounds. These models ought to be further utilized.

As researchers focus design attempts on drugs aimed at specific targets, Dr. Brownlee identified high throughput screening for new therapeutic targets and agents as the new wave of the future.

Dr. Brownlee noted that the DCCT/EDIC symposium's emphasis on multidisciplinary research and collaboration between areas of expertise strongly suggests that dual Principal Investigator grants and exploratory research programs that promote discovery and innovative research should be a priority. In conclusion, he listed the following areas as the most important and most promising research opportunities:

- Development of a mechanism for real discovery and innovation.
- Multidisciplinary efforts fostered through dual investigator grants between researchers in complementary fields to produce innovative work.
- Funding for non-patented therapeutic agent trials.

Dr. Fradkin closed the session with the comment that the meeting produced not only intriguing ideas in the area of diabetes research, but identified available resources for carrying them to fruition.